



The hygiene hypothesis: immunological mechanisms of airway tolerance

Eline Haspeslagh^{1,2,3}, Ines Heyndrickx^{1,3}, Hamida Hammad^{1,3} and Bart N Lambrecht^{1,3,4}

The hygiene hypothesis was initially proposed as an explanation for the alarming rise in allergy prevalence in the last century. The immunological idea behind this hypothesis was a lack of infections associated with a Western lifestyle and a consequential reduction in type 1 immune responses. It is now understood that the development of tolerance to allergens depends on microbial colonization and immunostimulatory environmental signals during early-life or passed on by the mother. These environmental cues are sensed and integrated by barrier epithelial cells of the lungs and possibly skin, which in turn instruct dendritic cells to regulate or impede adaptive T cell responses. Recent reports also implicate immunoregulatory macrophages as powerful suppressors of allergy by the microbiome. We propose that loss of adequate microbial stimulation due to a Western lifestyle may result in hypersensitive barrier tissues and the observed rise in type 2 allergic disease.

Addresses

¹Laboratory of Immunoregulation and Mucosal Immunology, VIB Center for Inflammation Research, Technologiepark 927, B-9052 Ghent (Zwijnaarde), Belgium

²Department of Biomedical Molecular Biology, Ghent University, Technologiepark 927, B-9052 Ghent (Zwijnaarde), Belgium

³Department of Internal Medicine, Ghent University, De Pintelaan 185 K12, B-9000 Ghent, Belgium

⁴Department of Pulmonary Medicine, ErasmusMC, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands

Corresponding authors: Hammad, Hamida (Hamida.Hammad@UGent.be), Lambrecht, Bart N (bart.lambrecht@ugent.be)

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Introduction

Allergic sensitization is characterized by the presence of allergen-specific immunoglobulin E (IgE) in serum. Exposure to allergens via inhalation, ingestion or contact with the skin can lead to diseases such as asthma, hay

fever, eczema and, in some cases, to systemic anaphylaxis. During the last 150 years, allergies have emerged in a very rapid way and their prevalence is still on the rise. Nowadays, more than 30% of children are allergic, up to 10% of children suffer from asthma and allergic rhinitis, and 5–7% of children have developed food allergy. It is still not entirely clear why asthma prevalence is so high, but the rapid time frame of its origination and expansion suggests that environmental or behavioral changes in Western lifestyle are involved.

A modern lifestyle is associated with dysbiosis

An important evolution of the last 150 years is a successful decrease of infectious disease burden, due to the massive introduction of hygiene measures, antibiotics, and vaccines. In 1989, Strachan observed that growing up in large families with more older siblings decreased the chances of developing hay fever or eczema [1]. He postulated that the recent increase in allergy incidence was a result of ‘declining family size, improvements in household amenities, and higher standards of personal cleanliness’, which had reduced ‘the opportunity for cross infection in young families’. The original ‘hygiene hypothesis’ was thus introduced. Since then, this hypothesis has been supported by numerous studies, especially in murine models, showing that exposure to bacteria, viruses, helminths or microbe-derived products could protect from allergy (reviewed in [2], [3**]). However, it should be kept in mind that not all pathogens are protective; for instance, respiratory syncytial virus (RSV) or rhinovirus are associated with a higher risk to develop wheeze and asthma up to adulthood [4].

Changes in lifestyle can also heavily influence the composition and diversity of the microbiome at several mucosal surfaces. These microbial communities have co-evolved with and within the human body for millions of years, and, consequently, the human immune system has been calibrated and fine-tuned so to maintain and shape symbiotic relationships with them (reviewed in [5]). Two theories, the ‘Old friends’ and the ‘Biodiversity’ hypotheses, have been proposed by Rook and by Haahela as a more accurate, or at least complementary, explanation for the recent allergy pandemic [6,7]. They stipulate that the reason for the increased incidence in allergic disorders is a reduced exposure to such beneficial symbiotic bacteria or parasites. Indeed, several studies have

reported that alterations in the composition of the skin, the nose or the gut microbiome are associated with eczema, asthma and food allergy [8–10]. These changes do not affect a single commensal, but rather reflect a reduced total microbial diversity [11], and they may be caused by several factors, including sibling order in the family [12], exposure to animals [13], and other early-life events [14]. The importance of a healthy microbiome in controlling allergies was further substantiated in mice, with germ-free mice being especially prone to develop overt allergic (airway) disease, a phenotype reverted by microbial recolonization [15,16]. However, other studies showed that germ-free mice are not universally more susceptible to house dust mite driven asthma, and that only selected strains of lung microbiota seem to suppress asthma [17]. During the last 30 years, the body of correlative epidemiological studies has expanded vastly, and is the subject of many excellent reviews. Here, we will zoom in on recent advances in the search for the underlying immunological mechanisms explaining the observed effects.

