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European Journal of Immunology

Protection against allergies: microbes, immunity and the farming effect

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Keywords: allergy, asthma, microbiome, diet, farm effect

Abstract

The prevalence of asthma and other allergic diseases has rapidly increased in 'Westernized' countries over recent decades. This rapid increase suggests the involvement of environmental factors, behavioral changes or lifestyle, rather than genetic drift. It has

Received: 01 13, 2021; Revised: 07 08, 2021; Accepted: MONTH DD, YYY

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/eji.202048938.

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become increasingly clear that the microbiome plays a key role in educating the host immune system and thus regulation of disease susceptibility. This review will focus on recent advances uncovering immunological and microbial mechanisms that protect against allergies, in particular within the context of a farming environment. A whole body of epidemiological data disclosed the nature of the protective exposures in a farm. Current evidence points towards an important role of the host microbiome in setting an immunological equilibrium that determines progression towards, or protection against allergic diseases. Conclusive mechanistic insights on how microbial exposures prevent from developing allergic diseases in humans is still lacking but findings from experimental models reveal plausible immunological mechanisms. Gathering further knowledge on these mechanisms and confirming their relevance in humans is of great importance in order to develop preventive strategies for children at risk of developing allergies.

Introduction

From the moment of birth, we are exposed to a variety of allergens via inhalation, ingestion or contact with the skin. For reasons that remain mostly undetermined, certain individuals get sensitized to harmless allergens, like house dust mite (HDM), cockroach or grass pollen, and this is then characterized by the presence of allergen-specific Immunoglobulin E (IgE) in serum. Sensitization to allergens is a risk factor to develop diseases such as allergic asthma, hay fever, atopic dermatitis or food allergies [1].

The incidence of allergies is 20 times higher in affluent Westernized countries compared to the incidence in low-income countries [2]. The original hygiene hypothesis, first described by Strachan in 1989, proposed that the rise of allergic diseases was caused by the reduced infection pressure, which resulted from increased hygiene and reduced household size [3],[4]. Even though this was demonstrated for certain non-lower tract respiratory infections, not all infections prevent the development of allergies [5]. For instance, children with recurrent lower respiratory tract infections caused by rhinovirus or respiratory syncytial virus have a higher chance to develop allergic asthma later in life [6]. These conflicting epidemiological data has led to confusion about the hygiene hypothesis. Meanwhile, there was a growing understanding that the increased incidence of allergies might be caused by a change in lifestyle rather than merely by reduced infection rate. Indeed, the Western lifestyle, partly characterized by an indoor living environment, high sugar and high fat diet and reduced physical activity, causes microbial dysbiosis in the gut and skin [7]. Therefore, as a refinement of the original hygiene hypothesis, Rook launched ‘the old friends hypothesis’ and Haahtela the ‘biodiversity hypothesis [8],[9]. Both hypotheses state that a loss of symbiotic relationships with a diverse range of beneficial microbes is responsible for inadequate immune regulation and increased incidence of allergic diseases. To unveil the nature of protective versus risk exposures, multiple association studies have been performed in areas where the incidence of asthma is still low, mainly in rural areas of Europe and the US. This has led to a better understanding about the mechanisms and possible species involved in protection or exacerbation of allergies in children that are at risk to develop allergic diseases. For instance, the risk for asthma development caused by a genotype in the 17q21 locus is confined to children with a history of respiratory viral infections [10]-[12] whereas the same genotype confers protection from asthma in farmer’s children [13]. The intriguing fact that one locus can either drive the disease or confer protection to the disease

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depending on environmental pressure, highlights the importance of environmental exposures as a regulator for the development of asthma.

A farming environment protects children from developing asthma

Population based studies have consistently shown strong inverse associations between growing up on traditional farms and the prevalence of childhood asthma. In an early study by Riedler et al. conducted in rural areas in Austria, a low prevalence of asthma, hay fever and allergic sensitization was found among the farm children compared to the nonfarm children [14]. Other studies followed, for example in Bavaria and the UK, confirming a strong 'farm effect' in the prevalence of allergies [15],[16]. In the UK, Perkin and Strachan compared rural nonfarm and farmers' children and showed that farmers' children, when compared to the rural nonfarm children, had significantly less current wheeze (adjusted odds ratio (aOR) 95% confidence interval (95% CI): 0.67 (0.49-0.91)) [16]. A longitudinal study from Canada determined not only the prevalence but also the incidence of asthma among 13.524 asthma-free children aged 0-11 at baseline [17]. The 2-year cumulative incidence was 2.3% among children living in farm environments, 5.3% in children form rural nonfarm environments and 5.7% from non-rural environments [17]. Many more studies have confirmed these findings showing that the 'farm effect' is a very robust epidemiological observation. The strongest effect was seen among Amish children who live a most traditional dairy farm lifestyle, whereas Hutterite children, which are genetically related to Amish children but upraised in families of industrial farmers, are not protected from allergies and asthma [18].

