Phenotypic and Genetic Aspects of Epithelial Barrier Function in Asthma.

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Source of support : Medical Research Council (MRC) (UK), National Centre for Reduction Refinement and Replacement of Animals in Research (NC3Rs) (UK), Asthma UK, Asthma Allergy and Inflammation Research Charity (AAIR), National Institute for Health Research (NIHR) (UK), Biotechnology and Biological Sciences Research Council (BBSRC).

Running header: The epithelial barrier in asthma

ABSTRACT

The bronchial epithelium is continuously exposed to a multitude of noxious challenges in inhaled air. Cellular contact with most damaging agents is reduced by the action of the mucociliary apparatus and by formation of a physical barrier that controls passage of ions and macromolecules. In conjunction with these defensive barrier functions, immunomodulatory cross talk between the bronchial epithelium and tissue-resident immune cells controls the tissue microenvironment and barrier homeostasis. This is achieved by expression of an array of sensors that detect a wide variety of viral, bacterial, and non-microbial (toxins and irritants) agents resulting in production of many different soluble and cell-surface molecules that signal to cells of the immune system. The ability of the bronchial epithelium to control the balance of inhibitory and activating signals is essential for orchestrating appropriate inflammatory and immune responses and for temporally modulating these responses to limit tissue injury and control the resolution of inflammation during tissue repair. In asthma, abnormalities in many aspects of epithelial barrier function have been identified. We postulate they play a causal role in immune dysregulation in the airways by translating gene-environmental interactions that underpin disease pathogenesis and exacerbation.

Number of words = 189

Key words : asthma, tight junction, innate immunity, cytokine, homeostasis.

Abbreviations:

Adherens junctions (AJs); A Disintegrin and Metalloprotease-33 (ADAM33); aryl hydrocarbon receptor (AhR); bronchial hyperresponsiveness (BHR); cadherin-related family member 3 (CDHR3); dendritic cells (DCs); double stranded (ds); dual oxidase 1 (DUOX1); epidermal growth factor receptor (EGFR); expression quantitative trait loci (eQTLs); extracellular matrix (ECM); forkhead box J1 (FOXJ1); genome-wide association studies (GWAS); glutathione S-transferase (GST); granulocyte-macrophage colony-stimulating factor (GM-CSF); hedgehog interacting protein (HHIP); histone deacetylases (HDACs); innate lymphoid cells (ILCs); intercellular adhesion molecule (ICAM); interferon (IFN); interleukin (IL); interleukin-1 receptor associated kinase M (IRAK-M); major

histocompatibility (MHC); natural killer (NK); NOD-like receptors (NLRs); Orosomucoid like 3 (ORMDL3); pathogen-associated molecular pattern (PAMP); polyaromatic hydrocarbons (PAHs); programmed death-ligand 1 (PD-L1); patched homolog 1(PTCH1); protocadherin 1 (PCDH1); retinoic acid-inducible gene-I-like receptors (RLRs); rhinovirus (RV); single nucleotide polymorphisms (SNPs); sodium-ascorbate cotransporters (SVCT2); signal transducer and activator of transcription (STAT); suppressor of cytokine signalling 1 (SOCS1); tight junctions (TJs); toll-like receptors (TLRs); thymic stromal lymphopoietin (TSLP); transforming growth factor beta (TGF-β); unfolded protein response (UPR);

Asthma heterogeneity

Asthma is a common, chronic inflammatory disorder of the conducting airways which undergo distinct structural and functional changes leading to non-specific bronchial hyperresponsiveness (BHR) and variable airflow obstruction. Recruitment and careful clinical characterization of large cohorts of asthmatic subjects has established beyond doubt that asthma is a heterogeneous disease in terms of phenotype, endotype (ie. underlying pathogenic mechanism), response to treatment and/or long term clinical outcomes[1]. Cluster analysis has enabled identification of 4-5 phenotypic clusters that have differences in gender, asthma onset, lung function, atopic status, asthma control, healthcare utilization and exacerbation frequency[2-5]. Molecular phenotyping of blood, induced sputum and epithelial brushings has identified additional heterogeneity especially in severe asthmatic subjects[6-9] who are a major economic burden on the healthcare system due to poor responses to traditional asthma medications. Some of the differences in asthma clusters may reflect underlying genetic differences: for example, there are differences in genetic risk in early-onset compared with later-onset asthma[10], while others may reflect differences in environment and lifestyle or, perhaps most likely, a combination of both gene and environment effects[11]. Many, but not all, asthmatics have Th2 inflammation in their airways and clinical trials using monoclonal antibodies to interleukin (IL)-5, IL-13, or IL-4 receptor (alpha chain) have identified a Type 2 endotype[12]. Thus, patient stratification using Type-2 relevant biomarkers has enabled effective targeting of these treatments to subsets of moderate and severe asthma[13-17]. However, while clinical trials have shown Type 2 inflammation is an important disease modifier in some patients, they have also highlighted that non-Type 2 inflammatory pathways must contribute to certain forms of asthma[18]. These may include pathways associated with obesity or neutrophilia or with susceptibility to environmental factors such as infection and air pollution, but disease mechanisms/endotypes are not well

understood. We postulate that a dynamic interaction between a genetically susceptible epithelium and environmental risk factors for asthma is important for the development of asthma and its sub-phenotypes[19].

Bronchial epithelial barrier structure and function

Given the multitude of challenges imposed on the airway epithelium, it is not surprising that it combines structural and functional protective mechanisms together with innate immunological mechanisms to maintain healthy barrier homeostasis and to minimize inflammation and cellular dysregulation. Structurally, the bronchial epithelium is pseudostratified, comprising mainly columnar multiciliated cells, secretory (goblet) cells and undifferentiated cells that overlie smaller basal cells that have the capacity for self-renewal[20]. Rare cell types include pulmonary neuroendocrine cells[21;22] and brush (tuft) cells[23] that may have neurosensory or chemosensory functions but information on these cells is limited.

On the epithelial surface, the mucociliary apparatus is a crucial primary innate defence mechanism that protects the lungs from deleterious effects of inhaled pollutants, allergens, and pathogens. Surface epithelial cells and submucosal glands produce secretions comprising a superficial gel or mucous layer and a layer of periciliary fluid that contacts the surface of the epithelium. Mucus contains hydrated gel-forming mucins and a range of host defence and cytoprotective molecules, including defensins, IgA, lactoperoxidase, catalase, superoxide dismutase and low molecular weight antioxidants[24]. The viscoelastic properties of the mucus are dictated in large part by the oligomeric secreted mucins MUC5AC and MUC5B[25], multifunctional glycoproteins that provide the structural framework of the mucous barrier. These bronchial secretions shield the epithelial surface, detoxify noxious agents and trap

many inhaled particles allowing clearance by the action of the mucociliary escalator. MUC5B may also contribute to immune homeostasis by direct regulation of leukocyte functions[26;27].

In addition to secreting mucus, the bronchial epithelium forms a sheet-like structure that acts as a physical barrier to protect the internal milieu of the tissue. Individual epithelial cells contact each other through a range of cell-cell adhesion complexes (tight junctions (TJs), adherens junctions (AJs) and desmosomes) that control the permeability of the epithelial sheet and link with the cytoskeleton to resist mechanical stress (Figure 1); in addition, gap junctions directly connect the cytoplasm of adjacent cells allowing cell-cell communication[28-30]. The apical-most adhesive complexes are the TJs formed by transmembrane and intracellular proteins that link to the actin cytoskeleton[31] (Figure 1B). TJs seal the epithelium, regulating paracellular passage of ions, water, and various macromolecules. They also maintain cell polarity by preventing lateral diffusion and intermixing of molecules in the apical membrane with those in the lateral membrane. Proteins of the TJ include tricellulin and occludin that regulate the passage of macromolecules through the TJ[32] and claudins which are responsible for the size- and charge-selective conductance properties of the TJ paracellular pathway[33]. Expression of 'barrier' or 'sealing' claudins that selectively decrease paracellular cation permeability has been reported in normal human adult lung (claudin-1, -3, -4, -5 -7 and -18)[34] and the expression profile varies with anatomical location and function[35;36]. Claudin-2, a 'pore-forming' claudin is also detected in the lung and its presence is thought to increase ionic permeability by acting as a cation selective pore[36].