Microbes induce protective regulatory DCs and T cells

Allergies are generally aberrant immune reactions to innocuous antigens, orchestrated by T helper 2 (Th2) cells and type 2 innate lymphoid cells (ILC2s). In the case of asthma, this type 2 cell activity leads to mucus hypersecretion, goblet cell hyperplasia, smooth muscle cell hyperreactivity, and the infiltration and/or activation of eosinophils, mast cells and basophils, ultimately culminating in breathing difficulties and airway remodeling [18]. Dendritic cells (DCs) are always found at the body's barriers, and because they express a wide range of pattern recognition receptors (PRRs), they can sense the environment for the presence of danger signals [19]. Our group has shown that Th2 responses to house dust mite (HDM) allergens were induced by IRF4-dependent cDC2s in the lungs and in the skin [20,21*] (Figure 1). These cDC2s capture the HDM allergens in the airways and migrate to the draining lymph nodes, requiring ILC2-derived IL-13, where they present the allergens to naïve T cells [22]. It is easy to imagine that environmental changes sensed at the level of the lungs, the skin but also of the gut will modify the context of allergen recognition by DCs, and either protect against or enhance allergic responses.

Chronic *Helicobacter pylori* infection has been inversely linked to asthma in humans and can effectively protect mice from OVA-induced asthma [23,24]. In mice, *H. pylori* infection induced the accumulation of CD103+ cDCs in the lungs, which were required for the protection, as was their IL-10 production [24]. In a recent study, semi-therapeutic *H. pylori* extract treatment also reduced airway allergy, shifted the CD11b+/CD103+ DC ratio in the lungs, and reduced the antigen processing by lung and

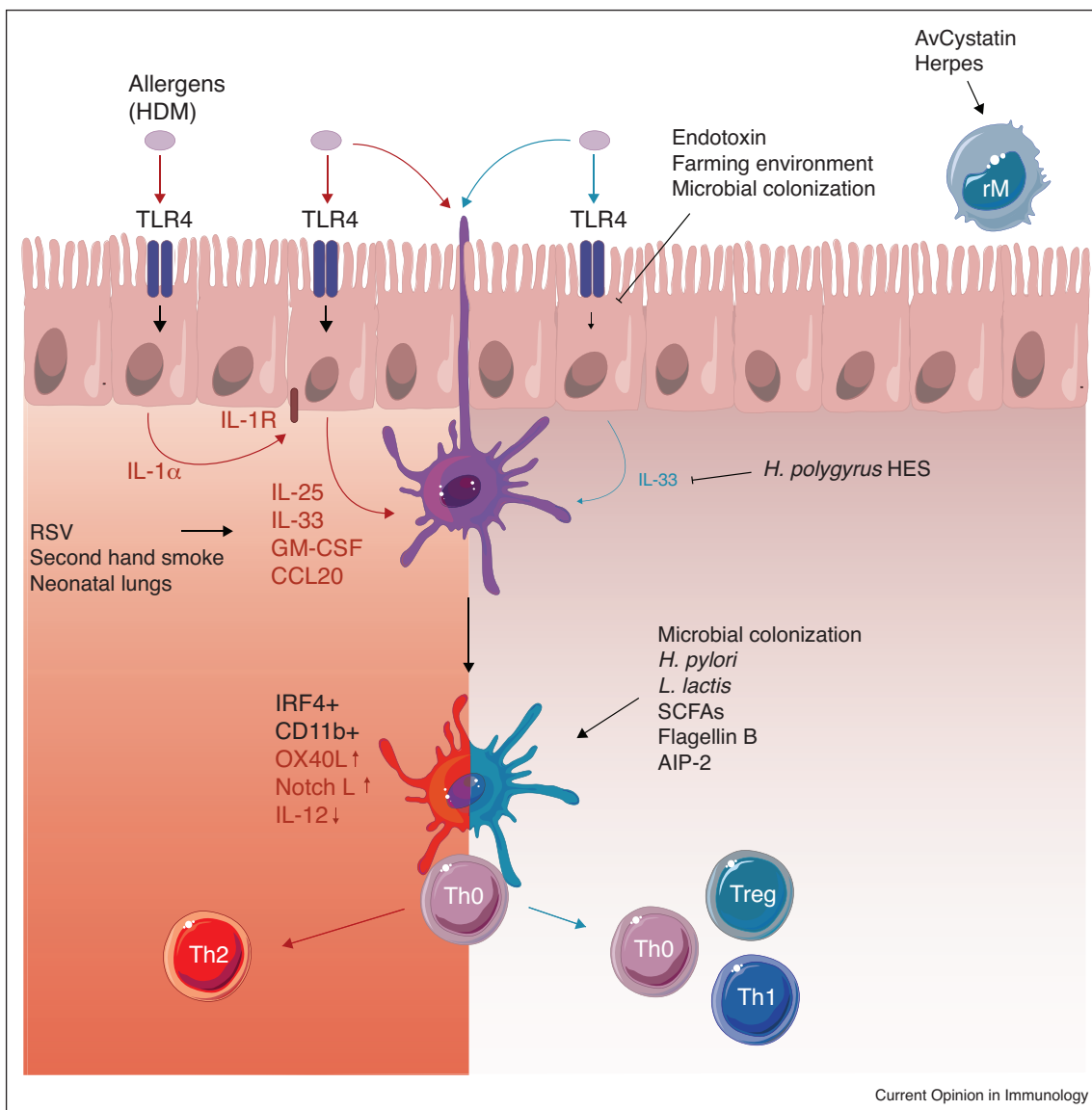
lymph node DCs [25]. Other studies demonstrated protective modulation of *in vitro* bone-marrow derived DC cultures (BMDCs). A synthetic TLR1/TLR2-agonist induced LPS-tolerance and IL-10 production in BMDCs, whereas the cowshed *Lactococcus lactis* instigated a Th1-polarizing program, both rendering the BMDCs unable to sensitize mice to OVA-allergen upon adoptive transfer [26,27*].

