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REVIEW

# Does airway smooth muscle express an inflammatory phenotype in asthma?

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In addition to hyperresponsiveness in asthma, airway smooth muscle (ASM) also manifests an inflammatory phenotype characterized by augmented expression of mediators that enhance inflammation, contribute to tissue remodelling and augment leucocyte trafficking and activity. Our present review summarizes contemporary understanding of ASM-derived mediators and their paracrine and autocrine actions in airway diseases.

**LINKED ARTICLES**
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**Abbreviations**
5-LO, 5-lipoxygenase; *Af*, *Aspergillus fumigatus*; AHR, airway hyperresponsiveness; ASM, airway smooth muscle; CAM, cell adhesion molecule; COPD, chronic obstructive pulmonary disease; EGF, epidermal growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GPCR, G-protein coupled receptor; IL, interleukin; LPS, lipopolysaccharide; LT, leukotriene; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase; $PGE_2$, prostaglandin-$E_2$; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; RANTES, regulated upon activation normal T-cell expressed and secreted; RGS, regulator of G-protein signalling; RTK, receptor with intrinsic tyrosine kinase; TDI, toluene diisocyanate; TGF-β, transforming growth factor β; Th1, T-helper 1; Th2, T-helper 2; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor

## Introduction

Asthma, a heterogeneous disease, manifests by chronic airway inflammation associated with infiltration of eosinophils and T-helper 2 (Th2) lymphocytes, airway hyperresponsiveness (AHR), remodelling, mucus hypersecretion and, in part, reversible airway obstruction. While imbalance in T-helper 1 (Th1) and Th2 inflammatory responses may underlie asthma pathogenesis, evidence suggests that AHR persists in the absence of airway inflammation implying that structural cells likely play a role in development of the disease. In this context, studies have identified airway smooth muscle (ASM) as a pivotal tissue promoting the asthma diathesis. Similar to other airway structural tissues, ASM expresses a broad class of chemokines/cytokines, eicosanoids and prostaglandins that modulate airway-health and -disease (Table 1). Although much work has been performed *in vitro*, contemporary studies have identified ASM to express aforementioned molecules *in vivo*. Previous reviews by us and others have elaborated on the immunomodulatory faculties of ASM (Hershenson *et al.*, 2008; Dekkers *et al.*, 2009; Tliba and Panettieri, 2009). Our focus here encapsulates the evolving role of ASM as a modulator and effector population with an overarching role in airway-inflammation, -remodelling and -irreversible obstruction.

## Modulation of inflammation via paracrine and autocrine pathways

Tumour necrosis factor-α (TNF-α) as a key cytokine mediates pathogenic mechanisms of a number of chronic inflammatory diseases, including rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, psoriasis and asthma (Russo and Polosa, 2005). In addition to cross-sectional studies showing enhanced prevalence of TNF-α in the airway of asthmatics, the development of AHR post-TNF-α inhalation in normal individuals reiterates its singular relevance towards airway

**Table 1**
Inflammatory mediators and airway smooth muscle (ASM) function

| Mediator | Synthesized by ASM | Autocrine effects on ASM | Modulates ASM function |
|---|---|---|---|
| IL-1 | + | + | +++ |
| IL-4/13 | +/– | +/– | +++ |
| IL-6 | +++ | +/– | – |
| IL-8 | +++ | – | – |
| IL-9 | – | – | ++ |
| $PGE_2$ | +++ | +++ | +++ |
| $LTD_4$ | +/– | +/– | +++ |

+++, substantial effect; +, modest effect; –, little or no effect.

dysfunction in asthma (Bradding *et al.*, 1994; Thomas and Heywood, 2002). Similarly, murine-isolated tracheal rings incubated with TNF-α show exaggerated responses to contractile agonists, including carbachol, bradykinin and serotonin (Brightling *et al.*, 2008). In airways of individuals with asthma, mast cells are prominent contributors of TNF-α secretions, and ASM bundle-resident mast cell numbers positively correlate with the degree of AHR and with the bronchoconstrictor response to a deep inspiration, leading some to speculate that their juxtaposition facilitates rapid mast cell-derived TNF-α activation and myocyte shortening (Carroll *et al.*, 2002; Slats *et al.*, 2007; Brightling *et al.*, 2008). Overlaying its pathological effects on airway mechanics, localized TNF-α abundance has other disease-specific connotations in asthma. Several *in vitro* studies have elucidated an ability of TNF-α to elicit airway neutrophilia and eosinophilia, mucus hypersecretion, T-cell activation, expression of cell adhesion molecules (CAMs), and maturation and differentiation of structural tissue (Matera *et al.*, 2010). As demonstrated extensively, TNF-α effects on ASM are elaborate, and the following text elucidates its biological role in the context of inflammation, infection and remodelling.