Located below the TJs are the AJs that link to the actin cytoskeleton[37;38], desmosomes that link to the intermediate filaments[39] and hemidesmosomes[40] containing $\alpha_6\beta_4$ integrins that facilitate attachment to the basement membrane (Figure 1A). AJs and desmosomes are critical for providing the adhesive force to ensure the integrity of the cell layer. Cadherin-catenin complexes comprise the core of the AJ, bridging neighbouring cells and the actin-

myosin cytoskeleton, contributing to mechanical coupling between cells. In addition to its adhesive function, E-cadherin physically interacts with several receptor tyrosine kinases and impacts their signalling abilities. Similarly, β-catenin which is an integral structural component of AJs, is also the key nuclear effector of canonical Wnt signalling in the nucleus[41]. This coupling of cell-cell adhesion with signalling functions, ensures that AJs can be extremely plastic allowing the cell to adapt rapidly to its changing environment. Like AJs, the TJ plaque also contains many signalling molecules[42;43], allowing proteins in involved in cell-cell and cell-matrix adhesion to integrate and co-ordinate epithelial responses[44]. Perturbation in the turnover and concentration of junctional proteins is therefore likely to have important implications for the maintenance and stability of the epithelium and the permeability barrier.

Junctional adhesion molecules also serve as sites for interaction of the epithelium with cells involved in immune surveillance. For example, TJ proteins interact directly with dendritic cells (DCs) to allow them to sample the airway lumen without disruption of the epithelial barrier[45;46] while E-cadherin is a ligand for $\alpha_E\beta_7$ integrin (CD103) expressed T cells[47;48] and DCs[49]. In addition to structural adhesion molecules, the bronchial epithelium expresses inducible adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and -2 which have essential functions in the clearance of T cells from the lung during resolution of inflammation[50].

Airway epithelial cells express an array of pattern-recognition receptors (PRRs) including toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene-I-like receptors (RLRs), and a variety of natural killer (NK) cell receptor ligands. These enable detection of a wide variety of microbial and non-microbial agents resulting in production of many different soluble and cell-surface molecules, collectively termed the "epimmunome"[51] (cytokines, chemokines, damage-associated molecular pattern (DAMP) molecules, and major

159 histocompatibility (MHC) gene products) that recruit and activate cells such as macrophages
160 and neutrophils involved in inflammation and the induction of adaptive immunity. Together
161 these responses enable many infections to be controlled by the immune system with limited
162 damage to host tissues, however it is important to note that both innate and adaptive immune
163 signaling events are involved in mediating tissue damage[52]. For example, macrophages,
164 neutrophils and eosinophils release a range of molecules, including cytotoxic cytokines,
165 cationic proteins, lipid mediators, metalloproteinases and reactive oxygen species that induce
166 tissue damage or malfunction. Therefore, the ability of the epithelium to control the balance
167 of inhibitory and activating signals is essential not only for initiating an appropriate immune
168 response to environmental challenges, if required (Figure 2), but also for temporally
169 orchestrating these responses to limit tissue injury and control the resolution of inflammatory
170 reactions via cell surface molecules and release of inhibitory cytokines and lipids during
171 tissue repair.

172 *In vitro* and *in vivo* studies have shown that epithelial cells can modulate a variety of immune
173 cells. For example, epithelial derived transforming growth factor (TGF)-β is chemoactive for
174 innate lymphoid cells (ILCs)[53] which may provide early defences against pathogens and
175 intervene in the repair of damaged tissues. TGF-β secreted by bronchial epithelial cells has a
176 direct inhibitory effect on T lymphocyte proliferation and epithelial cell-conditioned T
177 lymphocytes show increased differentiation towards IL-10-producing Tr1 cells[54]. Epithelial
178 cell secretions also inhibit proinflammatory responses of monocytes, macrophages and
179 dendritic cells, increase dendritic cell expression of the negative regulatory programmed
180 death-ligand 1 (PD-L1, CD274), decrease the ability of dendritic cells to induce T
181 lymphocyte proliferation[54] and suppress human lung mast cell histamine secretion[55].
182 Epithelial cells express CD200 which binds to the inhibitory immune receptor, CD200R,
183 expressed at high levels on lung macrophages. This not only maintains a strong threshold for

response in the context of inhaled, but non-pathogenic antigens[56] but also dampens macrophage responses in the context of infection. Thus, in CD200 knock out mice there is increased macrophage activity and severe immune-mediated lung damage following influenza infection[57]. The activation status of NK cells is also controlled by the balance of various inhibitory and activation receptors[58;59]. For example, the NK cell activating receptor, NKG2D, is ligated by molecules such as MHC class I polypeptide-related sequences A and B or UL16-binding proteins which are only expressed on stressed airway epithelial cells[60;61], resulting in the killing of the target cells, ultimately leading to protection from infection. The importance of NK cells and NKG2D in allergic airways responses has been suggested by the findings that mice that lack NKG2D are resistant to induction of allergic inflammation; while adoptive transfer of wild-type NK cells was able to restore the response, granzyme B deficient NK cells could not[62].

One common link between both infectious and non-infectious triggers of Type 2 immunity is that many induce some level of physical trauma that breaches the protective barrier of the body. Tissue damage, at least in the absence of strong type 1-promoting pathogen-associated molecular pattern (PAMP) signaling, appears to be a potent mechanism driving Type 2 immunity. This involves rapid release of several epithelium-derived cytokine alarmins, such as IL-1, IL-33, thymic stromal lymphopoietin (TSLP), and IL-25, all of which can drive downstream Type 2 immunity[63]. These cytokines invoke an immune response, involving mast cells, basophils, eosinophils, type 2 innate lymphoid cells (ILC2s) and alternatively activated macrophages that has evolved to respond to a parasitic infection by generating pro-inflammatory mediators, toxin-neutralizing enzymes, and helminth-killing toxins, that also have endogenous tissue damaging properties. A number of studies have identified many environmental agents linked to asthma that have the potential to cause epithelial barrier disruption and tissue injury in the airways including the house dust mite allergen Der p 1[64],

fungal allergens[65], rhinovirus[66], cigarette smoke[67;68] and air pollutants[69;70]. Nonetheless, a key question arising from these observations is: '*Why are the airways of asthmatic subjects more susceptible than normal to these relatively ubiquitous agents*?' As detailed below, it is likely that the explanation lies in a combination of (i) decreased epithelial barrier defences lowering the threshold for epithelial damage, (ii) dysregulated innate immune or immunoregulatory responses that contribute to ongoing barrier dysfunction and (iii) impaired epithelial barrier repair leading to failure to resolve inflammatory responses.

Dysregulation of the Epithelial Barrier in Asthma

Targeted studies of the bronchial epithelium have demonstrated a range of abnormalities at many levels of barrier function and innate immunity (Figure 3). However, unbiased transcriptomic approaches are now enabling in-depth analysis of epithelial gene expression profiles[8;9] to provide evidence of molecular mechanisms that may eventually define specific epithelial endotypes of asthma. We will first summarise key abnormalities identified in the epithelial barrier in asthma and then put these into the context of the newer clusters that have been identified and how these relate to genetic susceptibilities.

The mucociliary apparatus is modified in asthma as evidenced by an increase the number of goblet cells with increased mucin gene expression, an increase in MUC5AC protein relative to MUC5B and a reduction in ciliated cell number[71-73]. In addition, decreased ciliary beat frequency, dyskinesia, and ciliary disorientation have been reported in severe asthma[74]. Together, mucous hypersecretion and ciliary dysfunction in asthma may result in stimulation of neural receptors that result in cough[75] and mucous plugging which, over time, can lead to severe airflow obstruction.