Trompette *et al.* recently found that feeding mice a fiber-rich diet changed the composition of the lung and gut microbiome, the latter metabolizing the fiber into circulating short-chain fatty acids (SCFA's) [28]. The increased SCFA levels protected the mice from allergic lung inflammation. Mechanistically, the SCFA's altered DC precursor generation in the bone marrow, and the DCs subsequently seeding the lungs had a higher phagocytic capacity and were impaired in polarizing Th2 cells. Additional studies have supported the protective effect of dietary fiber supplementation on allergic asthma development in mice [29], and on wheeze in human infants when the fiber was given to the pregnant mother [30].

One mechanism by which the DCs in microbe-exposed animals can confer protection, is by inducing the generation of regulatory T cells (Tregs). Microbial colonization in 2-week old mice was shown to be necessary for the transient upregulation of PD-L1 on lung CD11b + DCs, and the expansion of a specific pulmonary Treg subset [31]. PD-L1 blockade in neonates resulted in exaggerated responsiveness to HDM through adulthood, suggesting a crucial role for this microbial-induced DC–Treg axis for immunological tolerance. In another mouse model of *H. pylori*-mediated asthma protection, the *Helicobacter* infection inhibited TLR-induced DC maturation and reprogrammed the DCs towards a FoxP3+ Treg-polarizing phenotype [32]. The bacterial component flagellin B, given semi-therapeutically together with allergen, could also inhibit murine allergic asthma symptoms in a DC– and CD25+ Treg-dependent manner [33*].

Although helminths are prototypical inducers of type 2 immunity, they have been correlated with reduced allergen skin prick test reactivity, and to some degree with asthma protection (reviewed in [34]). A general explanation for this non-intuitive association is that helminths induce a so-called 'modified Th2' response, with immunoregulatory cells such as Tregs complementing the Th2-arm of immunity, and regulating the response to bystander antigens such as aeroallergens. Therefore, several groups have tried to find helminth-derived products with immunomodulatory properties that could be used to suppress Th2 immunity. For instance, an anti-inflammatory protein (-2; AIP-2) from the parasitic hookworm was identified to suppress murine airway allergy in a DC-dependent and Treg-dependent manner [35]. In another study, the helminth-derived immunomodulator