Protective effects that are inherent to a farming environment

Farm lifestyles differ significantly from nonfarm households and therefore different aspects of farm life i.e. exposure to animal barns and fodder, and nutritional factors such as the consumption of unprocessed raw cow's milk have been scrutinized. In the GABRIEL Advanced Studies, Illi and colleagues performed stratified analyses based on types of farms and a child's exposure to specific farm characteristics [19]. Interestingly, protection was mostly associated with dairy farming in combination with cultivation of grain and corn. Furthermore, by investigating individual exposures of farm children, these authors identified important protective effects from 'contact with cows and straw', 'exposure to manure' and 'consumption of unprocessed raw cow's milk' [19]. The importance of individual farm characteristics was further investigated by Ege *et al*. in the PARSIFAL study, showing protective effects from keeping pigs, frequent stay in the animal sheds, child's involvement in haying and again, the consumption of unprocessed cow's milk [20]. In the ALEX study, protection was associated with exposure to animal sheds and consumption of unprocessed raw cow's milk, but mostly if these exposures occurred in the 1st year of life (aOR (95% CI): 0.14 (0.04-0.48)) hinting at the importance of early life exposures [14]. These cross-sectional observations have been confirmed by findings in the prospective PASTURE birth cohort study [21]. In the first year of life mothers were asked to keep a weekly diary documenting nutritional factors, symptoms and illnesses of the child as well as it's exposure from month 2 to month 12. Loss *et al*. reported that exposure to animal sheds significantly reduced the risk

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of wheeze in the first year of life. This reduction in risk was see for the children at risk of developing asthma, i.e. in carriers of the risk allele of chromosome 17q21 [13].

A farming environment protects children during an early window of opportunity

Whether this early 'window of opportunity' expands to prenatal life was studied by Douwes *et al.* [22]. The child's and maternal exposure during pregnancy to animals and/or grain and hay reduced the risk of asthma symptoms. A combination of prenatal and current child's exposure was strongly inversely related to use of asthma medication (OR (95% CI): 0.50 (0.30–0.82)) and asthma ever (OR (95% CI): 0.50 (0.33–0.76)). These findings suggest that prenatal exposure may contribute to the asthma protective effect, but that continued exposure may be required to maintain protection. The role of maternal exposures have furthermore been addressed by Pfefferle *et al*. by measuring cytokine levels in cord blood of children born to farming and nonfarming mothers in the PASTURE cohort [23]. Maternal exposure to farming activities and farm dairy products during pregnancy affected cytokine production patterns in the offspring by increasing levels of TNF-alpha and IFN-y. Schaub *et al*. showed that farm exposure during pregnancy resulted in increased number and function of cord blood Treg cells [24]. These findings show that farming exposure during pregnancy can shift the relative distribution towards increased Th1 cells (IFN-y producing) and decreased pro-allergic Th2 cells (IL-5 producing) and additionally reinforce protection from allergies by enhancing Treg cell function [24].

Microbial exposures in a farming environment

The aforementioned 'farm effect' studies highlight the importance of the consumption of raw cow's milk and exposure to microbes through plants, animals, their fodder and bedding. The dietary effects of raw cow's milk have been recently reviewed elsewhere [25] and will not be discussed further. An important marker of bacterial exposure in nonfarm and farmers' children is the indoor level of endotoxin (a.k.a. bacterial lipopolysaccharide), which is a cell-wall component of gram-negative bacteria. Children carry their microbial exposures from the outside (and the animal sheds) into their beds where mattresses are individual reservoirs of bacterial exposures. Braun-Fahrländer *et al*. reported that endotoxin levels from the child's mattress were inversely related to the occurrence of asthma [26]. The levels and determinants of bacterial endotoxin, mold, β-(1,3)-glucans and fungal extracellular polysaccharides (EPS) in indoor dust samples of farm children, Steiner school children and reference children were in turn evaluated by Schram-Bijkerk *et al*. [27]. Furthermore, Karvonen *et al*. investigated microbial exposures as predictor of asthma using a birth cohort [28]. The study showed no significant associations of single microbial markers with risk of asthma. However, the sum of indicators for fungi, Gram-positive bacteria, and Gram-negative bacteria showed a significant inverted-U-shaped association with the incidence of asthma. The highest risk was found at medium levels and the lowest risk at the highest level. Taken together these analyses showed that farm children are not only exposed to more bacteria and endotoxin but also to increased levels of fungal substances and that increased levels of exposure were inversely associated with the risk of developing childhood asthma [28].

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These investigations were further refined by culturing fungi and applying SSCP methods to detect specific bacteria from indoor sources. Ege *et al.* showed that not one single exposure but the diversity of bacterial and fungal exposure was inversely related to asthma [29]. This increased diversity is mainly attributable to richness, i.e. the presence of many, mostly low abundance cattle-associated taxa in farm homes. When considering taxa with higher relative abundances, a farm-like microbial compositional structure is also found in nonfarm homes [30]. Such exposure was equally associated with protection from asthma in the nonfarm homes suggesting that the inverse association between bacterial exposure in farm environments is not a peculiar exposure limited to very few people still living a traditional farm life but is generalizable to other indoor environments.

If environmental microbial exposures play an important role in the protection from childhood asthma, then the question arises whether the environmental microbiome shapes a child's microbiome and thereby affects disease risk. Depner *et al*. showed that the farm exposure was positively associated with bacterial diversity found in the mattress dust samples and in nasal swabs of 86 school age children [31]. Alterations in nasal microbiota but not of throat microbiota were associated with asthma. Asthmatics had lower α- and ß diversity of the nasal microbiota as compared to healthy control children. Hence, the nasal bacterial phylogenetic trees showed a higher similarity within asthmatic patients compared to those of healthy controls (ß diversity parameter) and asthmatics had a lower bacterial richness within the nasal sample (α diversity parameter). Furthermore, asthma was positively associated with a specific OTU from the genus Moraxella in children not exposed to farming, while in farm children Moraxella colonization was unrelated to asthma [32]. These findings suggest that the environmental microbiome shapes the nasal microbiome, which in turn contributes to the asthma protective effect. Recently, Depner *et al.* showed that also the gut microbiome contributes to asthma risk and is shaped by environmental exposures [33]. A number of facets were relevant: the compositional structure at age 2 months, the speed of maturation until age 12 months and production of the bacterial metabolites butyrate and propionate at age 12 months. The environment (farm, cats) and lifestyle factors such as nutrition, antibiotic use, sibship size, smoking and delivery mode all influenced the gut microbiome development [33].