The increase in MUC5AC relative to MUC5B seen in asthma has been postulated to affect mucous clearance, reduce eosinophil apoptosis[76] and/or contribute to abnormal innate immune responses[57]. Reprogramming of epithelial differentiation towards a hypersecretory phenotype has been linked to increased expression of the epidermal growth factor receptor [72], and to the activity of Th2 cytokines including IL-13 and IL-9[77;78]. Consistent with this, the 'Th2 high' asthmatics have significantly increased airway mucin gene expression[79]. Th2 cytokines also significantly decrease epithelial expression of the antimicrobial peptide, human beta-defensin 2 *in vitro* and mice with allergic airway inflammation have significantly more viable bacteria in their lungs after infection[80]. In contrast, atopic asthmatic subjects with Type 2-high asthma have been reported to harbor significantly lower bronchial bacterial burden[81] and, in severe asthma, no taxa were associated with a Th2-related epithelial gene expression signature[82]. These differences may reflect long-term changes and treatment effects and contrast with the acute responses seen after infection of mice with allergic airways inflammation[80].

There is considerable evidence for an association between levels of particulate pollutants and asthma exacerbations[83-85], asthma pathogenesis and poorer lung function outcomes[86-88]. Exposure to air pollutants can lead to oxidative stress in the airways and there is compelling evidence that asthmatic airways are deficient in antioxidant defences[89]. Furthermore, the antioxidant capacity of the lungs is inversely related to asthma severity[90]. In addition to lower levels of superoxide dismutase and catalase[89], it has recently been shown that goblet cells express the high affinity, sodium-ascorbate cotransporters (SVCT2) which is involved in vitamin C uptake into cells and that expression of SVCT2 is inversely related to lung lining fluid vitamin C levels[91]. There is also considerable evidence that polymorphisms in glutathione cycling enzymes may result in increased susceptibility to air pollution[92-94]. Glutathione S-transferase (GST)-pi is predominantly expressed in airway epithelial cells, and

expression is decreased in the airways of children with asthma[95]. In view of the increased susceptibility of the asthmatic bronchial epithelium to oxidant-induced apoptosis *in vitro*[96], and the observation that increased levels of oxidants can reduce the anti-inflammatory effects of budesonide, an inability to control oxidative stress may not only drive epithelial damage, but also confound treatment responses[97].

Polyaromatic hydrocarbons (PAHs) are a key toxic component of air pollution. PAHs are raised in the plasma of asthmatic children and linked to a number of markers of asthma[98]. The aryl hydrocarbon receptor (AhR) which plays a key role in the detoxification of environmental pollutants also regulates multiciliogenesis[99]. Importantly, whereas air exposure triggers AhR targeting of genes important for multiciliogenesis, toxic AhR ligands induce detoxifying cytochromes, with no overlap in target gene induction. These mutually exclusive responses suggest a potential pathophysiological mechanism whereby AhR ligands in air pollutants disrupt AhR-mediated ciliogenesis to contribute to disruption of barrier defences in asthma[99].

Epithelial fragility[100] and epithelial shedding[101] in asthma have been recognized for many years, but this remains a controversial area[102]. Nonetheless, through use of specific markers of response to injury such as increased expression of the epidermal growth factor receptor (EGFR), epithelial damage has been confirmed in bronchial biopsies from asthmatic adults[103] and children[104]. Many studies have reported disruption of adhesive mechanisms in asthma including loss of tight junction proteins[67;105;106], reduction in adherens junction proteins[105] and a reduction in desmosome length[107]. The membrane expression of caveolin-1, a stabilizer of AJs is significantly lower in airway epithelia of asthmatic subjects and, *in vitro*, loss of caveolin-1 causes loss of junctional E-cadherin and β-catenin expression and disrupted epithelial barrier function[108]. Consistent with reduced adhesion, functional studies comparing epithelial cultures from asthmatic or normal donors indicate that there is increased

permeability and sensitivity to environmental stressors in asthma[67] and increased susceptibility to oxidant stress[96]. Increased barrier permeability may not only promote allergic sensitization, but also reduce the threshold for epithelial damage and activation of a Type 2 response which itself may affect barrier function. Thus, in addition to their effects on goblet cell differentiation, Th2 cytokines have a disruptive effect on epithelial barrier function[109] and lead to a distinct profile of epithelial gene expression, both *in vitro* and in Th2-high asthmatic subjects *in vivo*[79]. Claudin-18, a lung specific 'barrier' claudin has been shown to be expressed in bronchial epithelium and is reduced in asthma, being lowest in Th2-high asthmatics[106]. In the same studies, IL-13 down-regulated claudin-18 *in vitro* and targeted knock-down of claudin-18 increased epithelial permeability. Furthermore, claudin-18 null mice had significantly higher serum IgE levels and increased airway responsiveness following intranasal aspergillus sensitization suggesting loss of claudin-18 may promote sensitization and airway hyperresponsiveness[106]. As mast cells are important sources of IL-13 and are in close proximity to the bronchial epithelium in asthma[110], it is noteworthy that IL-33 activated mast cells, as well as ILC2s, are able to drive a predominantly IL-13-regulated pattern of gene expression in normal human bronchial epithelial cells *in vitro*[111]. Furthermore, ILC2s have been shown to directly impair epithelial barrier integrity via IL-13[112] whereas T_H2 cells cause barrier leakiness via IL-4 and IL-13, an effect that can be prevented by inhibition of histone deacetylases (HDACs)[113].

Consistent with the evidence of epithelial disruption in asthma, epithelium-derived cytokine alarmins, such as IL-33, TSLP, and IL-25 are increased in asthma[114;115]. IL-33, a member of the IL-1 cytokine family has gained prominence in Type 2 immunity by virtue of the genetic association of both *IL33* and its receptor, *IL1RL1* (ST2), with asthma[10;116] and by its functional effects on ILC2 cells, Th2 cells, mast cells, basophils and alternatively activated macrophages[117]. IL-33 is normally localized in the nucleus where it is a transcriptional

regulator[118] and can act as an extracellular cytokine by binding to its receptor, ST2[119]. Full length IL-33 binds ST2 and is biologically active, although activity can be increased after cleavage by inflammatory proteases[120], whereas caspase cleavage leads to inactivation[121]. IL-33 can be released by non-programmed cell death, or it can be actively secreted via vesicular transport from the Golgi complex[122]. Stimulation of bronchial epithelial cells with allergen or ATP results in active release of IL-33 which depends on the NADPH oxidase dual oxidase 1 (DUOX1)-mediated activation of src and EGFR signalling through cysteine oxidation[123]. Nasal epithelial cells from asthmatic subjects display enhanced DUOX1 expression, as well as allergen-induced IL-33 secretion compared with healthy controls, suggesting that increased expression and activation of DUOX1 might be an important feature of enhanced IL-33 secretion in asthma[123]. In addition to full length IL-33, alternative splicing of the IL-33 transcript can result in deletion of exons 3 and 4 (Δ exon 3,4) to confer cytoplasmic localization and facilitate extracellular secretion without cell death, while retaining signaling capacity. Analyses of epithelial brush RNA suggest that Δ exon 3,4 is strongly associated with airway Type 2 inflammation, whereas full-length IL33 is not[124]. These results suggest that therapeutic IL-33 inhibitors will need to block all biologically active isoforms.