Together with TNF-α, interleukin (IL)-1β identifies as a key cytokine that modulates inflammatory cascades during exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (Howarth *et al.*, 2005; Hackett *et al.*, 2008). The pleiotropic nature of IL-1β results in the activation of a wide range of cells such as mast cells, T- and B-lymphocytes, epithelial cells and ASM. As a prominent mediator of asthma pathophysiology, IL-1β expression is increased in the airways of asthmatic individuals, and IL-1 receptor antagonist (IL-1Ra) administration reduces AHR induced by allergens in mice (Barnes, 2008). In ASM cells of human origin, IL-1β treatment enhances production of numerous inflammatory mediators including prostaglandin-$E_2$ ($PGE_2$), eotaxin, regulated upon activation normal T-cell expressed and secreted (RANTES), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-related oncogene-α, epithelial neutrophil-activating protein-78, nerve growth factor, monocyte chemotactic protein (MCP)-1,2,3, IL-8 and IL-11 (Pype *et al.*, 1999; Freund *et al.*, 2002; Jarai *et al.*, 2004; Issa *et al.*, 2006; Tliba *et al.*, 2008; Clarke *et al.*, 2009a). Besides contributing to pro-inflammatory milieu, IL-1β amplifies ASM responses by modulating receptor expression as with IL-5R and protease-activated receptor-2 expression in human myocyte cultures, and ASM-resident IL-1RI, TNF-RI,RII and 5-$HT_{2A}$ receptors in *ex vivo* cultures of murine tracheal rings, further amplifying its effects on ASM function (Hedges *et al.*, 2000; Cardell *et al.*, 2008). Numerous studies in ASM have identified COX-2 enzyme inducing pivotal IL-1-mediated responses. While IL-1β enhances bradykinin-induced responsiveness in tracheal ring segments, desensitization of the $H_1R$ via induction of $PGE_2$ renders bronchial rings less responsive to histamine (Pype *et al.*, 2001). Further, IL-1β synergizes with TNF-α to promote $\beta_2$AR hyporesponsiveness, a phenomenon that is ablated by selective COX-2 inhibition (Shore *et al.*, 1997). Evidence suggests that glucocorticoids render IL-1β and TNF-α mitogenic to ASM, via suppression of COX-2-dependent protein kinase A (PKA) activity (Misior *et al.*, 2009). As a constituent of IL-1 superfamily, IL-33 levels are also elevated and co-expressed with TNF-α within ASM bundles in endobronchial specimens from severe phenotypes of asthma (Prefontaine *et al.*, 2009). Interestingly, TNF-α-induced IL-33 was found to be resistant to the mainstay anti-inflammatory treatments in cultured ASM, implying a role for IL-33 in severe and refractory asthma. Besides being the effector population, ASM also secrete IL-1β. Exposure of ASM to IgE immune complexes induces IL-5 secretion. The secreted IL-5, in a paracrine manner, enhances eosinophil survival, and, in an autocrine fashion, augments IL-1β expression (Hakonarson *et al.*, 1999). Accordingly, ASM cells simulated with IgE complexes, IL-5 or IL-1β synthesize signalling molecules in the IL-1 axis, including IL-1α, IL-1β, IL-1β-converting enzyme, IL-1 receptor accessory protein, IL-1 receptor, IL-1 receptor-like 1 and IL-18 receptor 1 (Whelan *et al.*, 2004; Hershenson *et al.*, 2008). Among other functions, members of the IL-17 cytokine family also enhance influx of leucocytes and induce pro-inflammatory mediators in airway tissues. While IL-17 increases ASM-derived eotaxin-1 and CXCL-8 levels, IL-1β and TNF-α increase IL-17 receptors isoforms on ASM, further perpetuating inflammation.

## *Interleukin-4 (IL-4) and interleukin-13 (IL-13)*

Although asthma has been genetically linked to polymorphisms in the genes encoding IL-4Rα, IL-13Rα, STAT-6 and BCL6, the role of IL-4 or IL-13 in mediating airway inflammation or dysfunction remains unclear. Recent studies show that the presence of $IL\text{-}4^+$ and $OX40^+$ (a protein with implications in Th2 polarization and eosinophilic inflammation) cells among ASM correlates with asthma severity (Siddiqui *et al.*, 2010). As a consequence of IL-4 and TNF-α stimulus, ASM cells express FcεRI that further augments the selective CC (eotaxin-1 and RANTES, but not thymus activation-regulated chemokine) and IL-8, IP-10 chemokine secretion following IgE exposure (Redhu *et al.*, 2009). *In vitro* studies showed that IL-4 and IL-13 induce AHR in a STAT6-dependent manner and that carbachol-induced hyperresponsiveness was attenuated in $STAT6^{-/-}$ mice. Others showed that anti-IL-4Rα antibodies attenuate acetylcholine-induced responsiveness in IgE-sensitized ASM, supporting the role of IL-4Rα-STAT6 pathway in mediating AHR (Grunstein *et al.*, 2002). In response to IL-4 or IL-13, human ASM cells release

eotaxin *in vitro* that is inhibited by anti-IL-4Rα antibodies and antisense oligonucleotides to STAT6 (Hirst *et al.*, 2002; Peng *et al.*, 2004). In either IL-13$^{-/-}$ mice, IL-4/5/9/13$^{-/-}$ or wild-type mice, IL-13 was required for AHR, ASM hyperplasia and increases in IgE levels (Nath *et al.*, 2007). Interestingly, these reports stand in contrast to those reported in smooth muscle myosin heavy chain $^{cre}$IL-4Rα$^{-/lox}$ mice whose IL-4Rα showed little effect on allergen-induced AHR or expression of other inflammatory biomarkers as compared to wild-type mice (Kirstein *et al.*, 2010). Collectively, the role of IL-4 and IL-13 in allergic asthma remains unclear, and IL-4Rα-independent compensatory mechanisms may mediate AHR.

## *Interleukin-6 (IL-6)*

Disruption of IL-6-mediated responses by IL-6Rα-blocking antibody diminishes ovalbumin (OVA)-induced Th2 inflammation; others showed that *Aspergillus fumigatus* (*Af*)-mediated mucus secretion is substantially diminished in IL-6$^{-/-}$ mice, suggesting a role for IL-6 in mediating/resolving allergen-induced AHR and inflammation. Pursuing experimental models of allergen-induced AHR in transgenic mice, investigators show that airway inflammation and bronchial reactivity can be uncoupled with IL-6 overexpression enhancing pro-inflammatory cytokines, TNF-α and IL-1β, but diminishing carbachol-induced responsiveness (Elias *et al.*, 1997). Based on the demonstrated ability of several disease-specific mediators including TNF-α, IL-1β, transforming growth factor β (TGF-β), thymic stromal lymphopoietin, IL-17A, bradykinin, endothelin-1 (ET-1) and sphingosine-1-phosphate (S-1-P) to induce IL-6 secretion in ASM, airway myocytes may directly contribute to IL-6 production in asthma (Ammit *et al.*, 2001; McKay and Sharma, 2002; Iwata *et al.*, 2009; Tliba and Panettieri, 2009; Shan *et al.*, 2010). In a more complex role, TNF-α induces interferon (IFN)-β secretion from ASM, which by its autocrine actions alters TNF-α-mediated IL-6 and RANTES secretions (Tliba *et al.*, 2003). ASM in spatial proximity to epithelium also enhances basal or TNF-α-induced IL-6 and IP-10 secretions (Damera *et al.*, 2009b).