Figure 1



Proposed model of airway tolerance. In the absence of immunoregulatory pathways, epithelial barrier cells readily respond to allergen binding on their pattern recognition receptors, among which TLR4, by the secretion of inflammatory mediators (IL-1 α , IL-25, IL-33, GM-CSF, CCL20, and others). These mediators license antigen-bearing IRF4+ CD11b+ conventional dendritic cells (cDC2s) to polarize naïve T cells to T helper 2 (Th2) cells in the lung-draining lymph nodes. Neonatal and germ-free mice are especially prone to develop such Th2 responses. Respiratory syncytial virus (RSV) infection and second hand cigarette smoke, two known asthma risk factors, increase IL-33 secretion and may thereby stimulate this pathway. Exposure to endotoxin, farm dust or microbial colonization blunts the epithelial response by increasing the expression of negative regulators. Epithelial IL-33 release is also inhibited by helminth-derived excreted and secreted products (HES). DCs devoid of epithelial activation signals do not induce T cell activation (Th0). Other protective factors impede T cell activity by influencing the maturation, antigen presentation, or phagocytic capacity of DCs. Some protective factors induce DCs that provoke regulatory T cell (Treg) activity or T helper 1 (Th1) activity. Regulatory macrophages (rM) can also induce Tregs, or block DC-mediated Th2-polarization. Abbreviations: HDM, house dust mite; TLR4, Toll like receptor 4; IL-1R, IL-1 receptor; *H. pylori*, *Helicobacter pylori*; *L. lactis*, *Lactococcus lactis*; SCFA, short chain fatty acids; AIP-2, anti-inflammatory protein 2.

AvCystatin was demonstrated to induce regulatory macrophages that protected against experimental asthma upon adoptive transfer [36]. Regulatory alveolar macrophages from bone marrow origin were recently also implicated in long-lasting protection conferred by a latent

murine gammaherpesvirus infection, a model for Epstein–Barr virus infection in mice [37*]. The regulatory macrophages induced by the infection replaced the long-lived and self-replenishing alveolar macrophages that are generated shortly after birth, and became long-lived as well.

This potentially explains the long-lasting effects of microbial stimuli in the lungs on allergy suppression.

Allergic asthma is initiated by aberrant immune responses at barrier tissues

To initiate an allergen-specific Th2 response, cDC2s need to be instructed by barrier epithelial cells (ECs) lining the airways. Barrier ECs are permanently exposed to environmental insults or innocuous signals and, like DCs, they are well-equipped to integrate these signals via a range of PRRs (reviewed in [38]). Activation of PRRs on ECs by allergens induces NF- κ B activation and ROS production, resulting in the secretion of a wide range of inflammatory mediators, among which the cytokines IL-33, IL-25 and TSLP. DCs react to these cytokines by OX40L and Notch ligand upregulation, and downregulation of IL-12 production, an activation state that favors Th2 polarization in the lung-draining lymph node [39,40]. Interestingly, the barrier tissue of the skin also constitutes a possible entry route for aeroallergens [21*]. Thus, barrier cells act very upstream in the inflammatory cascade of events leading to allergic sensitization (Figure 1).

Our group has previously reported that the PRR toll-like receptor 4 (TLR4) on airway ECs was critically necessary to mount a Th2-mediated asthmatic response to HDM [41]. Strikingly, several HDM allergens have the intrinsic capacity to facilitate or amplify TLR4 signaling by binding directly to proteins of the TLR4 signaling complex or to its ligands [42,43]. However, TLR4 is best known as the receptor for LPS, also termed endotoxin, a component of gram-negative bacteria. It is difficult to reconcile how a receptor specialized in bacterial sensing can contribute to Th2 immunity and allergy, especially given the fact that high endotoxin levels in children's mattresses are protective against atopic sensitization and asthma in humans and mice [44–46]. Another study also associated house dust endotoxin levels with a significantly reduced risk of allergic sensitization or eczema, specifically in children with a polymorphism in the CD14 gene [47]. In fact, a body of epidemiological studies have convincingly correlated a traditional farming environment, where endotoxin levels are high, with protection against hay fever, allergic sensitization, and asthma (reviewed in [48]). In a hallmark study, children growing up on agricultural Hutterite and Amish farms in the US were compared, and the latter were found to have six times less chance of developing atopy and asthma [49**]. These two farming populations share a similar genetic ancestry and lifestyle. Farming practices, however, differ, and the Amish house dust contained almost 7 times more endotoxin than the house dust from Hutterite farms. Only the transfer of the Amish dust intranasally to mice inhibited subsequent experimental asthma development. We have recently confirmed that farm dust collected from Bavarian farms (in which farming practices resemble the Amish's ones), and LPS, conferred protection against experimental asthma. This

