Title: Immune Mechanisms of Respiratory Viral Infections in Asthma

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Abstract
The more severe pathology respiratory viral infections produce in asthma sufferers is a result of a dysregulated immune response. Excess type 2 inflammation is a well-described feature of virally-induced asthma exacerbations, with growing evidence that production of antiviral interferons may also be impaired. However the mechanisms underlying these are little understood. This review summarizes the current understanding and recent discoveries of the cellular and molecular events that follow viral infections in asthma. In particular, we discuss differences in viral sensing and intracellular signalling pathways upstream of interferon induction in asthma, and the role of epithelial-derived cytokines in orchestrating type 2 immunopathology, including type 2 Innate Lymhpoid Cells (ILC2s).

Highlights
- Viral sensing and intracellular signalling pathways are dysregulated in asthma
- Improving interferon responses, with anti-IgE treatment, reduces viral exacerbations
- Epithelial-derived cytokines orchestrate type 2 immunopathology in viral infections in allergic asthma

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Figure 1a: Immune response to respiratory viruses in asthma.

dsDNA = double stranded DNA; ICAM-1 = intercellular adhesion molecule 1; IgE = immunoglobulin E; IFN = interferon; IL = interleukin; SOCS1 = suppressor of cytokine signalling 1; TSLP = thymic stromal lymphoipoietin

Figure 1b: Modulation of cellular viral sensing and signalling pathways by IL-33 and type 2 cytokines.

TLR = toll-like receptor; RIG-I = retinoic inducible-gene I; MDA5 = melanoma differentiation-associated protein 5; MyD88 = myeloid differentiation primary response gene 88; IRAK1 = interleukin-1 receptor-associated kinase 1; IRF = interferon regulatory factor; NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells

Introduction

The pathology observed following respiratory viral infections is a product of virus and host factors. Thus influenza causes increased morbidity compared to rhinovirus, the common cold, and the same rhinovirus infection that precipitates a few days of upper respiratory symptoms in healthy individuals triggers lower airways symptoms and airway hyper-responsiveness in those with asthma, lasting up to two weeks. This is underpinned by differences in the immune response to viral infections in asthma, with two dominant themes emerging: an excess of type 2 immunopathology, characterised by the cytokines interleukin (IL)-4, IL-5 and IL-13; and deficient interferon (IFN) responses. Recently attention has focused on the mechanisms underlying these and whether they might be linked, which forms the subject of this review.

Interferon responses

A growing number of *in vitro* studies taking different cells (e.g. bronchial epithelial cells (BECs), peripheral blood mononuclear cells (PBMCs), etc.) from asthmatics and healthy controls and infecting them *ex vivo* with respiratory viruses have shown deficient induction of interferons (IFN) in asthma (reviewed in [1]). Most but not all studies have demonstrated this, with the finding appearing more robust in those with more severe asthma or allergies.

A recent *in vivo* study has also identified deficient IFNs in mice with allergic airway inflammation, following a viral infection [2]. They began from the premise that a 'dual hit' of allergic sensitization and viral infections in early life leads to asthma, as supported by the observation in a cohort of children that those sensitized to aeroallergens and with a history of rhinovirus-induced wheezing had the highest risk of developing asthma [3]. A sophisticated mouse model was designed to replicate this by exposing mice to cockroach extract (CRE) and pneumonia virus of mice (PVM) in early and later life [2]. Following PVM infection in later life, the animals developed type 2 immunopathology and deficient IFN-α and -λ, associated with increased viral loads. In a follow up preliminary study these animals were infected with rhinovirus four weeks later, mimicking a human asthma exacerbation, and again displayed deficient IFN-α [4].

The same has not been demonstrated in man, possibly because it is hard to disentangle the potentially confounding effects IFNs and viral loads have on each other – viral replication should be increased in the absence of a robust IFN response, but higher viral loads would induce greater IFN production, masking any deficiency. Indeed following experimental infection with rhinovirus, asthma subjects have increased levels of IFN-γ and -λ during exacerbation [5] accompanied by increased viral loads [6]. However an earlier rhinovirus challenge study showed that deficient IFN induction following *ex vivo* infection of bronchoalveolar lavage (BAL) cells taken at a baseline bronchoscopy, correlated with *in vivo* viral load and exacerbation severity when the same subjects were subsequently experimentally infected [7]. This is at odds with the observation of similar viral titres in subjects with and without asthma from two cohorts, children with naturally occurring infections and adults with experimental rhinovirus infection, although the groups were small and interestingly there was a non-significant trend towards reduced IFN-λ [8].