IL-17A, a CD4$^+$ cell-derived cytokine, also selectively augments TNF-α-induced IL-6 and IL-8 expression by altering transcript stability, while showing little effect on IL-1β-mediated responses (Henness *et al.*, 2004). Despite the lack of a canonical IL-6 membrane-bound receptor (mIL-6R) in ASM, IL-6 induces inflammation and vessel expansion by release of eotaxin and vascular endothelial growth factor (VEGF) via the soluble IL-6R (sIL-6Rα) receptor (Ammit *et al.*, 2007). Evolving evidence also shows that conditioned serum from ASM cells treated with cytomix (TNF-α, IL-1β and IFN-γ) promotes an eosinophilopoietic potential on CD34$^+$ bone marrow-derived cells that was ablated in the presence of neutralizing antibodies to IL-5 and GM-CSF (Fanat *et al.*, 2009). As a modulator of eosinophil trafficking, activation and the survival in asthma, ASM cells secrete GM-CSF in response to TNF-α/IL-1 alone or in combination with serum, or mast cell-derived tryptase. In response to histamine, ASM cell secretion of IL-1β-induced GM-CSF is increased while diminishing TNF-α-mediated RANTES output, an effect abrogated by selective antagonism at the $H_1$R (Chhabra *et al.*, 2007). As a facilitator and sustainer of leucocyte trafficking in airways, ASM stimulation with endothelin (ET-1) and TNF-α enhances both GM-CSF and ET-1 secretion by positive feedback loops, a mechanism impaired by bosentan and specific inhibition of either ET(A)R or ET(B)R (Knobloch *et al.*, 2009). Oncostatin M, another member of the IL-6 family, enhances IL-1R1 expression and augments IL-1β-mediated VEGF, MCP-1 and IL-6 secretion, or synergizes with IL-13 and IL-4 to increase eotaxin-1 expression in human ASM (Faffe *et al.*, 2005a,b). Collectively, these data suggest a dynamic, paracrine crosstalk modulating IL-6 secretion in asthma.

## *Interleukin-8 (IL-8)*

As with COPD, increases in neutrophilic inflammation in subjects with severe asthma correlate with production of CXCL-8 within the lungs. CXCL-8, found in bronchoalveolar lavage (BAL) fluid and in the supernatants of cytokine-stimulated ASM cells, activates CXCR-1 receptors promoting mast cell migration. Emerging evidence shows that enhancement in CXCL-8 secretion in asthma can increase binding of NF-κB, C/EBPβ and RNA Pol II elements to CXCL-8 promoter (John *et al.*, 2009) in ASM cells. Likewise, phenotypic differences in ASM have been suggested to augment IL-8-dependent AHR and to enhance IgE-mediated IL-8, IL-6 and IL-4 secretion from normal ASM (Govindaraju *et al.*, 2006; 2008). Human ASM cells produce IL-8 when treated exogenously with IL-1β, TNF-α or TGF-β (Chung, 2000). In a scenario reminiscent of COPD, pro-inflammatory stimuli, such as TNF-α and cigarette smoke, synergize to induce IL-8 secretion from ASM (Oltmanns *et al.*, 2005). Bradykinin directly elevates IL-8 secretion via the canonical bradykinin receptor-Gq-ERK cascade. Such effects are augmented by $\beta_2$-AdR-agonists and/or by cAMP-regulated Epac1, Epac2 and PKA (Pang and Knox, 1998) (Roscioni *et al.*, 2009). Acetylcholine induces $M_3$R-mediated neutrophil chemotactic responses and mucus hypersecretion in airway epithelial cells. Later studies show that $M_3$R agonists augment cigarette smoke-induced IL-8 secretion in airway myocytes (Gosens *et al.*, 2009). These data suggest that anticholinergics such as tiotropium bromide in COPD (Oenema *et al.*, 2010; Wollin and Pieper, 2010) may offer value in IL-8-dependent airway inflammation. In addition to inhibiting AHR, other $M_3$R antagonists such as aclidinium bromide have substantial pharmacological benefits by inhibiting airway eosinophilia in murine models of asthma (Damera *et al.*, 2010). Whether mast cell-derived histamine stimulates IL-8 secretion in ASM remains unknown; however, β-tryptase, another mast cell-derived serine protease, substantially enhances IL-8 via transcriptional and post-transcriptional mechanisms (Mullan *et al.*, 2008).

## *Interleukin-9 (IL-9)*

Overexpression of IL-9 in murine models evokes airway eosinophilia, mast cell hyperplasia, mucus production and AHR, reminiscent of the asthma diathesis. Gene mapping for bronchial hyperresponsiveness in humans suggested IL-9 and IL-9R are candidate genes for asthma. Enhanced expression of ASM IL-9R in biopsies from atopic asthma subjects as compared with that of non-asthmatic subjects has been shown. Early studies demonstrate that, in spite of its minimal effect in directly mediating ASM-derived cytokine secretion, IL-9 augments TNF-α-induced IL-8- or IL-13-induced eotaxin release in isolated human ASM (Baraldo *et al.*, 2003). Further,

IL-9 selectively and directly enhances eotaxin-1/CCL11 secretion that can promote airway eosinophilia (Yamasaki *et al.*, 2010).