TSLP is an interleukin 7-like cytokine that can trigger dendritic cell-mediated Th2 inflammatory responses[125] and Th2 cytokine production by mast cells[126]. A variety of stimuli including double stranded (ds)RNA and allergens stimulate TSLP expression in bronchial epithelial cells and this is enhanced by inflammatory cytokines[127]. Challenge of cultured epithelial cells from asthmatic donors with dsRNA results in a skewed response favoring more TSLP and less Type 1 interferon compared with healthy cells[128]. Allergen-specific T cells also enhance TSLP production by epithelial cells from asthmatic donors, suggesting T cell-airway epithelium interactions that may lead to maintenance and amplification of allergic

inflammation[129]. In a double blind, placebo-controlled study, treatment using a human monoclonal antibody to TSLP resolved airway inflammation and attenuated allergen-induced bronchoconstriction, findings consistent with TSLP as a therapeutic target in allergic asthma[130]. However, in addition to its effects on immune cells, it is noteworthy that TSLP drives an IL-13 dependent increase in bronchial epithelial cell proliferation[131] and increases TJ expression to enhance nasal epithelial barrier function suggesting a role for TSLP in restoration of epithelial barrier integrity[132]. In contrast, TSLP has been reported to disrupt TJs in 16HBE bronchial epithelial cells[133]. Furthermore, a short, constitutively-expressed form of TSLP (sfTSLP) has been detected in skin and gut; this variant cannot activate signal transducer and activator of transcription (STAT)5, but has potent antimicrobial activity[134]. Recent studies suggest that sfTSLP can protect against bronchial epithelial barrier disruption *in vitro* and house dust mite- or toluene diisocyanate-induced airways inflammation in vivo[133;135]. Consequently, optimal therapeutic antibody targeting may need to be directed specifically to the long form of TSLP.

IL-25 belongs to the IL-17 cytokine family and is secreted by Th2 cells, mast cells, basophils and eosinophils, as well as epithelial cells[136]. It can drive airway remodelling in allergic models of airway inflammation[137], and in combination with IL-33, it can promote the development of Type 2 ILCs that appear critical in the early initiation of the Th2 response[138]. Expression of IL-25 has been reported to be increased in epithelial cells from subjects with asthma, and can be induced further by rhinovirus infections[139]. Others have found increased systemic levels of IL-25 in subgroups of patients with asthma with Th2 high asthma[140]. Furthermore, the IL-25 receptor (IL-17RB) is upregulated on myeloid and plasmacytoid dendritic cells in blood and sputum 24 hours after allergen challenge[141]. IL-25 up-regulated TLR9 expression by plasmacytoid (p)DCs and orchestrated the responses to TLR9 ligation, suggesting that IL-25 may act as a link between adaptive and innate immune responses[141].

Respiratory viral infections, especially rhinovirus (RV) infection are the main triggers of asthma exacerbations[142;143]. Several[144-146], but not all[147;148], studies have shown the bronchial epithelial cells from asthmatic donors respond abnormally to RV infection involving an insufficiency of interferon (IFN)-β and -λ. This has been linked to increased $TGF\beta_2$ production by asthmatic epithelial cells[149] and suppressor of cytokine signaling (SOCS1) expression[150], however it is also of interest that RV-induced EGFR activation can suppress IFN-λ production and increase viral infection[151]. The importance of decreased anti-viral immunity in asthma has been tested in a clinical trial using inhaled interferon-beta: the drug was found to improve asthma control and reduce exacerbations in difficult-to-treat asthmatics[152].

It is well known that mechanical forces are critical to lung development and that abnormal mechanical stresses can lead to pathological lung injury[153] In asthma, constriction of the bronchial smooth muscle during an acute asthma attack causes the airway wall to buckle resulting in folding and compression of the bronchial epithelium[153]. *In vitro* studies have shown that airway epithelial cells respond rapidly and robustly to compressive stress with changes in goblet cell numbers and production of profibrogenic growth factors[154;155]. The relevance of these findings has been demonstrated *in vivo*, where induction of bronchoconstriction using methacholine caused airway remodelling involving goblet cell metaplasia and sub-epithelial fibrosis without evidence of inflammation[156]. While these changes may simply be due to the hyper-responsive properties of the bronchial smooth muscle in asthma, there is evidence bronchial epithelial cells from asthmatic donors respond abnormally to compression, with increased release of TGFβ and granulocyte-macrophage colony-stimulating factor (GM-CSF)[157], suggesting that bronchoconstriction may skew epithelial innate immune responses in asthma. Since the asthma susceptibility gene, *A Disintegrin and Metalloprotease-33* (*ADAM33)*, has been linked to BHR[158] and has been

shown to cause bronchial smooth muscle contraction[159], there is potential for multifactorial, indirect genetic effects on epithelial barrier function.

Increased expression of the EGFR in bronchial biopsies from asthmatic adults[103] and children[104] is consistent with an ongoing response to injury and this is highly correlated with epithelial IL-8 expression[160]. However, expression of the cyclin dependent kinase inhibitor, $p21^{waf}$ [104;161] may be indicative of impaired proliferation or ongoing epithelial stress in asthma. During epithelial repair, neighbouring epithelial cells become migratory in response to growth factors such as TGF-β or EGF. This 'repair' phenotype is characterized by down regulation of TJs and increased expression of matrix metalloproteases and extracellular matrix (ECM) components, as observed in asthma. Studies using cultures of epithelial cells from asthmatic children, suggest that the airway epithelium displays a dysregulated repair response taking longer to repair mechanically induced wounds[162] and undergoing a more extensive epithelial-mesenchymal transition in response to TGF-β than cultures from non-asthmatic donors[163]. It has recently been reported that IL-22 can promote a repair phenotype in the presence of TGF-β_1, causing a marked reduction in E-cadherin, but only in cells obtained from severe asthmatic donors[164].

Epithelial clusters and asthma heterogeneity

The use of large scale transcriptomic approaches in large cohorts of well characterised asthmatic and healthy control volunteers has enabled unbiased, in-depth analysis of gene expression profiles in epithelial brushings and allowed clustering into distinct phenotypes. Analysis of transcriptomic data from 155 donors in combination with exhaled nitric oxide has identified five molecularly defined and clinically distinct subject clusters (SCs) with distinct expression of gene clusters (GCs)[8], summarized in Figure 4. The majority (73%) of all healthy controls were located in SC1 which was distinguished by high expression of GCs

involved in processes including ‘innate immunity/antibacterial function’ and ‘Notch signalling’ and low expression of genes clusters including ‘interferons/stress’ and ‘Type 2 immunity’. In contrast, the largest group of severe asthmatics (SC2) showed a diametrically opposite pattern with low expression of both ‘innate immunity/antibacterial function’ and ‘Notch signalling’ GCs and high expression of ‘interferon/stress’ and ‘Type 2 immunity’ GCs. In addition, ‘cilia structure and function’ was low in the severe asthma SC2. It is interesting to note an apparent paradox that gene signatures for both cilia-related gene and Notch signalling are reduced in SC2. As Notch signaling inhibits ciliated cell differentiation *in vitro* by repressing multicilin and forkhead box J1 (FOXJ1)[165], low levels of Notch might suggest increased ciliogenesis, but this was not the case. However, it has been shown that IL-13 inhibits ciliated cell differentiation independent of Notch signalling[166] suggesting two distinct signaling pathways can affect ciliated cell differentiation which may be of relevance in the different subject clusters of severe asthma. The other subject clusters showed some overlap with SC2, but each exhibited distinct profiles illustrating the heterogeneity of the epithelial gene signature across the spectrum of asthma severity. Further analysis of the same data using weighted gene co-expression network analysis highlighted that genes in modules linked to epithelial growth and repair and neuronal function were markedly decreased in severe asthma[9]. Of particular note, low expression of epithelial growth and repair and neuronal function genes was more strongly associated with severe asthma than Type 2 inflammation, suggesting that epithelial integrity and related processes are of primary importance to the development of asthma and severe asthma.

Assuming that these phenotypes are stable, rather than fluctuations due to disease activity, these data illustrate the complexity of the epithelial phenotype. Reinforcement of these findings with longitudinal studies should provide a basis for hypothesis-driven research that allows precise definition of epithelial endotypes in asthma. Nonetheless, based on the

evidence to date, further consideration of strategies that promote epithelial repair and restore epithelial homeostasis may provide novel therapeutic approaches for treatment of asthma[24]. For example, the protective effects of growth factors such as EGF have been recognized for many years (reviewed in [24]). However, novel strategies include potential use of the macrolide antibiotic azithromycin which has been shown to decrease ionic permeability of human airway epithelia by changing the processing of tight junction proteins[167] or HDAC inhibition using JNJ-26481585 which has been shown to ameliorate the effects of T_H2 cells on barrier function[113].