Together, epidemiological data from numerous studies show that growing up on a farm reduces the risk from developing asthma and allergic diseases and the microbial exposures that are inherent to a farming environment play an important role in this protective effect.

The microbiome defines host susceptibility to allergic diseases

The microbiome is well established as a key modulator of immune development and disease susceptibility. Indeed, over the past decade numerous reports have described the constituents of the microbiome at our body barrier surfaces, and their respective associations with diseases, including asthma and other allergic disorders. These studies have been the focus of in-depth reviews [34],[35] and will not be covered here in detail, rather, we will highlight key recent findings and some of the principles that are coming to the fore.

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Healthy versus disruptive airway microbiome

For many years the general consensus has been that the diversity of the microbiome is associated with health or disease; in essence, higher diversity is linked with health, while a lower diversity can be considered 'dysbiotic' with an over-representation of microbes that promote disease. This broad conclusion still holds true; however, as studies have become more sophisticated, the field has been able to start to interrogate whether: the process of microbial community diversification (maturation) is important; the presence of single highly immunomodulatory bacteria strains is important; or whether certain 'keystone' strains of bacteria provide the structure for community development?

Studies from mouse models have clearly shown that microbes play a central role in susceptibility to allergy. In the most extreme scenario, i.e. the complete absence of microbes, axenic mice exhibit exacerbated allergic responses, which can be reverted to 'normal' when they are colonized [36]. The timing and nature of this colonization looks to be important, as gnotobiotic mice that have a delayed formation of their microbiome in early life, can exhibit a hyper-IgE syndrome [37]. Although the mechanisms are yet to be determined, it is likely that sequential colonization of microbes at barrier surfaces goes hand-in-hand with immune maturation at key developmental steps. Evidence for this has been provided in the lung [38], gut [39] and skin [40], where discrete immune maturation windows have been reported, particularly within the first post-natal month. An important new study indicates similar principles are relevant in humans [33]. Specifically, a substantial proportion of the protective effect against allergies linked to farming environments (discussed above), can be explained by the Estimated Microbial Age (EMA) of the gut microbiome during the first year of life. It is well established that there are substantial changes to the constituents of the gut microbiota in the first year of life, particularly due to changes in diet and environment [41]. EMA represents the constituents of the gut microbiota that are abundant at different ages. This study highlighted the association of the genera Roseburia and Coprococcus with protection against asthma, and their production of the short chain fatty acid (SCFA), butyrate (discussed below). Earlier studies have also suggested the presence of SCFA-producing microbes is important during the formation of the microbiome in the first year of life [42],[43]. Overall, the evidence is clear that early life formation of the microbiome is key to immune maturation and protection, or susceptibility, to allergies and asthma; however, how this could be controlled or shaped as a general preventative approach remains to be determined. A key question requiring further investigation is whether individual microbes, for example SCFA-producing members of the Roseburia genus, could be effective against asthma. Or, might the most effective means of establishing a beneficial microbiome be through creating an environment that suits a community of microbes? A deeper understanding of microbial ecology is an important step in this direction. Network analysis and the identification of microbial hubs, as performed in the Depner *et al.* study [33], that contain keystone species of microbes will be central for translating observations to preventative and therapeutic approaches. The most effective microbial intervention strategy might need to involve the formation of microbial communities, which in combination elicit sustained health-promoting benefits.

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Less is understood about the potential of harnessing the airway microbiome to prevent asthma. As compared to the gut, the airway microbiome already matures within the first 2 postnatal months in humans [38], possibly linked to the maturation of the airway environment and the lack of pressure from dietary changes. Both in mice and in humans, this process is linked with immune maturation [38],[44]. Whether the early life airway microbiome can be manipulated and whether 'inhaled probiotics' could be used to prevent asthma remains to be seen. Currently, inhaled microbial components, such as endotoxin and those present in farm dust [45], can elicit protection against allergic inflammation in mouse models, and remain at the forefront of potential development. A better understanding of microbial metabolites (discussed below) might also lead to novel inhaled therapeutics or preventative strategies.

The role of fungi, viruses and helminths

The bulk of current understanding surrounds the role of bacteria in protection or susceptibility to asthma. Emerging evidence, however, is starting to shed light on the potential role of the other members of the microbiome–fungi, viruses and in some settings, helminths.