### *Prostaglandins and leukotrienes*

Activation of ASM COX-2 enzymes generates arachidonic acid metabolites including $PGE_2$. $PGE_2$ stimulates EP1 receptors that mediate $Ca^{2+}$ mobilization, EP3 receptors that inhibit adenylate cyclase, and EP2 and EP4 receptors that activate adenylate cyclase. Overall, physiological responses to $PGE_2$ were thought to depend on selective receptor expression on target tissues, and recent reports suggest that $PGE_2$ expression is also mediated by receptor sensitivity (Rolin *et al.*, 2006). In murine models of asthma, evaluating pharmacological consequences of prostaglandin depletion with COX inhibitors or chimeric knockouts of COX show augmented airway eosinophilia, enhanced Th2 cytokine profile and AHR. A wide range of stimuli including bradykinin, IL-1β, TNF-α, IFN-γ, ozone, cigarette smoke extract, lipopolysaccharide (LPS), mechanical stretch and cAMP-elevating agents potentiate $PGE_2$ production by ASM cells (Clarke *et al.*, 2009b). ASM-derived $PGE_2$ can modulate a wide variety of outcomes such as mast cell mediator release, epithelial barrier function, neutrophil and eosinophil chemotaxis, lymphocyte-derived IgE production and structural cell mitogenesis. Leptin, an obesity-linked hormone, inhibits platelet-derived growth factor (PDGF)-mediated human ASM proliferation and migration and IL-13-induced eotaxin production via the production of $PGE_2$ (Nair *et al.*, 2008). Environmental pollutants such as ozone induce structural cell-derived IL-6 via early enhancement of $PGE_2$ (Damera *et al.*, 2009b). While cytokine treatment induces $\beta_2$AR desensitization in murine tracheal rings, mice deficient in EP2 receptor appear resistant to such effects, implying that $PGE_2$-mediated mechanisms serve as modulators of both inflammation and airway $\beta_2$AR responsiveness. Others postulate a protective role for $PGE_2$ concerning airway inflammation. For instance, mice lacking EP3 receptors show exaggerated airway inflammation and hyperresponsiveness in OVA-induced models of AHR (Kunikata *et al.*, 2005). In individuals with atopic asthma, inhaled $PGE_2$ abrogates early- and late-phase responses to antigen challenge (Gauvreau *et al.*, 1999). Similarly, individuals with aspirin-sensitive airways disease show depleted $PGE_2$ production in a variety of cell types, and inhalation of $PGE_2$ reversed aspirin-induced airflow obstruction (Sestini *et al.*, 1996). In addition to $PGE_2$, other prostaglandins such as prostaglandin-$D_2$ and thromboxane are bronchoconstrictors and could also exaggerate allergen-mediated airway eosinophilia (Allen *et al.*, 2006; Boyce, 2008).

The activation of 5-lipoxygenase (5-LO) enzyme generates bioactive leukotrienes (LTs) from arachidonic acid. Two groups of LTs, the dihydroxy LT, $LTB_4$, and the cysteinyl LTs (Cys-LTs), composed of $LTC_4$, $LTD_4$ and $LTE_4$, act through distinct receptors (Hallstrand and Henderson, 2010; Singh *et al.*, 2010). CysLT production is increased in asthma subjects, particularly during an exacerbation or upon challenge. Indeed, CysLT production is increased in some asthma subjects, particularly during exacerbation or upon provocative challenge (Montuschi *et al.*, 2006). Accordingly, 5-LO inhibition by zileuton or antagonism of $CysLT_1$ receptor by zafirlukast improves $FEV_1$ measurements in individuals with asthma (Drazen, 1998). Subsequent studies also show a positive correlation between CysLTs in the exhaled breath condensate and reticular basement membrane thickening in asthma (Lex *et al.*, 2006). *In vivo* studies investigating OVA sensitization and challenge in $5\text{-LO}^{null}$ and $LTC_4S^{null}$ mice reported reduced BAL fluid eosinophil numbers, diminished methacholine-induced AHR, and attenuation of total IgE and OVA-specific IgG in serum (Bosse *et al.*, 2009; Mehrotra and Henderson, 2009). Treatment of ASM with atopic serum or IL-1 can activate the 5-LO pathway. Post *Af* sensitization and challenge, Th2-type inflammation and AHR to methacholine were associated with enhanced $LTD_4$-$CysLT_1$ receptor interactions in ASM tissue (Kim *et al.*, 2006). While $CysLT_1$ receptor enhancement drives IL-13 and $LTD_4$ mediated ASM proliferation, Cys-LT directly or in conjunction with histamine mitigates ASM contractility. Others have shown that enzyme inhibitors 5-LO and LT receptor antagonist (pranlukast), but not an $LTB_4$ receptor antagonist, diminished DNA synthesis in a rat model of allergic asthma (Salmon *et al.*, 1999). The outcomes were further substantiated, when treatment with montelukast substantially inhibited development of ASM cell hyperplasia in another murine model (Henderson *et al.*, 2002). Others showed that $LTD_4$ modulates myocyte hyperplasia and AHR via paracrine mechanisms involving epithelial cell-derived TGF-β1 (Espinosa *et al.*, 2003; Bosse *et al.*, 2008).

Current evidence is compelling to suggest that ASM secretes numerous cytokines/chemokines, eicosanoids and prostaglandins that may alter autocrine and paracrine function of structural and trafficking leucocytes in the airways. The specific mechanisms that induce expression of such molecules, in part, differ from that of other cells. Future studies focusing on the inhibition or the stimulation of such molecules and structural cells may offer new therapeutic targets in the treatment of asthma and COPD.