Further evidence in favour of an IFN deficiency in asthma is the demonstration that restoring the IFN responses of PBMCs corresponds to reduced exacerbations *in vivo* [9]. In a randomized controlled trial, children with a recent history of exacerbations and treated with omalizumab (anti-IgE) 4-6 weeks before the September rhinovirus epidemic had significantly fewer exacerbations. Experiments on PBMCs taken from a subset of these children before and during treatment showed omalizumab increased the amount of IFN-α induced by *ex vivo* rhinovirus infection in the presence of IgE cross-linking. Moreover those with above average increases in IFN-α *in vitro* had greater reductions in exacerbation frequency *in vivo*. Also, treatment with inhaled IFN-β within 24 hours of cold symptoms in moderate-to-severe asthma patients with a history of exacerbations led to reduced symptoms and improved lung function in a subgroup with moderately severe disease (the only subjects who developed a significant increase in asthma symptoms following viral infection) and a trend towards

lower sputum rhinovirus virus loads [10].

How anti-IgE treatment leads to improved IFN-α responses to rhinovirus is unclear. It may be through modification of viral sensing, as an *in vitro* study showing virus-induced IFN-α production by plasmacytoid dendritic cells (pDCs), the main source of antiviral IFNs in PBMCs, was impaired by cross-linking of the IgE receptor also found downregulation of Toll-like receptor (TLR) 7 on pDCs [11]. TLR7 is one of several pattern recognition receptors (PRRs) that recognise newly synthesised viral nucleic acids, the first step in the immune response to viruses.

Virus sensing and intracellular signalling in asthma

There have been variable reports of reduced expression of PRRs in asthma, particularly TLR7. TLR7 expression was reduced on the bronchial mucosa of moderate-to-severe asthma [12] [13] and in alveolar macrophages from severe asthmatics [14]. Other studies reported unchanged expression of various PRRs and signalling molecules, although these analysed different cell types from patients with asthma of varying severity, as defined by the proportion taking inhaled corticosteroids and baseline spirometry (see table [15] [16] [17]).

Table 1: Expression of pattern recognition receptors and downstream signalling molecules in asthma

Study	Asthma severity	Cell type	Molecules assayed	Findings in asthma
Shikhagaie 2014 [12]	Moderate-to-severe	Bronchial mucosa	TLR5 TLR7	↓ ↓
Hatchwell 2015 [13]	Moderate-to-severe	Bronchial mucosa	TLR7	↓ in eosinophilic asthma
Rupani 2016 [14]	Severe	Bronchoalveolar lavage cells (86% macrophages)	TLR7	↓
Sykes 2012 [15]	Mild-to-moderate	Bronchoalveolar lavage cells	13 in total including TLR3, TLR7, TLR8, RIG-I, MDA5, IRF3, IRF7	↔
Parsons 2014 [16]	Moderate-to-severe	Bronchial epithelial cells	TLR3, MDA5, RIG-I	↔
Pritchard 2014 [17]	Mild-to-moderate	PBMCs	TLR7, IRF1, IRF7, STAT1 TLR8, IRF5, IFNAR	↓ after rhinovirus ↔ vs controls

TLR = toll-like receptor; RIG-I = retinoic inducible-gene I; MDA5 = melanoma differentiation-associated protein 5; IRF = interferon regulatory factor; STAT1 = signal transducer and activator of transcription 1; IFNAR = interferon-α/β receptor; PBMCs = peripheral blood mononuclear cells

It may be that PRR expression is only reduced in people with asthma with ongoing type 2 inflammation, i.e. those with moderate-to-severe asthma suggestive of continuing disease activity or after viral challenge when inflammation rises. In support of this, pre-treating BECs from healthy subjects with IL-4- and IL-13 reduces TLR3 expression [18], and airway eosinophilia suggestive of active inflammation is associated with reduced TLR7 expression [13]. Co-culturing BECs with eosinophils enhances the effect of transforming growth factor (TGF)-β in suppressing IFN production after *ex vivo* infection [19]. Rhinovirus infection also induces FOXA3, amongst other inflammatory changes, which in turn downregulates expression of several PRRs and their downstream transcription factors [20].

Even in the studies where there were no differences in expression of PRRs or intracellular signalling molecules in asthma, induction of antiviral IFNs was impaired following rhinovirus infection. This suggests dysregulaton of signalling pathways downstream of PRRs. Moreover as PRRs and intracellular signalling molecules are themselves IFN-stimulated genes (ISGs), reduced IFN release ultimately results in deficient induction of this antiviral cellular machinery [21].

