



From Amish farm dust to bacterial lysates: The long and winding road to protection from allergic disease^{*}

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ABSTRACT

Allergic diseases typically begin in early life and can impose a heavy burden on children and their families. Effective preventive measures are currently unavailable but may be ushered in by studies on the “farm effect”, the strong protection from asthma and allergy found in children born and raised on traditional farms. Two decades of epidemiologic and immunologic research have demonstrated that this protection is provided by early and intense exposure to farm-associated microbes that target primarily innate immune pathways. Farm exposure also promotes timely maturation of the gut microbiome, which mediates a proportion of the protection conferred by the farm effect.

Current research seeks to identify allergy-protective compounds from traditional farm environments, but standardization and regulation of such substances will likely prove challenging. On the other hand, studies in mouse models show that administration of standardized, pharmacological-grade lysates of human airway bacteria abrogates allergic lung inflammation by acting on multiple innate immune targets, including the airway epithelium/IL-33/ILC2 axis and dendritic cells whose *Myd88/Trif*-dependent tolerogenic reprogramming is sufficient for asthma protection in adoptive transfer models. To the extent that these bacterial lysates mimic the protective effects of natural exposure to microbe-rich environments, these agents might provide an effective tool for prevention of allergic disease.

1. Introduction

In September 2002, a seminal review in *The New England Journal of Medicine* summarized strong epidemiologic evidence demonstrating that allergic disease incidence in developed countries had steadily risen over the previous three decades [1]. The author, Jean-François Bach, noted that “concomitantly, there has been an obvious decrease in the incidence of many infectious diseases ... as a result of antibiotics, vaccination, or more simply, improved hygiene and better socioeconomic conditions.” In support of his point, he highlighted studies performed in Austria, Germany, and Switzerland in which children whose parents were farmers and who lived on their parents’ farms were found to be less subject to allergies than other children from the same rural region who were not raised on a farm [2,3]. These studies also showed that allergies

were less frequent when the children were exposed early and for a prolonged period to farm animals and cow’s milk [4]. Building on these results, a paper published in the same issue of the *Journal* demonstrated an inverse relation between endotoxin levels in bedding (an index of microbial exposure) and the incidence of atopic diseases among children living in rural areas [5]. Bach commented that “there is a certain irony in the fact that we must now search for new ways to reproduce the infectious diseases against which we have been fighting with great success over the past three decades.” An accompanying Editorial (“Eat Dirt – The Hygiene Hypothesis and Allergic Diseases”) acknowledged that “eating dirt or moving to a farm are at best theoretical rather than practical clinical recommendations for the prevention of asthma”, but recognized the powerful translational and mechanistic implications of this body of work and stated that “the challenge will be to elucidate the immune

Abbreviations: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; CoA, coenzyme A; DC, dendritic cell; GPR, G protein-coupled receptor; HDM, house dust mite; LPS, lipopolysaccharide; OR, odds ratio; OVA, ovalbumin; PASTURE, Protection against Allergy: Study in Rural Environments; PBL, Peripheral blood leukocyte; TLR, Toll-like receptor; Treg, T regulatory; SCFA, short-chain fatty acid.

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mechanisms involved and to determine the extent of exposure that will have the desired outcome — the development of a healthy child with a very low risk of disease” [6].

Much has been learnt in the years after these articles were published. Our understanding of “infections” has especially been transformed by our deepening knowledge of microbes, their metabolic products, and their relations with immunity and health. Nevertheless, the link between microbial exposures, immune function, and allergic disease holds steady and is in fact becoming stronger. This article seeks to outline a long, winding scientific journey that is elucidating allergic disease pathogenesis while (hopefully) bringing us closer to allergic disease prevention.

2. The “farm effect”

Few epidemiologic findings demonstrate the potential protective effects of environmental exposures on allergic disease risk as eloquently as the “farm effect” [7]. Children raised on traditional farms are strongly protected from asthma and allergies. Indeed, the first large study of farm children in Alpine Europe showed that exposure of children younger than 1 year to stables and consumption of farm milk was associated with lower frequencies of asthma (1% vs. 11%), hay fever (3% vs. 13%), and atopic sensitization (12% vs. 29%) compared to a reference group of age-matched, non-farm children [4]. This protection was largely explained by the child’s contact with farm animals in early life [5,8]. The original observations made in traditional European dairy farms were later extended to children with shared ethnic background but diverging environmental living conditions, such as rural and urban Mongolia, rural and urban China, Russian and Finnish Karelia (reviewed in [7,9]), demonstrating the consistency and robustness of these protective effects.