Although fungi are substantially less abundant than bacteria, they are influential members of the microbiome. Studies from mouse models have shown that the disruption of the gut fungal community by amphotericin B, or fluconazole, leads to an exaggerated allergic airway inflammatory response following allergen exposure [46]. Opportunistic growth of certain resistant fungi, including *Wallemia mellicola*, was highlighted as being a particularly key member of a dysbiotic mycobiome [46],[47]; although it should be noted that disrupting fungal communities changes the microbial ecosystem overall, including, albeit indirectly, the bacterial constituents of the microbiota. A recent study using gnotobiotic mice with defined bacterial and fungal communities has further highlighted the impact of fungi on immune development and susceptibility to allergies [48]. Recent reviews have provided in-depth commentaries on these developments [49],[50]. Numerous questions remain to be investigated with respect to the impact of fungi in promoting health. The mycobiome may be transient and is heavily influenced by environmental exposures–more so than bacteria; experimental mice housed in specific pathogen free conditions harbour very low levels of fungi as compared to wild mice, certain colonies [51] and gnotobiotic models [48]. Thus, although fungi unquestionably can impact immune status, there remains a great deal to unravel in order to clearly define their role in immune homeostasis and disease susceptibility.

Viruses remain, in large, limited to causing asthma exacerbations and pathogenesis, rather than protection. Exposure to viruses does hold the potential to 'educate' the immune system, such that counter-regulatory type 1 responses act to suppress type 2 responses against subsequent allergen challenge [52]; however, the impact of resident viruses on immune status is less well understood. Data from mouse models have shown that infection with norovirus can elicit immune maturation in the gut in the absence of bacteria [53], largely through the induction of type I interferons [54]. This is an intriguing and exciting

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development, but the implications for allergies and asthma are unknown. With metagenomic sequencing approaches becoming more widely implemented in human cohort studies, in the foreseeable future, it will become clearer whether viruses exert a sustained pressure on the microbiome and consequently influence its development, or whether their role is limited to their well-established detrimental effects.

Helminths and their products have profound immunomodulatory effects, which were recently reviewed [55]. Helminth-derived products hold great potential, and risk, as approaches to prevent allergies and asthma. As helminths have co-evolved with humans, they have developed means to modulate the immune system, for example with cytokine mimics and binding proteins [56]-[58]. However, given their foreign nature, they do carry the risk of becoming the target of host immunity, and thus being neutralized. These microbial products have been finely tuned during evolution and could be considered as templates for the development of novel biologics [59]. It is noteworthy, that beyond direct effects of helminths and their products, they have indirect effects by shaping the local tissue environment and consequently the microbiome. Infection of mice with Heligmosomoides polygyrus has been shown to modulate the development of allergic airway inflammation by altering bacterial communities and their metabolites, in particular, SCFA [60]. The presence of SCFA, such as butyrate and propionate, is a reoccurring theme in many settings (consumption of dietary fibre, helminth infection, farming environment [61]), and is linked with protection against asthma. Thus, this microbial metabolite pathway holds some of the greatest promises with respect to the development of preventative and intervention strategies. Few other microbial metabolites have thus far been linked with protection against asthma [61]. Recently, P-cresol sulphate (PCS), a product of L-tyrosine metabolism, has been shown to be elevated in the plasma of non-asthmatics as compared to asthmatics in two child cohort studies [62],[63]. High levels of PCS are found in individuals with chronic kidney disease, due to an inability to filter this metabolite, and in that context it is considered a toxin [64]. However, within healthy limits, PCS is linked a reduced incidence of asthma, and data from mouse models have recently shown that microbial metabolism of L-tyrosine to PCS protects against allergic airway inflammation [65]. Notably, L-tyrosine is metabolized by the gut microbiota, however PCS is distributed systemically and exerts its function directly on epithelial cells of the airways to reduce inflammation [65]. Further studies, identifying and validating microbial metabolites in the gut and airways holds great potential for the discovery of new therapeutic approaches.

Allergies are caused by aberrant immune responses at barrier sites

Allergies such as asthma and atopic dermatitis are regarded as typical type 2 immune responses. Under normal conditions, type 2 effector responses are incited to fight helminth infections and control tissue repair and barrier functions of the mucosa or skin. They are orchestrated by a range of innate type 2 immune cells, such as ILC2s, mast cells, basophils, eosinophils and alternatively activated macrophages. However, when allergic individuals are sensitized to harmless allergens they can mount aberrant type 2 responses that are driven by adaptive allergen-specific type 2 helper T-cells (Th2). Th2 cells and ILC2s produce cytokines like IL-4, IL-5, IL-13 and IL-9 that coordinate the type 2 immune responses in the

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lung, such as B-cell class switching to IgE, eosinophil expansion and activation, mucus overproduction and hyperreactivity of smooth muscle cells (Figure 1). Altogether, these events cause the clinical symptoms that are typically seen in asthma [66].

Sensitization to allergens occurs prior to disease manifestations and remains unnoticed because this stage is asymptomatic. Allergic sensitization involves a multifactorial process that eventually leads to the generation of allergen-specific Th2 cells (Figure 1). A whole body of evidence from experimental models for allergic diseases revealed that type 2 conventional dendritic cells (cDC2s) are crucial for allergic sensitization, both in the skin and in the lungs (reviewed in [67]). cDC2s reside in barrier tissues where they take up allergens and migrate to the draining lymph node to induce Th2 responses. Nevertheless, cDC2s cannot establish Th2 responses without receiving instructions from epithelial cells and tissue-derived factors that create an environment that is favourable for allergic sensitization. For a long time, epithelial cells were believed to exclusively function as barrier cells but it is clear now that they actively participate to allergen-induced immune responses by producing cytokines (e.g. IL-33, IL-25, TSLP, IL-1α, M-CSF and GM-CSF), chemokines (e.g. CCL2 and CCL20) and danger signals (e.g. ATP, Uric acid and HMGB1) (reviewed in [68]).