## Toll-like receptor-mediated immunomodulation and ASM

Respiratory infections evoke asthma exacerbations mediated, in part, by the expression and activation of toll-like receptor (TLR) by microbial components that promote airway inflammation. Resolution of infection depends on the specificity of the 11 known TLRs to bind distinct products of microbial-derived molecules and to elicit downstream signalling (Phipps *et al.*, 2007; Drexler and Foxwell, 2010). ASM cells express transcripts for most TLR isoforms, although quantitative disparities exist among TLR isoforms (Chaudhuri *et al.*, 2007). ASM cells express functional TLR-2, TLR-3 and TLR-4 (Sukkar *et al.*, 2006). While *in vitro* infection of human ASM cells with respiratory viruses induces production of IL-1β, IL-6, IL-8 and IL-11, expression of TLRs is also increased after treatment with TNF-α, IL-1β and IFN-γ. These data suggest that ASM TLR activation may amplify ongoing inflammatory processes (Damera *et al.*, 2009a). Similarly, bacterial endotoxins or autologous proteins with conserved TLR recognition sequences can also enhance synthesis of leucocyte chemotractant mediators including eotaxin-1, CXCL-8 or GM-CSF (Issa *et al.*, 2008). LPS-induced secretion of mono-

cyte chemotractants such as CCL2 (MCP-1) are also conditionally expressed by ASM when co-cultured with peripheral blood mononuclear cells (Morris *et al.*, 2005). Besides mediating TLR-4-mediated induction of IL-6 secretion, LPS alters agonist-mediated constrictor and relaxant responses (Shan *et al.*, 2006). Likewise, tracheal organ culture incubated with LPS or polyinosinic polycytidylic acid [poly(I:C), a TLR-3 agonist] enhances bradykinin- and [des-Arg(9)]-bradykinin-induced contractions (Bachar *et al.*, 2004). Surprisingly, TLR effects in human precision-cut lung slices did not modulate agonist-induced bronchodilation (Cooper *et al.*, 2009).

Besides activating TLR receptors on airway epithelial cells and immune cells, Der p2 (house dust mite) allergen has intrinsic enzymatic activity that enhances epithelial permeability and detachment, potentially exposing the submucosa to allergens (Roche *et al.*, 1997). Recent studies show that Der p2 activates TLR-2/MyD88 in ASM cells that induce secretion of MCP-1 and IL-6 (Chiou and Lin, 2009). Respiratory syncytial virus (RSV) ssRNA are potent activators of TLR-7/8, and RSV infection enhances airway epithelial barrier permeability, potentially leading to enhanced passage of RSV ssRNA to the submucosa (Wang *et al.*, 1998). However, the consequences of TLR-7/8 activation in specific airway tissues remain unclear; some studies show that activation of TLR-7/8 induces pro-inflammatory mediators and enhances mucus hypersecretion, while others demonstrate a substantial reduction in immune cell-mediated increases in airway myocyte mass, inflammation and hyperresponsiveness in murine models.

## ASM modulates leucocyte transmigration

As leucocyte transmigration is crucial in mediating immune responses, elucidating molecular mechanisms may offer new therapeutic targets for mitigating airway inflammation. Leucocyte migration and retention, initially evoked by selectins on endothelial cells, subsequently express CAMs presented by 'primed' airway structural cells. Studies *in vitro* and *in vivo* show that expression of CAMs mediates cell–cell interactions implicated in inflammation and tissue remodelling (Kelly *et al.*, 2007). ICAM-1 expression in ASM cells is mediated by pro-inflammatory mediators including cytokines, bacterial endotoxins and viral proteins, implying a broader role in chronic airway diseases (Tliba *et al.*, 2008). Surface expression of CAMs and their interactions with immune cells play a role in mediating ASM-leucocyte function interactions in asthma.

TNF-α and IL-1β induce ICAM-1 and VCAM-1 in ASM. Additionally, independent of replication, rhinovirus (RV-15) induces ASM-derived IL-5 and IL-1β secretion via interactions with ASM-resident ICAM-1 molecules (Grunstein *et al.*, 2001; Oliver *et al.*, 2006). In ASM cells, TNF-α activation and nuclear translocation of p65 NF-κB are sufficient to induce VCAM-1 but not ICAM-1, implying signalling diversity in CAM expression (Zerfaoui *et al.*, 2008). Reinforcing the role of NF-κB-independent mechanisms in CAM modulation, IL-1-mediated expression of ICAM-1 is attenuated by $PGE_2$, forskolin and short- and long-acting $\beta_2$-AdR-agonists in a PKA-dependent and NF-κB-independent manner (Kaur *et al.*, 2008). While functionally inconclusive, expression of CAMs could mediate T-cell adherence to airways potentially altering Ach-induced bronchoconstriction and isoprenaline-mediated bronchodilation (β2-AdR) (Hughes *et al.*, 2000; Hakonarson *et al.*, 2001). While toluene diisocyanate (TDI) administration potentiates AHR and airway inflammation including ASM-resident CAM expression in mice, administration of peroxisome proliferator-activated receptor (PPAR)γ agonists or adenovirus carrying PPARγ2 cDNA reversed TDI-mediated induction of cytokine and CAMs, implying a protective role of PPARγ in the pathogenesis of asthma (Lee *et al.*, 2006). Studies using antibodies blocking lymphocyte function-associated antigen 1 (LFA-1) and Very Late Antigen-4 (VLA-4) on activated T cells or antibodies against ICAM-1 and VCAM-1 on ASM cells showed greater attenuation of T-cell adherence to ASM compared to either anti-ICAM or anti-VCAM treatment alone (Duplaa *et al.*, 1997). Addition of anti-CD44 Abs to combination of mAbs against LFA-1, VLA-4, synergistically, reduces the binding of activated T cells to the level observed for resting T cells (Lazaar *et al.*, 1994). Suggestive of a broader role of CAMs in leucocyte trafficking, anti-ICAM-1 or anti-VCAM-1 attenuates eosinophil and neutrophil adherence to ASM. Expanding the role of CAMs, more recent studies suggest that mast cell infiltration of ASM cells in asthma occurs via expression of a heterophilic adhesion molecule, tumour suppressor in lung cancer-1 (TSLC-1) (Yang *et al.*, 2006). Studies by Ramos-Barbón *et al.* showed that adoptive transfer of $CD4^+$ T cells from sensitized rats induces proliferation and attenuates apoptosis of ASM in naive recipients after successive antigen challenges. Concomitantly, modified $CD4^+$ T cells expressing enhanced green fluorescent protein were localized in juxtaposition to ASM cells conferring that cell–cell interaction participates in airway remodelling. In an expanding role for CAMs in airway inflammation, studies also determined a critical role for a β-galactoside-binding lectin, Galectin-3 (Gal-3), in eosinophil trafficking and recruitment in allergic inflammation (Ramos-Barbón *et al.*, 2005). Comparative studies in transgenic mice revealed that $gal3^{-/-}$ mice showed significantly less AHR, lower Th2 responses after challenge and altered expression of other CAMs when compared to $gal3^{+/+}$ mice (Zuberi *et al.*, 2004).