In support of this hypothesis, treatment with TLR agonists to overcome dysregulated TLR signalling leads to amelioration of airway inflammation in allergen-sensitised mice (e.g. with resiquimod (a TLR7/8 agonist) [22], TLR7 [23] and TRL9 agonists [24]). It remains to be seen if such an approach is effective in virally-induced exacerbations.

Other potential mechanisms of IFN impairment: SOCS1

Suppressor of cytokine signalling (SOCS) 1 is a negative regulator of interferon production. SOCS1 can be induced in BECs by rhinovirus and IL-4 and IL-13. In rhinovirus-infected BECs, levels of SOCS1 are negatively correlated with IFN-β, -λ1, and –λ2/3 mRNA [25]. Studies using $SOCS1^{-/-}$ mice confirm that SOCS1 directly suppresses virus induction of IFN in BECs as well as IFN-induced amplification of IFN induction *in vivo*. Moreover SOCS1 expression in bronchial biopsies is related to asthma severity and atopic status, providing evidence for this pathway in man.

Epithelial susceptibility to viral infection in asthma

Airway epithelium forms a protective barrier and is, for most respiratory viruses, the primary site of replication. Allergen exposure decreases airway epithelial tight junction proteins [26], facilitating virus and allergen penetration. Consistent with this, mouse- and house dust mite-specific serum IgE levels are positively correlated with virus-induced exacerbation severity in children [27]. Viral infection also compromises epithelial integrity [28], compounding exposure to allergens. Taken together with IgE-mediated impairment of pDC IFN production, this suggests asthmatics with seasonal allergies (including house dust mite [29]) are more vulnerable to virus-induced exacerbations in the autumn and spring, corresponding to spikes in rhinovirus infection that are also associated with school terms. This also potentially explains why not all respiratory virus infections lead to asthma exacerbations.

Other changes in asthma also facilitate epithelial viral infection. For example, expression of the receptor for major group rhinoviruses, intercellular adhesion molecule (ICAM)-1, is increased on airway epithelia in asthma [30]. Exposure to IL-4 and IL-13 upregulates ICAM-1 expression [31] providing a plausible mechanism.

Epithelial-derived cytokines

As well as being a source of antiviral IFN, epithelial cells produce other cytokines including three that may be pivotal in promoting type 2 inflammation: IL-33, IL-25 and thymic stromal lymphopoietin (TSLP). Each of these can initiate a cascade of type 2 inflammation, activating group 2 innate lymphoid cells (ILC2s) and T helper 2 (Th2) cells to release IL-4, -5 and -13. *In vitro*, there appears to be a hierarchy with IL-33 the most potent activator [32].

Human rhinovirus challenge studies support the role of IL-25 and IL-33 in the asthmatic response to virus. IL-25 is increased in asthma at baseline, and following rhinovirus infection both IL-25 and IL-33 rise to a greater extent in subjects with asthma [6,33], with levels of IL-33 correlated with viral load, IL-5 and IL-13. BECs from subjects with asthma infected with rhinovirus *ex vivo* exhibit increased capacity for IL-25 production, while IL-25 receptor blockade restricts type 2 immunopathology and mucus production in a mouse model of virus-induced asthma exacerbation [33]. Similarly, supernatants from *ex vivo*-infected BECs induces IL-4, IL-5 and IL-13 production by human T cells, and IL-5 and IL-13 production by human ILC2s, in an IL-33-dependent manner [6].

Less is known about TSLP, with much knowledge coming from animal models. The recruitment of ILC2s, a potent source of type 2 cytokines, following Respiratory Syncytial Virus (RSV) infection in mice is dependent on TSLP and IL-33 [34]. TSLP and IL-33 have also been implicated in the development of Th2 cells, at the expense of regulatory T cells (Treg). Simultaneous exposure to rhinovirus and ovalbumin prevents the development of ovalbumin tolerance in mice and is associated with a shift in the balance of Treg to Th2 cells, but TSLP receptor-deficient mice and mice treated with an IL-33 receptor-blocking antibody have tolerance restored [35]. TSLP may be important in steroid-resistant asthma, as levels are increased in BAL fluid of people with asthma and this correlates with an inability of dexamethasone to inhibit type 2 cytokine production by ILC2s purified from BAL [36]. This study additionally identified novel therapeutic targets in the form of molecules upregulated by TSLP that are not inhibited by dexamethasone.