From asthma genes to function

Genome-wide association studies (GWAS) of asthma have identified novel risk alleles and loci, with many of the asthma susceptibility genes being expressed in the airway epithelium[168]. Among susceptibility factors for asthma, the genes for *IL1RL1/IL18R1*, *IL-33*, and *TSLP* have emerged as some of the most important associations for the development of the disease[10], linking epithelial-derived cytokines to Type 2 inflammation. Furthermore, a number of genes associated with epithelial homeostasis, differentiation or barrier immunity have been identified including *PCDH1*[169], *CDHR3*[170], *HLA-DQ*[10], *SPINK5*[171], G*PRA*[172], and *ORMDL3/GSDMB*[10] at the 17q12-21 locus. However, it should be noted that asthma-associated alleles have small effect sizes and account for little of the prevalence of asthma and it is likely that a significant portion of the genetic risk for asthma and its exacerbations results from genotype-specific responses to environmental exposures including allergens, pollution and viral infections, especially at particular stages of life[173-176;177]. Here, we have attempted to place some of the asthma susceptibility genes into the context of epithelial barrier dysregulation, with a view to highlighting potential epithelial endotypes of disease linked to reduced barrier defences, dysregulated immune responses and/or abnormal repair responses (Figure 5).

Epidemiological and genetic evidence have implicated epithelial susceptibility to environmental insults in asthma pathogenesis. However, clear functional relationships are not always easy to identify, perhaps reflecting the need for assessment in the context of an appropriate environmental trigger. For example, while two common deletion polymorphisms of the glutathione S-transferase genes *GSTM1* and *GSTT1* and the *GSTP1* Ile105Val polymorphism have been associated with asthma in children and adults, a meta-analysis has revealed extreme between-study heterogeneity[178] suggesting more focussed study in the context of environmental oxidative exposures would be more informative.

Genes such as the cadherin family members, *CDHR3* [170] and *PCDH1*[169] appear to play roles in adhesion. Several single nucleotide polymorphisms (SNPs) in *PCDH1* have been linked to asthma and BHR. These include Ala750Ala and IVS3_116 which are localized in the 3′UTR of exon 3 and may affect mRNA stability or splicing, whereas Ala514Thr is localized in the fifth cadherin repeat of the extracellular domain and may affect cell–cell adhesion[169]; however the functional consequences of this mutation has not been explored. Protocadherin 1 (PCDH1) co-localises with E-cadherin in airway epithelial cells and it has been implicated in the barrier enhancing properties of glucocorticoids[179] and the suppression of TGFβ[180] signalling. Since gene-by-passive-smoking interactions have been found to be relevant for the association of *PCDH1* with asthma[169;181], the contribution of *PCDH1* gene variants to asthma may only become evident in the context of smoke exposure[182]. *CDHR3* was originally identified as an asthma susceptibility gene linked to childhood exacerbation[170]. The asthma associated SNP (rs6967330) causes a non-synonymous mutation (G>A; C529Y) in the fifth cadherin repeat of cadherin-related family member 3 (CDHR3) which affects cellular localization[170]. Subsequent studies showed that CDHR3 is a receptor for Rhinovirus C (RVC), suggesting that the increased localization of Y529 CDHR3 on the bronchial epithelial

cell surface increases susceptibility for RVC infection and replication[183]. However, the normal cellular function of CDHR3 is still unknown.

Orosomucoid like 3 (*ORMDL3*) has been shown to be associated with early-onset asthma susceptibility in multiple independent genome-wide and candidate-gene association studies[173]. It is regulated by STAT6 and can be induced by IL-13 or IL-4[184] and SNPs in *ORMDL3* correlate with changes in Th2 cytokine levels[185]. ORMDL3 is found in the endoplasmic reticulum, and is involved in maintaining sphingolipid homeostasis and in the unfolded protein response (UPR)[186], but *in vitro* studies involving under or over-expression of ORMDL3 failed to show a significant role in modulating innate immune responses and the UPR[187]. However, in mice, overexpression of ORMDL3 decreases serum sphingolipids and increases inflammatory markers, airway remodeling and BHR in response to allergic stimuli[188]. Furthermore, pulmonary epithelial expression of ORMDL3 is sufficient for induction of *Alternaria*-induced allergic airways disease[189].

As already described, polymorphism in genes including *IL-33, IL1RL1* and *TSLP* have been linked with epithelial activation/damage and Type 2 immunity, although detailed studies are still revealing new levels of complexity involving alternative splicing[124]. In the case of TSLP, multiple SNPs are correlated with the expression levels of TSLP and some alleles are protective[190]. Of note, in subjects with one or more SPINK5 risk alleles, the absence of the TSLP protective minor alleles has been associated with a significant increase in asthma[191]. Thus, in addition to gene-environment effects, epistasis adds another level of complexity to asthma pathogenesis. Other immune regulators may be relevant to exacerbation prone asthma: these include Suppressors of cytokine signalling 1 (*SOCS1*)[192] and interleukin-1 receptor associated kinase M *(IRAK-M)*[193], both of which suppress IFN-β signalling and anti-viral responses[150;194].

The focus on epithelial repair genes in asthma has been limited to date, but promoter variants in *TGFB1* and *TGFB2* that increase TGFβ expression are associated with asthma[195;196] and airflow obstruction[197]. It is also interesting to note that genes like *HHIP* (hedgehog interacting protein) and *PTCH1* (patched homolog 1), that may play a role in epithelial repair have been identified through genetic association with reduced lung function[198], suggesting that impaired repair may drive ECM deposition and tissue remodelling.

Most of the asthma-associated SNPs identified by GWAS are not coding-change variants. Therefore, expression quantitative trait loci (eQTLs) analysis has been adopted to identify functional SNPs regulating expression levels of disease-associated genes in a cell-type specific fashion. Applying this analysis to bronchial epithelial cells has revealed SNPs in *TSLP*, *GSDMB*, *IL33*, *HLA-DQB1*, *C11orf30*, *DEXI*, *CDHR3*, and *ZBTB10* that affect asthma risk by allowing *cis*-regulation of its gene expression in an epithelial specific manner[190]. In the case of *IL-33*, all asthma-associated SNPs in this region of the genome are located in the 5′ or first intron of *IL33*, and eQTL analysis has revealed SNPs in the promoter region of *IL33* are correlated with IL-33 expression in bronchial epithelial cells. The same study identified an eQTL SNP for *CDHR3* (rs17152490) in bronchial epithelial cells which is in linkage disequilibrium (LD) with the GWAS SNP (rs6967330, G>A; C529Y) suggesting cis-regulation of *CDHR3* expression may also contribute to the asthma risk. SNPs in *PTTG1IP* (pituitary tumour-transforming 1 interacting protein) and *MAML3* (Mastermind-like 3) have been reported to be associated with BHR severity in adult asthma[199] and eQTL analyses indicate higher tissue expression with less severe BHR. These gene products may be particularly relevant to epithelial repair as PTTG1lP is co-expressed with vimentin and E-cadherin1, while MAML3 is co-expressed with MAML2 both involved in Notch signaling, a repair pathway that was deficient in the transcriptomic studies of severe asthma.