## ASM phenotype switching

Severe asthma represents a heterogeneous disease, with lung function that is, in part, irreversibly reduced in some (Moore and Peters, 2006; Chanez *et al.*, 2007). Among other features, a prominent feature in such patients includes increases in ASM mass, leading investigators to postulate that ASM growth, hypertrophy or hyperplasia could evoke irreversible airway obstruction. Proliferative responses in ASM are stimulated by epidermal growth factor (EGF), insulin-like growth factors (IGFs), PDGF and fibroblast growth factor-2. Some contractile agents such as histamine, ET-1, substance P, 5-HT, α-thrombin, thromboxane $A_2$ and $LTD_4$ also mediate ASM mitogenesis (Lazaar and Panettieri, 2005; Dekkers *et al.*, 2009). The precise role of cytokines, however, remains ambiguous concerning ASM growth. While IL-1β and IL-6 mediate hyperplasia in guinea pig ASM cells, such effects are less evident in human ASM. Similarly, while TGF-β1 and TNF-α induce proliferation or potentiate the effects of some

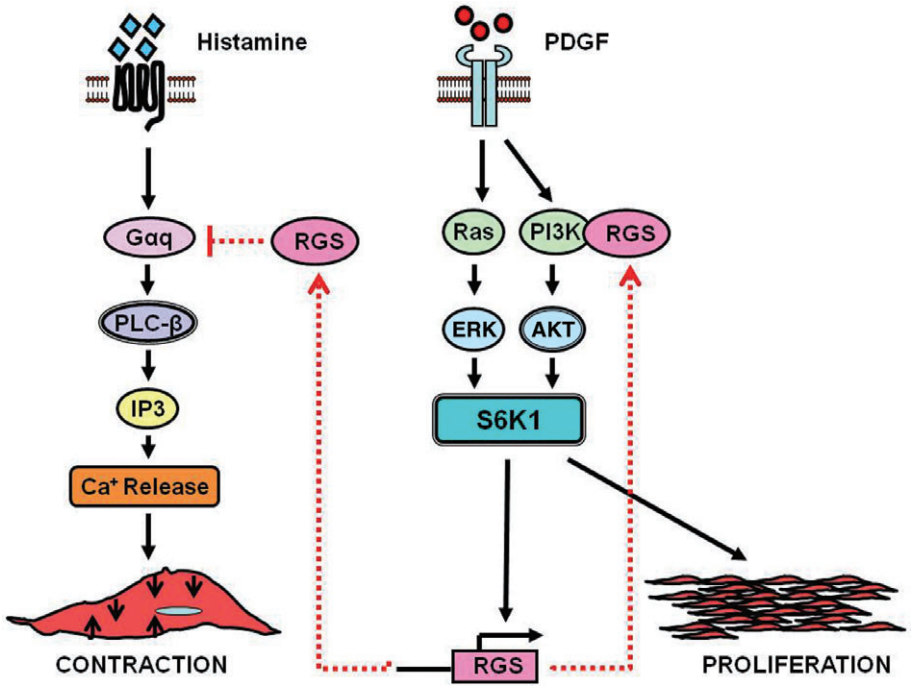
mitogens, some studies suggest that these cytokines inhibit mitogenesis. More definitive are the effects of IFN (type-1 and -2) and IL-4 that inhibit ASM proliferation by diverse signalling mechanisms (Amrani *et al.*, 2003; Tliba *et al.*, 2003).

Studies have identified signalling events that are both necessary and sufficient to promote ASM growth. Such events serve as critical checkpoints that modulate ASM mitogenesis. Mitogens stimulate their cognate receptors inducing activation of ERK, p21Ras (Ras), Src and PI3K leading to human ASM growth. As with most cell types, expression of D-type cyclins (D1, D2 and D3) is required for mitogen-induced cell cycle progression. In ASM cells, studies showed that cyclin D1 expression may serve as a possible surrogate for cell proliferation. BAL fluid derived from airways of subjects with asthma enhances ERK activation, cyclin D1 protein abundance, DNA synthesis and cell number of cultured human ASM cells (Naureckas *et al.*, 1999). In cultured ASM cells, activation of ERK is required for cyclin D1-mediated DNA synthesis. Likewise, studies also implicate involvement of Ras/Raf proteins in ERK-mediated ASM proliferation. For instance, anti-pan Ras neutralizing antibody attenuates DNA synthesis in human airway myocytes, and overexpression of a dominant-negative form of Ras inhibits PDGF-stimulated ERK activation (Page *et al.*, 1999). Selective overexpression of an activity deficient mutant of Raf-1 substantially diminishes endothelin-mediated ERK activation in rat airway myocytes, suggesting a role for Ras/Raf (Vichi *et al.*, 1999). Besides ERK, pharmacological inhibitors of PI3K also diminish cyclin D1 protein expression and DNA synthesis. Similarly, overexpression of active Rac1 or PI3K initiates transcription at the cyclin D1 promoter, and such mechanisms occur independent of ERK activation and remain insensitive to mitogen-activated ERK kinase inhibitors, suggesting that Rac1- or PI3K-mediated cell cycle progression occurs in parallel. Supportive of these outcomes, overexpression of Src or PI3K alone stimulated cell growth, and inhibition of PI3K abrogated mitogen-induced ASM proliferation. PI3K-dependent downstream events ultimately activate S6K1 that is associated with the efficiency of translation of mRNAs that are involved in mitogenesis and whose inhibition by rapamycin attenuates growth factor-induced DNA synthesis in airway myocytes (Scott *et al.*, 1996). Importantly, prolonged activation of PI3K and S6K1 at 12 h discriminated ASM mitogens from non-mitogenic agonists and mediators that otherwise equally activated ERK1/2 at 1 h (Krymskaya *et al.*, 2000). Activation of receptor tyrosine kinases (RTKs) can also initiate signalling cascades involving Rac and NADPH oxidase to produce reactive oxygen species leading to JAK-2/3-mediated cyclin D1 transcription (Simeone-Penney *et al.*, 2008).