Even though allergy is typically mounted by type 2 immune responses, evidence in humans and in animal models indicates a remarkable heterogeneity in T-cell responses to allergens. For instance, simultaneous occurrence of HDM-specific Th2 and Th17 are found in patients with severe atopic dermatitis [69], and asthmatic patients harbour fully functional HDM-specific Tregs alongside pathogenic HDM-specific Th2 cells [70]. The recent evolution of single cell analysis tools such as single cell RNA Sequencing and single cell TCR sequencing, allow researchers to perform full transcriptomics analysis of allergen-specific T-cells in humans and in animal models of allergic diseases [71]-[73]. These studies all show that T-cell responses to allergens are indeed very heterogeneous and the relative contribution of different types of allergen-specific T-cell might thus shape the immune response to allergens. As such, this could determine whether an individual develops tolerance to allergens or not, and how the disease will be clinically manifested. How these allergen-specific T-cells are formed remains an area of intensive investigation, and this is inextricably linked to the area of research how environmental triggers influence allergic diseases. The next section will discuss a selection of recent studies that described how environmental triggers could confer protection against allergies.

Protective triggers can target different key players in the pathogenesis of allergies

Epithelial cells

Barrier cells like keratinocytes in the skin and bronchial epithelial cells in the lungs and the gut are the first to encounter allergens but these are also constantly exposed to all sorts of environmental cues. Indeed, these tissues are heavily colonized by commensal microbes and changes in the microbial composition can be sensed by their innate immune system. Microbes and allergens, like pollen and HDM, contain pathogen-associated molecular patterns (PAMPs), danger associated molecular patterns (DAMPs) and proteases that can activate Pattern Recognition Receptors (PRRs) and Protease-Activated Receptors on epithelial cells. Intriguingly, Toll-like Receptor 4 (TLR4; a PRR that gets activated by

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endotoxin) expression on epithelial cells is indispensable for sensitization to HDM by stimulating epithelial cells to create a favourable type 2 milieu (Figure 1) [74]. However, certain dosing and timing of endotoxin administration hampers Th2 sensitization in murine models of allergic asthma, amongst others by TNF-mediated upregulation of Tbet in cDC2s and their subsequent production of IL-12 that counteracts Th2 skewing [75] and by inhibiting epithelial cells to produce type 2 skewing cytokines like GM-CSF and IL-33 [45]. Mechanistically, endotoxin or farm dust exposure to murine lungs induced the expression of *tnfaip3* in bronchial epithelial cells. TNFAIP3 (also known as A20) is a negative regulator of the NF-κB pathways resulting from activation of TLR, TNFR and IL-1R family members. A20 thus functions as a rheostat of cell activation by these particular PRRs. Mice lacking *tnfaip3* in epithelial cells are not protected from developing HDM-induced allergic airway inflammation upon endotoxin or farm dust administration [45]. This shows a central role of epithelial barrier cells in the farming-mediated protection against allergies.

Dendritic cells

Even though epithelial cells serve as an initial checkpoint that activates DCs and ILCs to determine the outcome of the allergic response, protection against allergies could also be conferred directly at DC level. Cowshed dust exposure induced the expression of CD86 and CD80 on cultured DCs and turned them into more 'regulatory' DCs that produced mainly IL-10 and were incapable to sensitize mice to allergens [76],[77]. Also a *Lactococcus lactis* isolate from cowshed exert a direct modulatory effect on DCs *in vitro* [78]. Upon uptake of the bacteria, endosomal acidification led to release of bacterial RNA, which in turn activated TLR signalling and induced expression of Th1 polarizing cytokines and co-stimulatory molecules. *Lactococcus lactis* stimulated DCs induced Th1 responses in co-culture with OT-II cells and upon *in vivo* transfer, they were unable to sensitize mice to OVA [78]. Administration of *Acinetobacter lwoffii,* another cowshed bacterial isolate, to murine neonatal lungs prevented the HDM-induced expansion in cDC2s and monocyte-derived DCs [79]. This correlated with reduced numbers of IL-13$^+$ CD4$^+$ T-cells, which predominated during neonatal age, resulting in an improvement of airway hyperreactivity (AHR) to HDM [79]. Another recent study showed that simultaneous HDM and endotoxin administration induces TNF in adult lungs, which in turn upregulates T-bet expression in cDC2s and skew toward Th1 immune responses [75]. Neonatal lungs require higher doses of endotoxin to circumvent the Th2-prone immune responses via this mechanism. Hence, both studies suggest that growing up on a farm, with increased microbial exposures, could protect children at young age from developing allergies by affecting DC populations in the lungs. Likewise, release of microbial metabolites in the gut, like SCFAs, was shown to affect bone marrow precursors of DCs and induced increased seeding of DCs in the lungs, with reduced Th2-skewing capacity [80]. Since the farming environment is strongly associated with the composition of gut microbiome [33] and subsequently altered exposure to immunomodulatory bacterial metabolites such as SCFAs, this pathway could be an additional mode of action to confer protection against asthma and allergies (Figure 1).