Concluding comments

Taken together, the evidence for epithelial dysregulation in asthma is compelling. Genomic studies have revealed the extent of epithelial heterogeneity in asthma and have provided considerable insight into expression profiles, pathways and processes that may drive epithelial dysfunction. Further understanding of asthma endotypes will come from integration of findings from these large datasets with the function and regulation of asthma genes and how these are modified by interaction with environmental factors, including the airway microbiome. However, the stability of the asthma phenotypes identified in molecular studies still needs to be addressed in longitudinal studies. In addition, the appreciation that changes in gene expression are also evident in epithelial cells harvested from peripheral airways of severe asthmatic subjects raises new questions about gene dysregulation in the smaller airways, which comprise the majority of the airway surface area and the need for better-targeted therapies for the peripheral airways[200]. Furthermore, there is a lack of critical information about epithelial heterogeneity and its role in childhood asthma. Crucially, we still lack detailed information about the functions of many asthma genes and how genetic polymorphism of these genes drives asthma susceptibility. The high costs of transgenic and gene-deletion mouse models has restricted progress in this area. Thus, it would be timely to investigate the potential of non-mammalian models such as *Drosophila* or zebrafish as tools to investigate gene function where the genetic tractability and low cost of rearing these organisms are major advantages[201;202]. Better understanding of epithelial dysfunction and its inter-relationship with airways inflammation and structural remodeling should help to define specific epithelial endotypes in asthma. Through development and use of therapeutic approaches that restore epithelial barrier homeostasis, it may be possible to prevent, or modify, the disease course by intervening close to the origin of the disease.

554 **References**

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Figure legends

Figure 1: A. Schematic representation of a pseudostratified bronchial epithelial cell layer (comprising a goblet cell, two ciliated cells and two basal cells) showing the junctional complexes and their interactions with the cytoskeleton or basement membrane to form a robust sheet-like structure. B. Illustration of the tight junction and adherens junction complexes showing how they mediate cell-cell contact and interact with the actin cytoskeleton. JAM= Junctional adhesion molecule, ZO – zonula occludens; p120, α, β, γ are all isoforms of catenin.

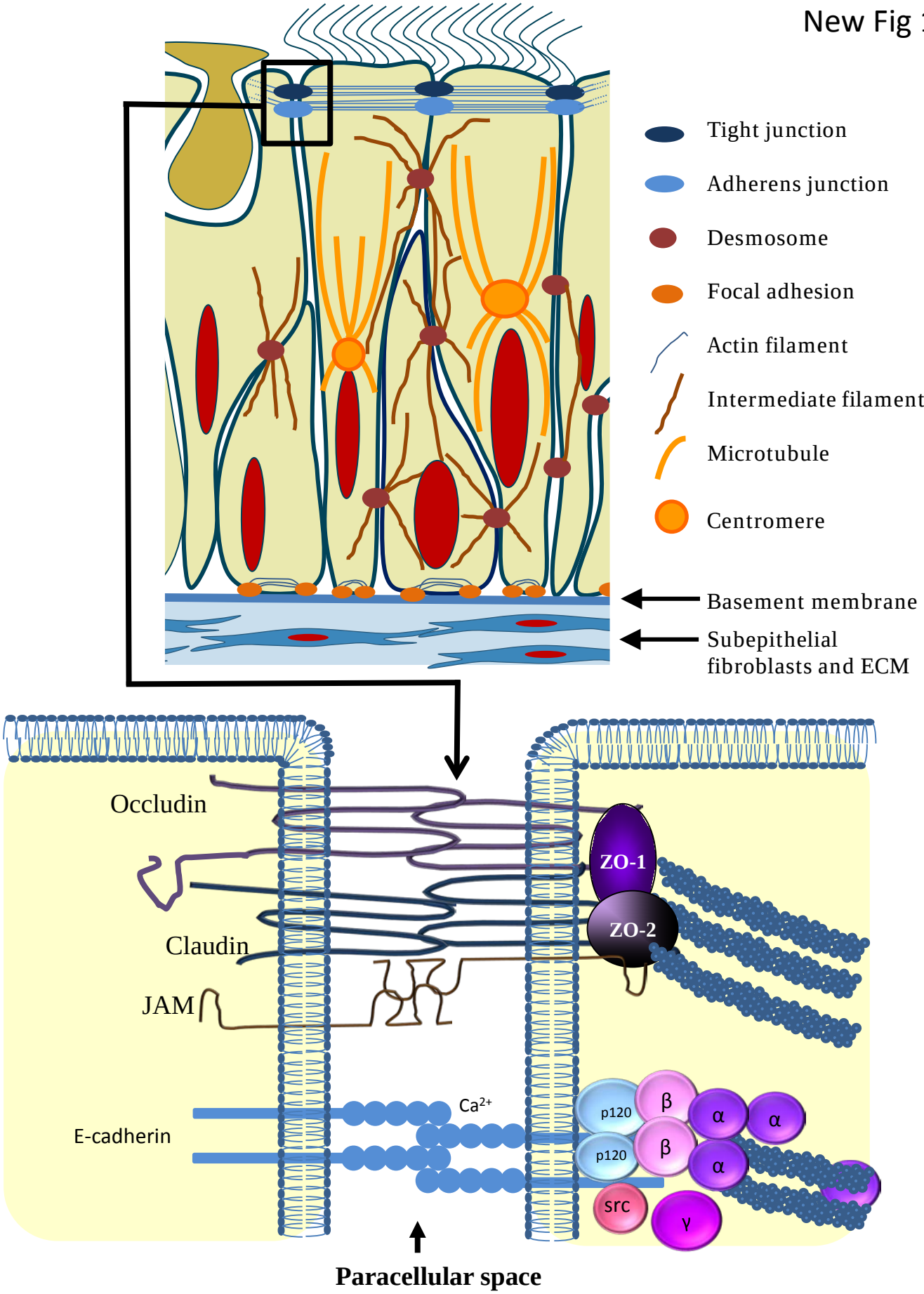
Figure 2: Schematic representation of epithelial barrier function illustrating protective and immune regulatory functions. Under basal conditions, the epithelium maintains homeostasis by limiting exposure of the airway tissue to components of the inhaled environment and by balancing immune regulatory signals. However, when compromised, the epithelium responds by releasing innate cytokines that help to orchestrate appropriate innate and adaptive immune responses. PM = particulate matter, $O^{\cdot}$ = oxygen radicals, NO_x = nitrogen oxides, CS= cigarette smoke.

Figure 3: Schematic representation of the epithelial barrier in asthma highlighting abnormalities in protective and immune regulatory functions (grey boxes). Persistent airway inflammation most likely arises as a consequence of impaired barrier defences (altered cytoprotective secretions and reduced cell-cell adhesion) leading to epithelial susceptibility to injury and dysregulated immune responses. In parallel, impaired repair may contribute to maintenance of epithelial activation and chronicity of responses. The relative contribution of each aspect of barrier dysfunction is likely to influence the overall phenotype of the epithelium and may manifest as distinct subgroups of asthma. PM = particulate matter, $O^{\cdot}$ = oxygen radicals, NO_x = nitrogen oxides, CS= cigarette smoke.