Among mitogenic responses elicited by growth factors and contractile agonists, important disparities exist. Mitogenesis stimulated by growth factors is mediated by RTK activity whereas G-protein coupled receptors (GPCRs) lack intrinsic kinase activity (Ritter and Hall, 2009). Growth induced by RTK activation appears to be Ras- and PI3K-dependent whereas that induced by GPCRs may be more diverse and use an array of small G-proteins and phosphotyrosine scaffolding proteins including Src (Chambard *et al.*, 1987; Kavanaugh *et al.*, 1988). In addition to differences between the proliferative mechanisms of growth factors and those of GPCR agonists, there exists substantial variability in the mitogenic capacity among agonists (Ritter and Hall, 2009). First, some, but not all, GPCR contractile agonists stimulate ASM proliferation, even though most induce comparable levels of PI hydrolysis and cytosolic calcium transients (Krymskaya *et al.*, 2000; Billington *et al.*, 2005; Kong *et al.*, 2006). Second, the mitogenic effects of endothelin and serotonin on vascular smooth muscle (SM), and thrombin on ASM, are coupled to *Pertussis* toxin-sensitive G-proteins, not *Pertussis* toxin-insensitive G-proteins that regulate agonist-induced ASM contraction (Kavanaugh *et al.*, 1988; Komuro *et al.*, 1988; Noveral *et al.*, 1992; Panettieri *et al.*, 1995; 1996). The differences in proliferative responses induced by growth factors and agonists (and among agonists) imply that other regulatory components are also involved.

ASM cells manifest phenotype plasticity, whereby contractile myocytes undergo switching toward a more proliferative phenotype in the presence of mitogens or when grown on distinct extracellular matrix (ECM) proteins (Halayko *et al.*, 2006; Dekkers *et al.*, 2007). Predictably, such alterations correlated with quantitative increases in synthetic pathways for protein and lipids and mitochondrial function with a diminished abundance of contractile proteins. In contrast to specific proteins, namely, smooth muscle myosin heavy chain, SM22, calponin and smooth muscle α-actin that mark pro-contractile phenotype, proliferating ASM show enhanced non-muscle myosin heavy chain, l-caldesmon, vimentin, α/β-protein kinase C and CD44 homing cellular adhesion molecule (Halayko *et al.*, 2008; Hirota *et al.*, 2009). Additionally, dystrophin glycoprotein complex expression and accumulation correlated with a contractile phenotype and diminished in proliferating ASM cells (Sharma *et al.*, 2008). Although investigators have examined the acute effects of growth factors on contraction of SM, few have addressed whether more prolonged exposure that likely occurs *in vivo* modulates agonist-induced force generation.

Our studies to identify intracellular mediators that modulate regulators of G-protein signalling (RGS) molecule identified this molecule as potential candidate that mediates ASM plasticity. RGS molecules act as canonical modulators of GTPase accelerating (GAP) activity and interact with the G-alpha subunits; RGS expression or depletion may define GPCR-mediated contractile outcomes in varied tissues (Hollinger and Hepler, 2002; Sethakorn *et al.*, 2010). Mice deficient in RGS2 develop a hypertensive phenotype that, in part, is reversed with an angiotensin II inhibitor, suggesting that loss of RGS2 increases angiotensin II-induced vasomotor tone (Heximer *et al.*, 2003). In cardiac muscle, RGS4 plays a critical role in regulating the chronotropic actions of Ach. Lack of RGS4 enhances sensitivity to carbachol-induced bradycardia and evokes arrhythmias (Neubig, 2008). Others report that increases in cardiac muscle expression of RGS4 decreases cardiac inotropy promoting heart failure (Owen *et al.*, 2001). More recent studies show that RGS molecules also have GAP-independent functions that mediate cell proliferation by interacting with components of PI3K. Druey *et al.* showed that RGS13 inhibits allergic responses by physically interacting with the regulatory p85α-PI3K subunit of phosphatidylinositol-3-OH kinase in mast cells, thus disrupting its association with an FcεRI-activated scaffolding complex (Bansal *et al.*, 2008). Similarly, RGS16 binding to the amino-terminal SH2 and inter-SH2 domains of p85α-PI3K




## Figure 1

Potential mechanisms involving regulators of G-protein signalling (RGS) proteins as modulators of airway smooth muscle plasticity. Airway smooth muscle (ASM) mitogens including growth factors and distinct contractile agonists mediate expression of RGS isoforms, which interact with (i) p85α subunit of PI3K to sustain AKT activity critical for proliferation, and (ii) Gα subunits of heterodimeric G-protein coupled receptors to attenuate agonist-induced ASM contractile responses.

inhibits interaction with the EGF receptor-associated adapter protein Gab1, altering proliferation. Among other events, newer studies now show that spatial positioning of downstream signal transduction molecules such as Grb2 and PI3K are critical for optimal RTK-mediated proliferation (Fukumoto *et al.*, 2009). In our studies, we showed that the kinetics of PI3K and S6K1 defined the cooperative mitogenic effects of GPCR and RTK activation in ASM cells. Although the agonist- and growth factor-induced amplitude and sustained activation of PI3K and S6K1 at 4 h predicted the magnitude of ASM mitogenesis, the specific mechanism remains elusive (Krymskaya *et al.*, 2000). We now speculate that the early activation of Src, PI3K and ERK1/2 that subsequently activates S6K1, which occurs at 0.5–2 h, increases expression of RGS proteins that then act to sustain activation of PI3K and S6K1 for 4–8 h, signals necessary to promote ASM cell proliferation through the G0/G1 checkpoint of the cell cycle. As illustrated in Figure 1, expression of distinct RGS isoforms and their interactions with PI3K and G-alpha subunits could define critical signalling events that mediate ASM plasticity in severe asthma (Liang *et al.*, 2009).