Mechanistic studies that explain whether and how farming could directly affect DC function *in vivo* are still needed. Nevertheless, Bosteels *et al*. recently described that cDC2s can switch on a different transcriptional program under inflammatory conditions, like viral infections or high dosage of HDM, that fades out the phenotypic and functional dichotomy between

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cDC1s and cDC2s [81]. Interestingly, this event relies on cell-intrinsic TLR- and type 1 IFN signalling, indicating that environmental triggers can directly change the transcriptional program of DCs, which reverses their predestined function [81]. Whether such a reversal in DC function could also occur in a milder setting compared to inflammation, such as environmental triggers from farms, remains to be investigated.

Adaptive immunity: T-cells and B-cells

One of the most studied mechanisms by which a farming environment can protect from developing allergies is the increased generation of Tregs that would outcompete pathogenic Th2 cells. Epidemiological data revealing that children from farm exposed mothers bear higher numbers of Tregs, indicate that this is indeed a plausible mechanism of protection [24],[82],[83]. Also, evidence from animal models reveal that microbial colonization is required for the generation of Tregs that could reduce susceptibility to allergic sensitization in neonates [44]. The expansion of protective Tregs relied on the interaction with PD-L1, which peaked on lung cDC2s at neonatal age (Figure 1) [44]. Multiple farm-derived agents from microbial source (polysaccharide A, SCFAs) or non-microbial source (N-glycolylneuraminic acid) induced Tregs in mice that could counteract allergic airway responses [42],[84]-[86]. How these farm-derived products can directly affect $CD4^+$ T-cells to induce Tregs is partially explained for SCFAs, like butyrate and propionate. Butyrate is a histone deacetylase (HDAC) inhibitor, ultimately resulting in increased histone acetylation at the *Foxp3* promoter and thus chromatin accessibility for transcription [87],[88]. Likewise, propionate signals via the SCFAs receptor G-coupled protein receptor (GPR)43 to induce IL-10 production and Foxp3 expression in colonic Tregs, presumably also via its HDAC inhibitor activity [89].

Bacterial colonization is often associated with type 17 immune responses in barrier tissues and this has also been reported in farm dust-exposed mice and in agricultural workers (unpublished observations and [90]). Ohnmacht et al. revealed that microbiome exposure induces RORγt expression in Treg cells and that these $ROR\gamma t^+$ Tregs, as well as microbiome-induced Th17 cells, counteract Th2-mediated immunity [91]. In the gut, these microbiome-induced $ROR\gamma t^+$ Tregs express high levels of CTLA4, which regulated CD80 and CD86 expression on gut DCs in order to restrain the population of Th2 cells [91]. A similar form of microbiome-mediated T-cell plasticity has been shown in the skin [92]. They found that *Staphylococcus epidermidis*-specific $ROR\gamma t^+$ $CD4^+$ and $CD8^+$ T-cells exhibit a poised type 2 effector program, that can be easily switched on upon injury, which results in the release of alarmins like IL-33, to promote tissue repair [92]. Microbiota-induced type 17 responses were shown before to enhance innate barrier function of the skin and might thus also affect allergic sensitization via the skin (Figure 1) [93]. However, Th17 responses to the microbiome can also induce inflammatory responses in the skin and aggravate Th2-mediated allergic airway inflammation, but only in adult mice [40]. As such, a fine-tuned balance between T-cell subsets appears to be important for tissue homeostasis and perturbations in the microbiome during a crucial time window may account for a deregulated type 2 responses leading to allergic diseases.

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Whether and how the environment affects humoral immunity is less extensively studied compared to the environmental effects on T-cells. Remarkably, in-depth protein-sequence analysis revealed that many allergens share similar linear and conformational epitopes with helminth-derived products [94]. This could partially explain how certain regions where the population is being subjected to endemic helminth infections are protected against allergies. Repeated exposure to these antigens from helminths may indeed induce immunoglobulin cross-reactivity and tolerize against allergens, similar to what is attained by allergen immunotherapy [95]. Two studies also showed that cross-reactive antibodies to bacteria in the gut or in the lungs and to allergens also emerge at neonatal age and these antibodies can prevent mice from developing allergies at later age. One of them showed that an IgA antibody to α-1,3-Glucan recognizes a specific strain of *Enterobacter cloacae* as well as Bla g 2, an important cockroach allergen [96]. Similarly, HDM allergens share epitopes with phosphorylcholine from *Streptococcus pneumoniae* [97]. Interestingly, immunization with these bacterial antigens *E. cloacae* or inactivated *S. pneumoniae* at neonatal age could protect mice from developing cockroach-induced or HDM-induced asthma, respectively, at adult age [97]. Also, an older study showed that antibodies that are reactive to polysaccharides, obtained from *S. pyogenes* immunized neonatal mice, from bacteria can also be reactive to chitin, a component of HDM, and *Aspergillus fumigatus* and dampened airway inflammation in mice [98]. These studies reveal that neonatal contact with bacteria are able to modulate the immune response to allergens via the generation of cross-reactive antibodies, suggesting that this could be an attractive approach to develop prevention therapies for children at risk to develop allergies.