IL-33 may modulate IFN responses in addition to promoting type 2 inflammation. In mice co-exposed to CRE and PVM (as described earlier), IL-33 receptor blockade or neutralizing anti-IL-33 antibodies reversed the deficient IFN-α and -λ responses to PVM, as well as the airway hyper-responsiveness, type 2 inflammation [2]. Further experiments with pDCs revealed that IL-33 mediates its effects by downregulating signalling molecules downstream of TLR7, interferon regulatory factor 7 (IRF7) and interleukin-1 receptor-associated kinase 1 (IRAK-1), leading to TLR7 hypo-responsiveness and deficient IFN-α [2]. This was corroborated in human samples by demonstrating that anti-IL-33 increased IFN-λ2 and decreased viral load in BECs from donors with and without asthma infected with rhinovirus *ex vivo* [4]. This supports a direct effect of IL-33 as type 2 cytokines are absent in a BEC monoculture system. One would therefore predict therapies targeting IL-33 to have anti-viral effects analogous to anti-IgE treatment.

Innate immune cells

The peak in symptoms and viral titres following experimental rhinovirus infection occurs within a few days – too rapidly for a *de novo* acquired immune response (although a common epitope may induce activation of pre-existing memory T cells [37]). Most research has focused on epithelial cells as the primary site of infection, but innate immune cells are also resident in the airways (e.g. alveolar macrophages, mast cells, dendritic cells) and able to initiate type 2 immunopathology.

These include the relatively recently discovered ILC2s, which are present in elevated numbers in asthmatic airways, particularly in subjects with severe asthma in whom they are the predominant source of IL-5 and IL-13 despite being less abundant than CD4+ T cells [38]. In a murine model of chronic asthma, ILC2s were necessary for the persistence of airway inflammation and hyper-responsiveness, whereas antigen-specific T cells were not, as shown by bone marrow chimeras using $Rag1^{-/-}$ (deficient in T and B cells) and $Rag2^{-/-}\gamma c^{-/-}$ mice (additionally deficient in ILCs) [39]. ILC2s are regulated by IL-33 [39] and negatively regulated by type I IFNs and IL-27 [40]. The absence of robust antiviral interferon induction therefore enables unchecked ILC2 expansion and activation.

Neutrophils are particularly increased in the airways following rhinovirus infection [41], and a recent study suggests they may also contribute to type 2 inflammation through the formation of neutrophil extracellular traps (NETs) [42]. Rhinovirus infection of mice sensitised to house dust mite (HDM) causes release of host double-stranded DNA (dsDNA), as well as producing the typical features of an asthma exacerbation. NETs contain dsDNA and degrading NETs with DNase or preventing their formation by inhibiting neutrophil elastase reduces type 2 inflammation, whereas injection of dsDNA alone (in place of virus infection) in allergic mice is sufficient to enhance type 2 inflammation. In human subjects experimentally infected with rhinovirus, dsDNA in nasal lavage samples is induced to greater levels in asthma, and correlates with type 2 cytokine levels and exacerbation severity [42].

Non-allergic asthma

Whilst the majority of asthma sufferers have allergic disease, the remainder are a heterogenous group that has been relatively neglected, although interest in these patients is growing. For example it has

been shown that, in severe asthma, sputum neutrophilia and high dose inhaled corticosteroids are independent predictors of impaired IFN-α induction following *ex vivo* infection of PBMCs with rhinovirus [43].

Another evolving story is that of IL-6, which was elevated in the sputum of two cohorts of asthma patients of varying severity and, in the severe asthma cohort, was associated with reduced lung function and more frequent exacerbations [44]. Whether defects in antiviral immunity underlie the frequency of exacerbations is unknown.

Conclusion

The immunopathology of virus infections in asthma is complex. Aberrant viral sensing and intracellular signalling in asthma, potentially following exposure to allergen and type 2 inflammatory proteins, results in deficient IFN responses to virus (Figure 1b). Epithelial-derived cytokines promote skewing towards a type 2 inflammatory response, particularly IL-33 which activates ILC2s, unopposed by type I IFNs (Figure 1a). Further investigation of exacerbations in non-type 2 asthma is also warranted, as the immunology may differ from that seen in exacerbations in type 2 asthma.