## Extracellular matrix- and matrix metalloproteinase-mediated airway remodelling

Chronic inflammation may promote airway remodelling manifested by increases in ASM mass, in goblet cell/mucous glands and matrix deposition. Surprisingly, some of these remodelling effects are independent of inflammation and often precede the onset of asthma symptoms (Benayoun *et al.*, 2003). ASM cells can play a role in airway remodelling by producing ECM components and matrix modifying enzymes, matrix metalloproteinases (MMPs). These molecules substantially alter proliferation, migration and contractile responses to mediators. In comparison to normal airways, ECM deposition is altered in individuals with asthma with enhanced deposition of collagens I, III and V, fibronectin, tenascin, hyaluronan, versican and laminin and decreased collagen IV and elastin deposition (Freyer *et al.*, 2001; Burgess *et al.*, 2009). Accordingly, isolated ASM from individuals with asthma produces a distinct array of ECM proteins, while ASM cells from healthy subjects manifested enhanced proliferation when cultured on a matrix provided by the asthma-derived ASM cells. Further, altered ECM deposition surrounding ASM cells may have a physiological impact by modulating airway rigidity and attenuating distensibility. ECM composition can alter contractile protein expression such as SM specific α-actin, myosin heavy chain and calponin in airway myocyte cultures. Studies *in vitro* showed that ECM produced by ASM isolated from individuals with asthma augments IL-13-induced eotaxin release, implying a potential role of ECM components in augmenting eosinophil chemotaxis. ECM can also modulate fibrotic signals by sequestering and influencing the effects of TGF-β, a cytokine whose levels correlate with basement membrane thickness in asthma.

Integral to the remodelling process, ECM components and mediators of inflammation could potentiate the production and activity of matrix modifying MMP enzymes that, in turn, modulate matrix-mediated signalling. Cell and matrix crosstalk occurs via the integrin family of transmembrane receptors, and proteolytic activity of discreet MMPs unmasks integrin-binding sites in the substrate altering cell function (Gueders *et al.*, 2006). While degrading ECM structure, MMPs also facilitate leucocyte migration through the ECM and endothelial cells and affect activation and survival by cleaving cytokines and their cognate receptors (Lagente and Boichot, 2010). Of the 25 mammalian MMPs, ASM-derived collagen modifying MMP-1,19, gelatinases MMP-2,9, stromelysins MMP-3,10, metalloelastase MMP-12 and membrane-bound MMP-14 are regulated transcriptionally and coordinate expression of endogenous tissue inhibitors of metalloproteinases (TIMPs) (McKay and Sharma, 2002; Elshaw *et al.*, 2004; Gueders *et al.*, 2010).

Transcriptional enhancement of MMPs appears to be selective to growth factors in MMP-2 and -9, LTs in MMP-1, endoproteases in MMP-2, and mechanical stress in MMP-1, -2, -3 and -14 (Rajah *et al.*, 1996; 1999; Foda *et al.*, 1999; Hirst, 2003; Xie *et al.*, 2005). Allergen exposure of mice enhances MMP-19, while MMP-19 gene deletion evokes tenascin-C accumulation in peribronchial areas. Accordingly, Th2-associated eosinophilic inflammation and AHR appear to be mediated in a MMP-19-dependent manner where tenascin could mitigate Th2 inflammation in allergic asthma (Gueders *et al.*, 2010). Enhancement of MMP-2 by thrombin or overexpression of MMP-14 also mediate ASM migration (Hasaneen *et al.*, 2005; Henderson *et al.*, 2007). Th2 cytokines including IL-4 and IL-13 induce MMP-1 that, in turn, alters collagen type I matrix and airway contractility (Ohta *et al.*, 2008). MMP-1 activation degrades insulin-like growth factor binding protein, releasing IGF and facilitating ASM proliferation and migration. Others show that mitogens including PDGF, TGF-β or combination up-regulate MMP-1 and MMP-3, and that silencing MMP-3 production decreases migration of ASM cells (Ito *et al.*, 2009). Epithelium is a prominent source of mitogens, cytokines and MMP; disruption of epithelial integrity could enhance MMP secretion affecting ASM migration and growth (Malavia *et al.*, 2009). Similar paracrine interactions exist between mast cells and ASM whereby activation of primary human mast cells by IgE receptor cross-linking stimulates contraction of ASM via expression of MMP-1 and MMP-2 (Margulis *et al.*, 2009). Concomitant and proportional secretion of TIMP-1 inhibits MMP-1, -2, -3 and -9 activity and TIMP-2 selectively complexes and attenuates MMP-2 proteolytic activity, and disruption in these ratios could modulate airway obstruction in asthma (Foda *et al.*, 1999; Johnson, 2001; Elshaw *et al.*, 2004).

## Conclusion

ASM plays a pivotal role in modulating bronchomotor tone and serves as an important therapeutic target for β-agonists in the promotion of bronchodilation. Contemporary thought identifies ASM as an immunomodulatory cell that may orchestrate and perpetuate airway inflammation. The molecular mechanisms that regulate secretion of chemokines and cytokines from structural cells appear to differ from that of trafficking leucocytes, and further studies to identify the critical pathways mediating such processes may identify novel therapeutic targets in the treatment of asthma and COPD.

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## Conflicts of interest

None.

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