An essential component of the farm effect is exposure that occurs prenatally or within the first 2–3 years of life [4,10], when the developing immune system is most amenable to modulation by exogenous cues. Indeed, farm exposure of the mother during pregnancy, rather than exposure of the infants after birth, was associated with increased expression of TLR2 and CD14 on blood cells [11], and levels of these innate immune receptors were 2.7- and 2.2-fold higher, respectively, in farm than non-farm children at school age [12]. Moreover, increased IFN- γ and TNF secretion and higher expression of TLR7 and TLR8 were detected cord blood mononuclear cells from prenatally exposed farm children, compared with non-farm children [13,14], and increased numbers and improved function of T regulatory (Treg) cells were found in neonates of farming mothers compared to neonates born to non-farm mothers [15]. Peripheral blood cells from farm children had high expression of other innate immune genes, such as IRAK-4 and RIPK-1, and enhanced expression of regulatory molecules such as IL-10, TGF- β , SOCS-4, and IRAK-2 [16]. All these data indicate that prenatal and/or early life exposure to a traditional farm environment upregulates innate immune responses.

It is noteworthy that the protection induced by farm exposure in early life can persist into adulthood. A study from Finland showed that contact with farm animals in early childhood reduces the risk of atopic sensitization, doctor-diagnosed asthma and atopic eczema at age 31 by 33%, 26% and 23%, respectively [17], while a study from Sweden found a lifelong protective effect of childhood farm living on the prevalence of allergic rhinitis [18]. Recent work in the Protection against Allergy: Study in Rural Environments (PASTURE) cohort added complexity to these temporal patterns by demonstrating that exposure to animal sheds in the first three years of life strongly protects farm children from hay fever at age 10.5 years [odds ratio (OR)= 0.26 compared to non-farm children]. However, optimal protection also required continuous consumption of farm milk from infancy to school age [19].

2.1. A suite of protective farm exposures

A traditional farming lifestyle involves multiple activities and diverse exposures that have complex effects on allergic disease phenotypes. The early farm studies demonstrated that exposure to stables and consumption of unprocessed cow’s milk have independent protective effects on asthma and atopy [4]. The protective effects of farming were consistent with respect to hay fever and atopy, but results for asthma were quite heterogeneous, raising the possibility that not all farms are created equal, and specific farm characteristics may have greater effect than others. A study based on a detailed questionnaire that documented not only the farm’s characteristics but also the child’s exposures showed that protection was typically associated with growing up on traditional farms that engage in cattle breeding and cultivation of grain and corn for animal feeding and bedding. Moreover, and most remarkably, just three exposures (contact with cows and straw, and consumption of farm milk) were sufficient to recapitulate the overall farm effect for asthma, while exposure to fodder storage rooms and manure explained protection for atopic dermatitis [20]. In contrast, the protective effect of farming on hay fever and atopic sensitization was only partially explained by the exposures tested in that study, suggesting that additional protective factors exist in a farming environment, and distinct mechanisms protect from asthma and atopy [20]. Recent work has reassessed the impact of raw cow’s milk consumption on the prevalence of asthma and allergies in farm children. A meta-analysis corroborated the protective effect of raw milk consumption early in life (<1–5 years) on asthma (OR, 0.58), hay fever or allergic rhinitis (OR, 0.68) [21]. Of note, the effect on asthma was observed not only in children raised on farms (OR, 0.62) but also in children living in rural areas but not on a farm (OR, 0.60), suggesting that the effect of farm milk consumption is independent of other farm exposures. Preliminary evidence attributes the effect to heat-labile molecules residing in the fat fraction [21].

Recent studies in China indicate that cows and their milk are not the only animal source of protection within traditional farms. Over the past three decades, China has undergone huge socio-economical changes. In parallel, there was a rapid rise in the prevalence of childhood asthma and allergic diseases [22]. Asthma symptoms were three times more common in 10-year-old children from Hong Kong, a highly urbanized, densely populated metropolis, than in mainland (Guangzhou and Beijing) children [23]. Moreover, a significantly lower rate of asthma was reported in Conghua, a rural county in southern China where agriculture predominates and farmers grow lychees and related crops, compared with urban Hong Kong children (2.5% vs. 5.3%). Interestingly, in that study, the protection conferred by living in a rural environment was mainly attributable to poultry exposure [24].