protection was mediated by an increased epithelial expression of TNFAIP3 (better known as A20), a negative regulator of the NF- κ B pathway, which blunted the epithelial cell response to HDM and downstream DC activity [50]. A similar tolerance to LPS mediated by A20 induction was demonstrated in intestinal ECs [51]. Interestingly, A20 expression was very low in neonatal rats, spontaneously increased shortly after birth coinciding with microbial colonization, and could be downregulated by treatment with antibiotics. It remains to be investigated if the gut or lung microbiota can similarly influence expression of A20 and other negative regulators in airway ECs. EC modulation has recently been demonstrated for *Heligmosomoides polygyrus*, a helminth often confirmed to protect against murine allergy [52]. Secreted and excreted products (HES) of this parasite inhibited IL-33 release by ECs and thereby suppressed Alternaria-induced airway allergy [3**]. Mechanistically, the *H. polygyrus* alarmin release inhibitor (HpARI), a 26 kDa protein, binds to activated IL-33 and at the same time tethers IL-33 to the DNA of necrotic cells, thus inhibiting IL-33 action in a dual manner and inhibiting innate eosinophilic airway inflammation [53**]. Other molecules secreted by the parasite are more related to TGF β and can induce a Foxp3+ Treg population with immunoregulatory potential [54].

A dysregulated immune response at the skin can cause atopic dermatitis (AD), which in itself is a risk factor to accumulate more allergies later in life, among which asthma, a process known as 'the atopic march'. In fact, AD and asthma share several risk factors. AD is strongly correlated with changes in the skin microbiome, the most well-known being pertinent *Staphylococcus aureus* colonization of allergic skin. In a recent study, IL-17R α -/- mice spontaneously developed AD with naturally occurring skin dysbiosis and a compromised skin barrier, and antibiotic treatment ameliorated skin inflammation [55]. A similar dysbiosis-AD axis has also been demonstrated in ADAM17-/- mice [56]. Topical treatment with non-pathogenic bacteria, on the other hand, can alleviate cutaneous inflammation in murine AD [57]. It has become clear in recent years that tonic sensing of skin commensals heavily shapes host DC and T cell functions [58–60]. It remains to be investigated what the relative importance is of passive barrier integrity and active signaling through keratinocyte PRRs, also poised to rapidly respond to innate immune ligands, in this microbe-immune cell cross-talk [61]. It will also be of great interest to study how environmental exposures influence the skin microbiome and/or the immune threshold of skin epithelium.

One intriguing observation is that allergies tend to develop early in life. In the same time window, and even *in utero*, protective effects of environmental factors and the microbiome are also the strongest [62,63]. We

recently demonstrated that the lung environment in neonatal mice is strongly type 2-skewed, with a gradual increase in IL-33 release by lung ECs, and with the spontaneous recruitment of several Th2-associated innate immune cells, peaking 2 weeks after birth [64**]. This spontaneous wave of early type 2 immunity is likely to be caused by the mechanical stress induced by the breathing patterns [65], but also by the constant remodeling necessary to build up new lung structures. Interestingly, this period is prone to favor stronger Th2 sensitization to inhaled allergens [64**], but also to favor lower immunity to bacteria [65]. Many environmental factors, like second hand smoking or RSV infection are known to facilitate Th2 sensitization in children. These triggers have in common that they induce high levels of IL-33 [66,67]. It is tempting to speculate that these risk factors act by prolonging or amplifying the epithelial cytokine response to allergens during early-life, and that combined early-life exposures thus define the final threshold for EC activation.

Conclusion

Effects of microbes on inducing Treg cells, Th1 cells and allergen cross-reactive antibody responses, are well-observed. In addition, we propose a model in which environmental and microbial stimuli are sensed and integrated by barrier tissues of the lung, the skin and the gut, resulting in a tonic DC activation status promoting either inflammatory or tolerogenic immunity. Together with direct effects on DCs and T cells, most protective stimuli thus seem to converge in the same central tolerogenic immune pathways. Fully understanding the fundamental immunological pathways underlying these protective triggers, their relative contribution, and how they interact, should hopefully allow us to pinpoint, modify or newly develop prophylactic or therapeutic therapies to cure asthma.

Conflict of interest statement

Nothing declared.

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