Innate immune cells

ILC2s are repeatedly reported to play an important role in allergic type 2 responses by producing key cytokines like IL-13, responsible for goblet cell hyperplasia and mucus production, and IL-5, which promotes tissue eosinophilia [99]. ILC2s are tissue-resident sentinels that rapidly react to tissue-derived factors like cytokines, chemokines and alarmins and they can therefore function as a local immune checkpoint [99]. Actually, the effector function of ILC2s, as well as the effector function of Th2 cells, relies on local cues from the tissue microenvironment that are produced by structural cells such as epithelial cells or stromal cells [100],[101]. It is therefore not unlikely that microbial products can modulate the function of ILC2s and as such, play a role in the prevention of developing allergies. In depth analysis of the transcriptional profile and chromatin landscape at single cell level showed that the transcriptional identity of ILCs in the small intestine is strongly affected by the microbiome [102]. A recent study showed that the microbial metabolite butyrate inhibited IL-5 and IL-13 production in murine and human ILC2s, presumably via its HDAC inhibitory activity, not by activating GPR41 or GPR43 [103]. This resulted in reduced airway hyperreactivity and relieved airway inflammation in a mouse model for allergic asthma [103]. Similarly, butyrate countered eosinophil functions in vitro, like adhesion to the endothelium, migration and survival, also independently of GPR41 and GPR43 [104]. So far, there is no conclusive evidence that eosinophils are intrinsically affected by the tissue microbiome although enteric eosinophil numbers and phenotype are affected in germ free mice [105].

Lately, there has been an increasing focus of the impact of microbiome on the memory capacity of innate immune cells, or so-called ‘trained immunity’ [106],[107]. Innate immune

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cells were historically considered to have no memory because most of them are relatively short-lived. However, the concept of 'trained immunity' describes that these cells can attain memory to previous infections and exposures by epigenetic and metabolic reprogramming of blood monocytes and tissue-resident macrophages (peripheral trained immunity) but also that of progenitor cells (central trained immunity) [108]. In the allergy field, recent evidence showed that innate immune cells could be modulated on the long term by prior exposures. For instance, allergen immunotherapy induced a sustained shift in the composition and heterogeneity of the innate immune compartment of treated allergic patients [109]. Also, in murine lungs, IL-33 exposure during the neonatal time window was shown to affect seeding and responsiveness of ILC2s later in life [110]. Interestingly, a longitudinal study found that DNA methylation profiles of immune cord blood cells are associated with innate immune responses that are linked with the risk to develop childhood asthma [111]. Another recent study links the upper airway microbiota to the risk to develop allergic rhinitis, and this is partly mediated by altered DNA methylation modules in mucosal airway cells [112]. These findings support the hypothesis that early microbial exposures may affect structural and innate cells in the long term. Nevertheless, more in-depth translational *in vivo* research is required to fully elucidate the mechanisms of microbiome-induced trained immunity and its role in the development of allergies.

Conclusion

The above-mentioned epidemiological data and the selection of mechanistic studies indisputably show that various environmental factors are able to impact important players in the pathogenesis of asthma and allergic diseases. These findings are promising for the quest to halt the rise in asthma and allergic diseases that is caused by a Western lifestyle. Nevertheless, in the interest of therapy development, there is a pressing need to identify the exact nature of protective triggers and to further unravel their mechanisms of action. From the whole range of microbiome-derived agents and metabolites, only a few have been studied so far. As such, a broader computational analysis of the microbiome metabolomics could reveal other important pathways in the protection against asthma and allergies. Additionally, not much is currently known about inter-microbial and microbe-host networks that could benefit the host. Consequently, a positive trend in the field is the shift towards the investigation of microbial communities and its interactions with the host immune system. Notably, most environment-driven immune regulations seem to occur at neonatal age when the host immune system is still being 'educated', meaning, whenever the susceptibility for asthma and allergic diseases is being set. Indeed, several studies in mice show that certain microbial products affect neonatal immunity whereas it becomes impossible to intervene with that same stimulus during adult age. Therefore, it remains very important to make that distinction based on age, and elucidate which exposures can affect the immune response at which time point. Certain environmental factors could have a potential in the clinic as a preventative strategy for children at risk while others would better be applied as an intervention therapy for established allergies that are already manifested by debilitating symptoms.

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Acknowledgements:

This work is supported by Longfonds (the Netherlands), by means of the AWWA (A World Without Asthma) consortium, of which the authors are part of. JD is supported by the Flanders Research Foundation (FWO grant number 12W0719N). BJM is an NHMRC Senior Research Fellow and VESKI Innovation Fellow.

Conflict of interest:

EvM reports to have received personal fees from PharmaVentures, OM Pharma, Springer-Verlag, Elsevier, Peptinnovate, Turun Yliopisto, Tampereen Yliopisto, Helsingin Yliopisto, European Respiratory Society, Deutsche Pharmazeutische Gesellschaft, Massachusetts Medical Society, the Chinese University of Hong Kong, European Commission, Boehringer Ingelheim, Universiteit Utrecht Faculteit Diergeneeskunde, Universität Salzburg, Georg Thieme Verlag, and Japanese Society of Pediatric Allergy and Clinical Immunology, outside of the submitted work. In addition, EvM has patent LU101064 pending, royalties paid to ProtectImmun for patent EP2361632, and patents EP1411977, EP1637147, and EP 1964570 licensed to ProtectImmun. The other authors declare no commercial or financial conflict of interest.