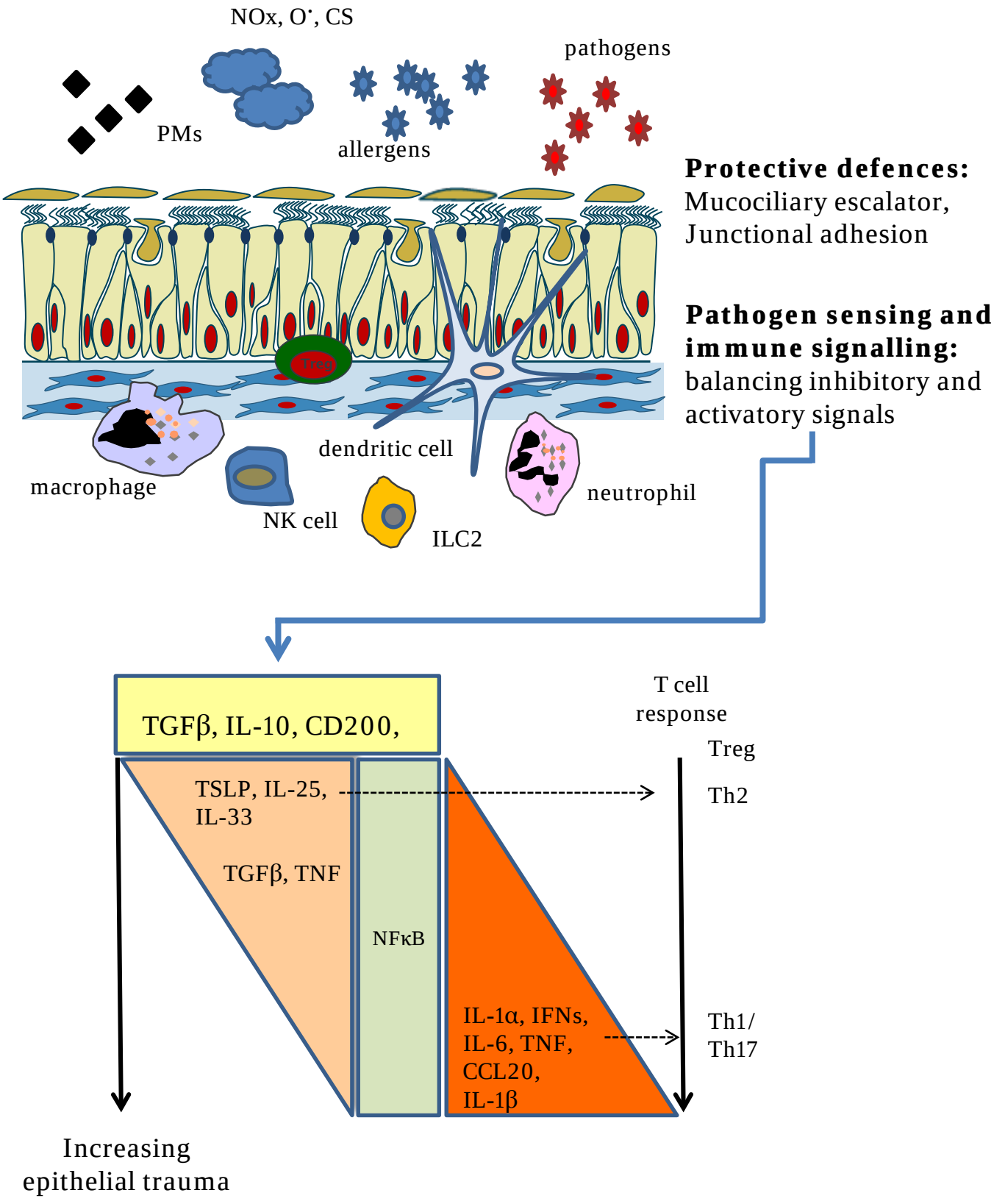
Figure 4: Pictorial representation of the subject clusters (SC) and gene clusters (GC) found in a transcriptomic analysis of epithelial brushings from 155 donors. Red indicates high, pink medium and blue low expression of genes within the cluster. The bar chart indicates the % of healthy controls, mild, moderate or severe asthmatics in each SC and the width of the bar is proportional to the number of subjects in the cluster. Findings are summarised from Modena et al[8].

Figure 5: Potential mechanisms of asthma defined by epithelial barrier dysfunction. Identification of potential links with asthma susceptibility genes and their interaction with environmental stimuli.

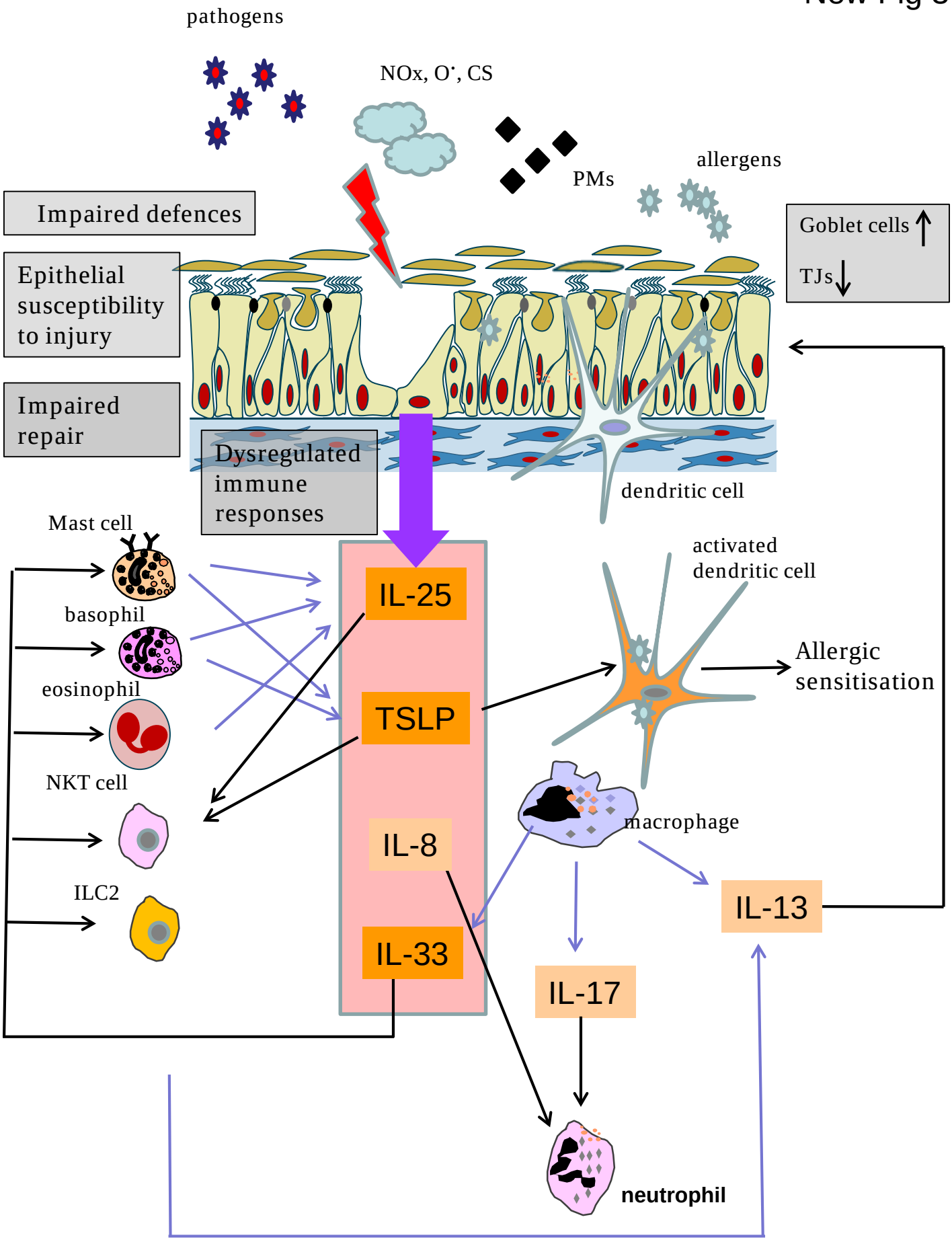
New Fig 1


Tight junction
Adherens junction
Desmosome
Focal adhesion
Actin filament
Intermediate filament
Microtubule
Centromere
Basement membrane
Subepithelial fibroblasts and ECM
Occludin
Claudin
JAM
E-cadherin
Ca2+
ZO-1
ZO-2
p120
β
α
α
p120
β
α
src
γ
Paracellular space