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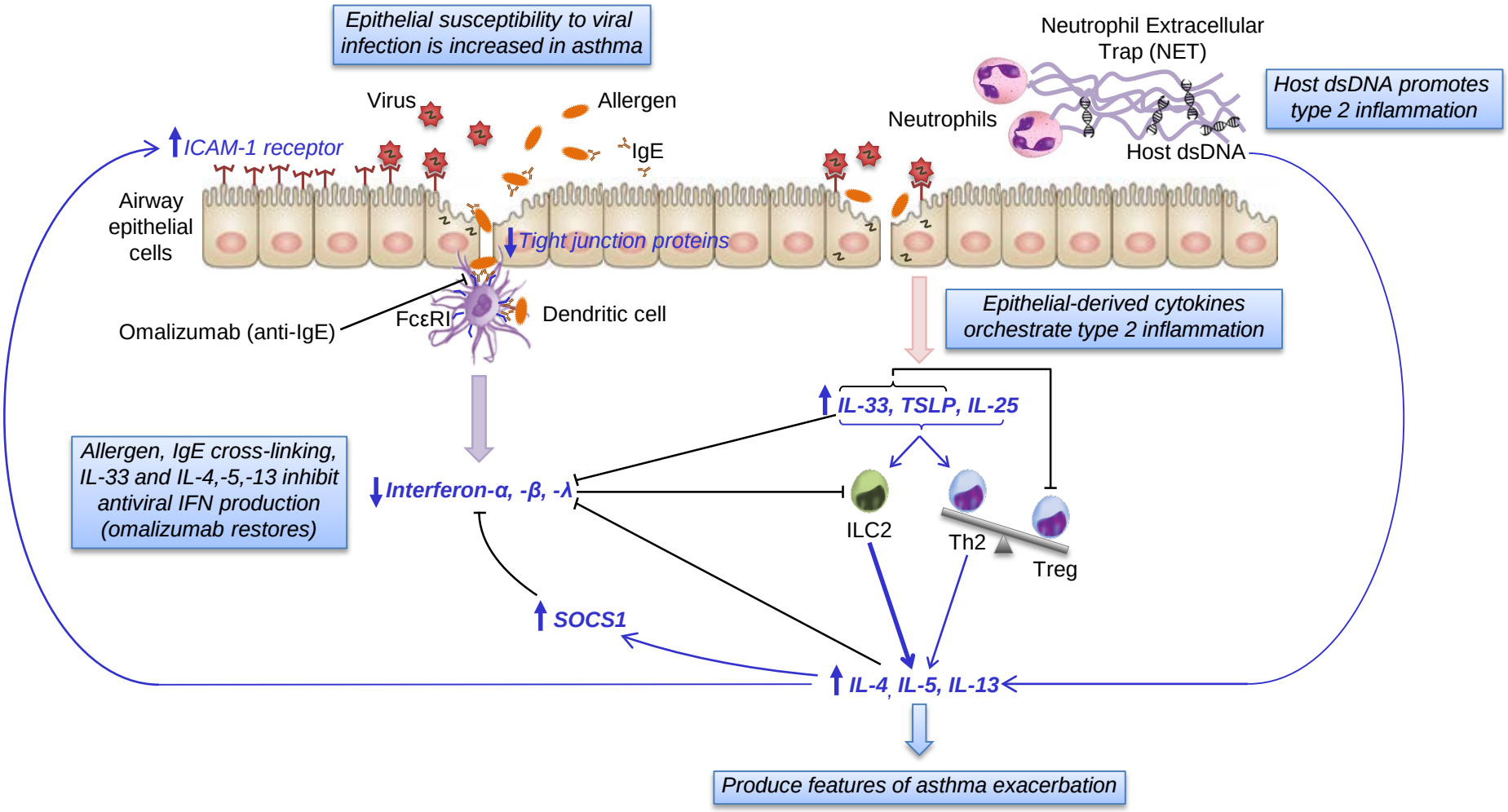
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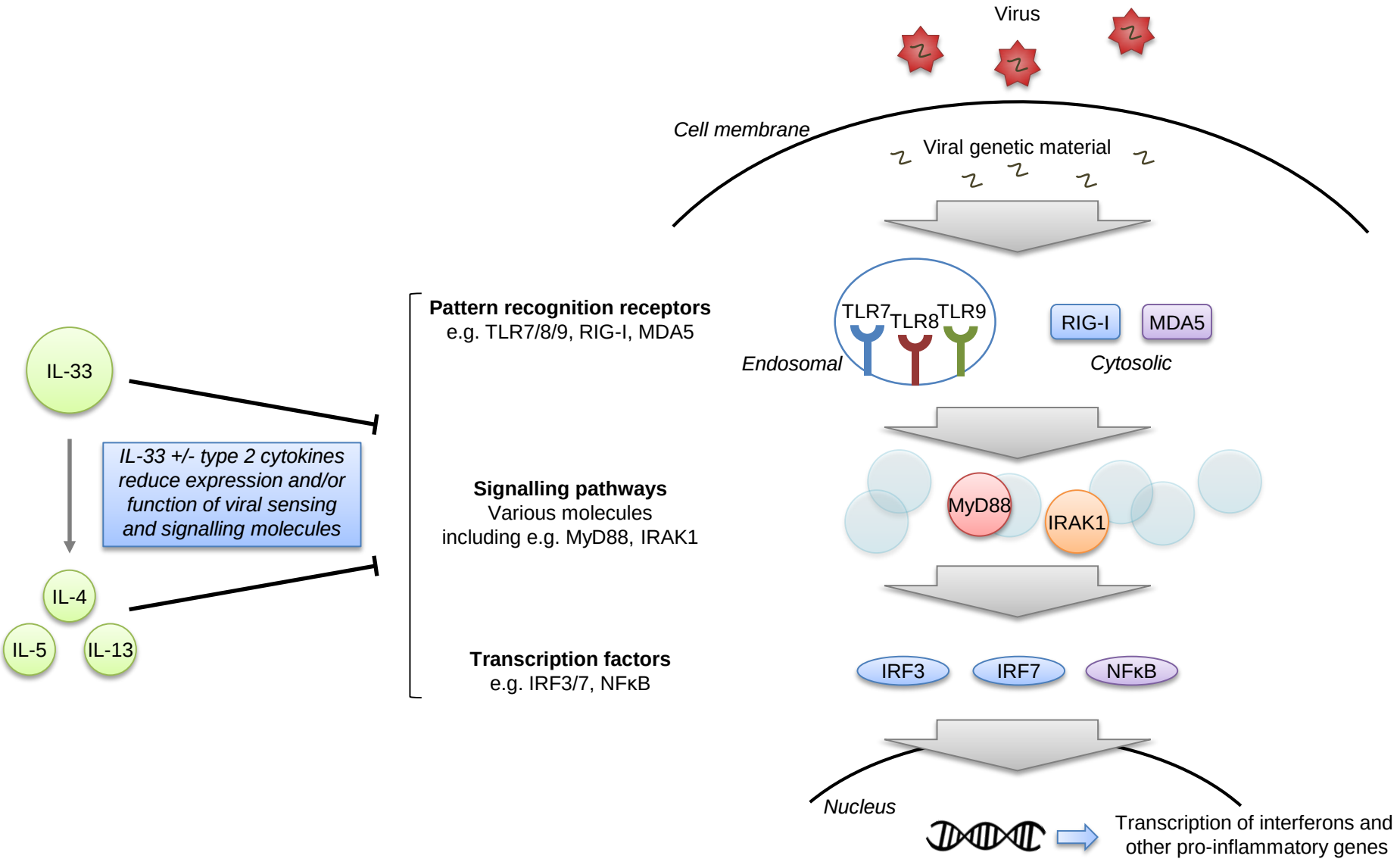
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Epithelial susceptibility to viral infection is increased in asthma
Neutrophil Extracellular Trap (NET)
Host dsDNA promotes type 2 inflammation
Virus
Allergen
IgE
Neutrophils
Host dsDNA
ICAM-1 receptor
Airway epithelial cells
Tight junction proteins
Omalizumab (anti-IgE)
FcεRI
Dendritic cell
Epithelial-derived cytokines orchestrate type 2 inflammation
IL-33, TSLP, IL-25
Allergen, IgE cross-linking, IL-33 and IL-4,-5,-13 inhibit antiviral IFN production (omalizumab restores)
Interferon-α, -β, -λ
ILC2
Th2
Treg
SOCS1
IL-4, IL-5, IL-13
Produce features of asthma exacerbation


Virus
Cell membrane
Viral genetic material
IL-33
IL-33 +/- type 2 cytokines reduce expression and/or function of viral sensing and signalling molecules
IL-4
IL-5
IL-13
Pattern recognition receptors
e.g. TLR7/8/9, RIG-I, MDA5
TLR7
TLR8
TLR9
Endosomal
RIG-I
MDA5
Cytosolic
Signalling pathways
Various molecules including e.g. MyD88, IRAK1
MyD88
IRAK1
Transcription factors
e.g. IRF3/7, NFκB
IRF3
IRF7
NFκB
Nucleus
Transcription of interferons and other pro-inflammatory genes