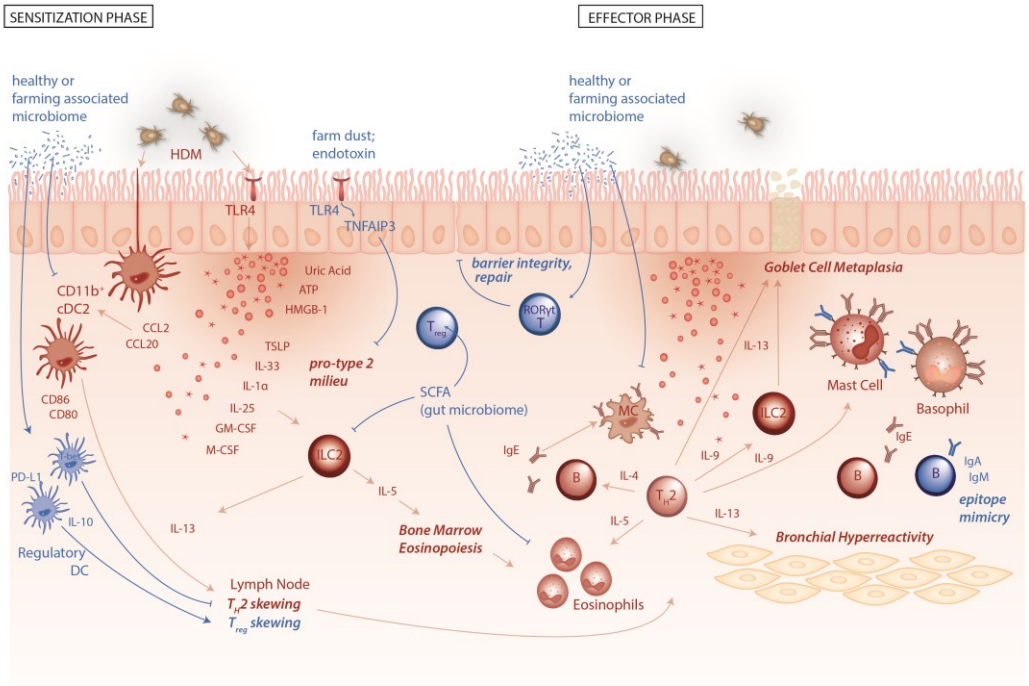

Figure 1: Proposed immunological mechanisms of microbial-mediated protection from asthma. The pathogenesis of allergic asthma involves a multi-faceted pathway (depicted in shades of red) that is initiated in epithelial cells, which are located at the interface between the barrier tissue and the external exposures. In the absence of immune regulation, allergens can induce allergen-specific type 2 helper T-cells (Th2), only via an intimate

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crosstalk between epithelial cells and type 2 conventional dendritic cells (cDC2s). Aeroallergens can be picked up by cDC2s that extend their dendrites into the airway lumen, but they need instructions from Toll-like receptor 4 (TLR4)-activated epithelial cells that create a favorable type 2 milieu by producing chemokines (CCL2, CCL20), cytokines (interleukin (IL)-1α, IL-33, IL-25, TSLP, GM-CSF, M-CSF) and danger-associated molecular patterns (DAMPs; Uric acid, ATP, HMGB1) to migrate to the draining lymph node and induce type 2 responses. These epithelial-derived factors also activates type 2 innate lymphoid cells (ILC2s) to exert an accessory role, important for type 2 immune responses during the sensitization phase as well as during the effector phase. Exposure to protective microbial triggers (depicted in blue) can affect multiple players in the pathogenesis of asthma (depicted in red), via different pathways (discussed in the main text). Abbreviations: HDM, house dust mite; SFCA, Short Chain Fatty Acid; TNFAIP, TNF alpha induced protein 3; Treg, regulatory helper T-cell; PD-L1, programmed death-ligand 1; MC, monocyte-derived cell, Ig, immunoglobulin. Arrows: 'induce' or 'counteract'.

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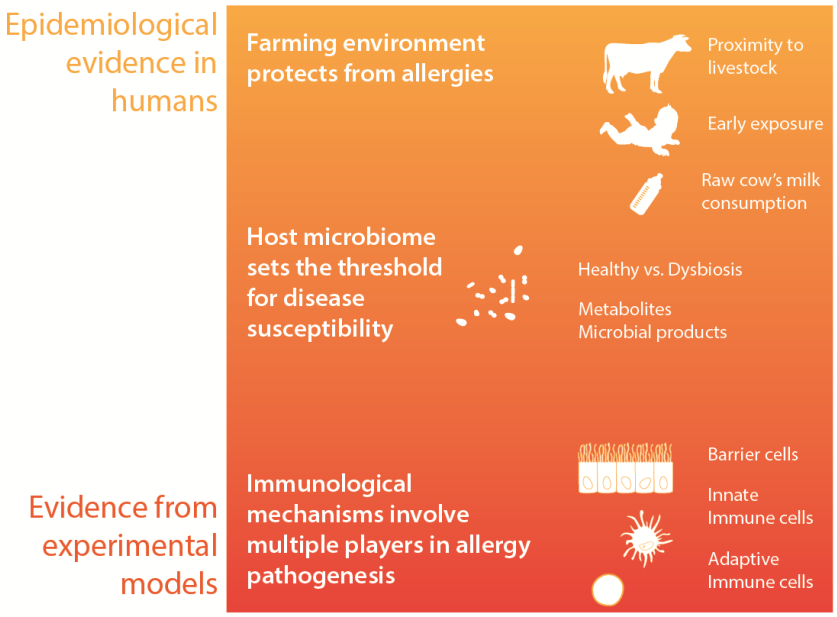
Graphical abstract



Epidemiological data has revealed that certain exposures in a farming environment protect children from developing allergies. Growing evidence highlights the importance of the host microbiome in setting the threshold of susceptibility to allergies and data emerging from experimental models is starting to elucidate the underlying mechanisms.

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