New Fig 2

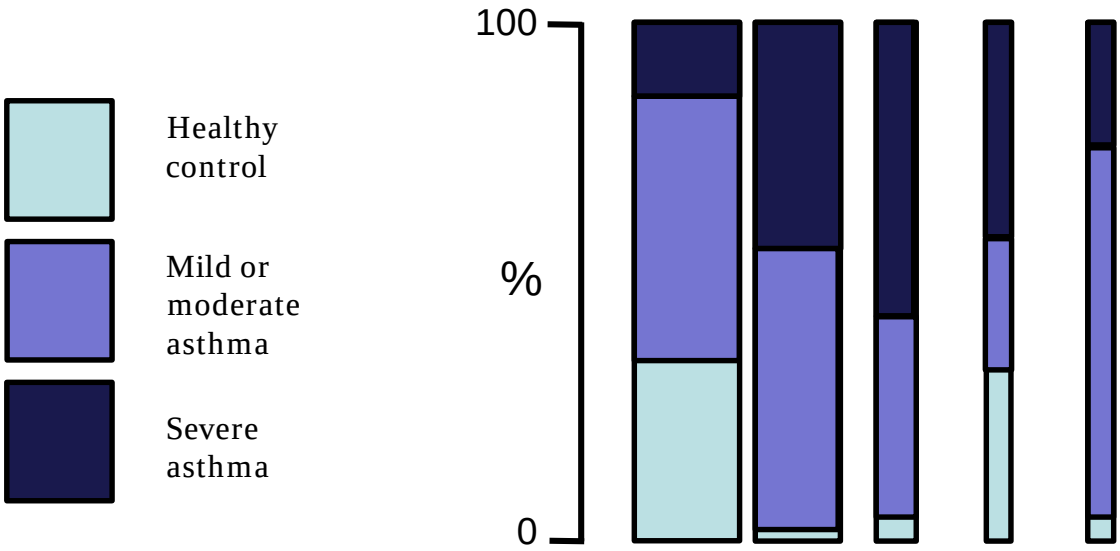

NOx, O˙, CS
pathogens
PMs
allergens
Protective defences:
Mucociliary escalator,
Junctional adhesion
Pathogen sensing and
immune signalling:
balancing inhibitory and
activatory signals
Treg
dendritic cell
macrophage
NK cell
ILC2
neutrophil
TGFβ, IL-10, CD200,
T cell
response
Treg
TSLP, IL-25,
IL-33
Th2
TGFβ, TNF
NFκB
IL-1α, IFNs,
IL-6, TNF,
CCL20,
IL-1β
Th1/
Th17
Increasing
epithelial trauma

New Fig 3

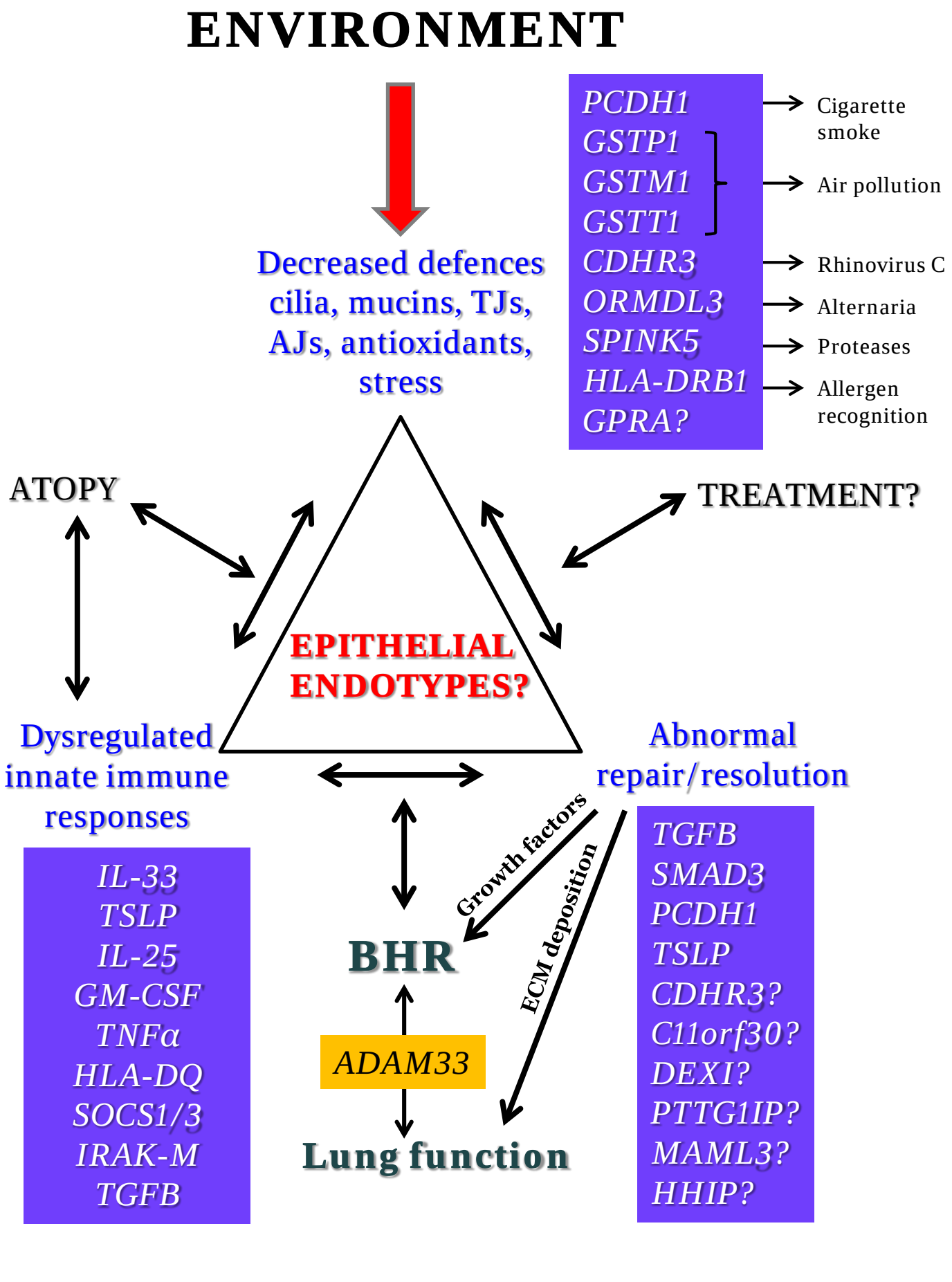

pathogens
NOx, O˙, CS
PMs
allergens
Impaired defences
Epithelial susceptibility to injury
Impaired repair
Goblet cells
TJs
Dysregulated immune responses
dendritic cell
Mast cell
basophil
eosinophil
NKT cell
ILC2
IL-25
TSLP
IL-8
IL-33
activated dendritic cell
Allergic sensitisation
macrophage
IL-13
IL-17
neutrophil

New Fig 4

Subject Cluster / Gene cluster	SC1	SC2	SC3	SC4	SC5
% mod to severe asthma	16	73	74	50	43
GC1 (Innate immunity/antibacterial function; Cell proliferation/apoptosis; Lymphocyte activation/migration)					
GC2 Cilia structure/function, other					
GC3TNF-α ; Muscle					
GC4 Notch signalling; Neuronal function; Dystrophin family; WNT family, Ion channels; Other					
GC5 ‘no obvious function’					
GC6 Microtubules; Mitochondrial; Actin related; Neuronal; Other					
GC7 Interferons; Apoptosis; P38 related; Keratins; Sialyl Lewis antigen; Cell matrix interactions; Other					
GC8 Cysteine metabolism; Mucins; Mast cells; Vasoconstrictors (possibly MC); Glycolipid antigen presentation; Other					
GC9; mitochondria; Intracellular trafficking; O-linked glycosylation; N-linked glycosylation; “Type 2 genes”; Other					



New Fig 5


ENVIRONMENT
Decreased defences
cilia, mucins, TJs,
AJs, antioxidants,
stress
PCDH1
GSTP1
GSTM1
GSTT1
CDHR3
ORMDL3
SPINK5
HLA-DRB1
GPRA?
Cigarette smoke
Air pollution
Rhinovirus C
Alternaria
Proteases
Allergen recognition
ATOPY
TREATMENT?
EPITHELIAL ENDOTYPES?
Dysregulated innate immune responses
Abnormal repair/resolution
IL-33
TSLP
IL-25
GM-CSF
TNFα
HLA-DQ
SOCS1/3
IRAK-M
TGFB
Growth factors
ECM deposition
BHR
ADAM33
Lung function
TGFB
SMAD3
PCDH1
TSLP
CDHR3?
C11orf30?
DEXI?
PTTG1IP?
MAML3?
HHIP?