2.2. The role of environmental and personal microbiota

The finding that exposure to farm animals, straw and unprocessed milk essentially accounted for the farm effect begged the question of what in those exposures induces protection. The possibility that environmental microbes are critical for allergic disease protection was raised by early studies that associated protection with exposure to a variety of microbial products such as endotoxin, muramic acid and β -glucans [5, 25,26]. These initial observations were confirmed by and extended in a seminal paper that assessed the microbial diversity to which farm children are exposed. Dust samples from the mattresses and bedrooms of farm and non-farm children were screened for bacteria and fungi with culture-independent or -dependent techniques. Microbial (bacterial and fungal) diversity was significantly higher in farmhouse dust and was strongly and inversely associated with the probability of an asthma diagnosis. Indeed, the microbial diversity score explained a significant proportion of the farm effect on asthma, while the association with atopy was weak, again suggesting that distinct mechanisms protect from atopy and asthma [10]. The patterns of cultivable microflora in dust from the bedrooms of farm children were similar to those from animal

sheds and barns, suggesting that viable microorganisms are transported from animal sheds and barns into the child's house [27]. Therefore, children living on a farm are exposed to microbes both when visiting animal sheds and barns and when indoors, and both exposures likely contribute to the protective effect of farming.

The asthma- and allergy-protective role of microbe-rich environments was further refined by modeling differences in house dust microbiota composition between farm and non-farm homes in Finnish birth cohorts. In children who grow up in non-farm homes, asthma risk decreased as the similarity of their home bacterial microbiota composition to that of farm homes increased [28]. Protection was independent of microbial richness and total bacterial load and was associated with reduced production of proinflammatory cytokines in response to bacterial cell wall components. These findings were replicated in rural German children whose asthma risk was lower if they lived in non-farm homes with indoor microbiota more similar to Finnish farm homes. Thus, indoor dust microbiota composition appears to be a robust predictor of asthma risk [28]. When combined across populations and locales, studies of this kind may converge on core sets of candidate environmental microbial communities suitable for interventional studies aimed at allergic disease protection.

It is noteworthy that both environmental and personal microbiota – especially those in the gut, which houses the largest microbial diversity and burden [29] – appear to influence the trajectory to (or away from) allergic disease acting trans-generationally [30]. Environmental exposures during pregnancy are thought to shape maternal microbiomes and immune function, which in turn influence the immune and microbiome profiles of the fetus by interacting with her/his genetic makeup. Relying on epigenetic mechanisms [31], these interacting influences train neonatal innate immunity and regulate its ability to interact with the “pioneer microbes” that are vertically transmitted from the mother and initially colonize neonatal body habitats [32]. Depending on their composition and functional properties [33], these maternal pioneer microbes shape immune function, which controls the rate and types of environmental microbes accumulated into these body habitats during the first year of life. The nascent gut microbiome then integrates dietary [29] and antibiotic [34] encounters that decisively affect microbiome membership, productivity and development, and gut microbial products influence immune function both locally and at remote mucosal sites [30]. In combination, these processes determine trajectories of microbiota development, innate and adaptive immune development, and ultimately risk of allergic disease (Fig. 1).

Consistent with this scenario, data from several independent birth cohorts demonstrate that early life gut microbiota perturbations (often described as dysbiosis) are associated with increased risk of atopy and/or asthma later in childhood [35–37]. Conversely, the farm studies decisively illuminate the link between microbiome development and asthma protection by showing that timely gut microbiome maturation during the first year of life significantly contributes to the protective effect of farming on childhood asthma. Modeling gut microbiome maturation between 2 and 12 months of age in the PASTURE birth cohort showed that the estimated microbiome age at 12 months was strongly associated with previous farm exposure and reduced risk of asthma at school age, and also mediated a substantial proportion of the asthma protection associated with the farm effect [38].

2.3. The gut-lung axis

Metabolites produced by gut microbiota are both key determinants of host-microbe mutualism and critical mediators of the host-microbe crosstalk that influences inflammation in peripheral tissues, such as the lung – a concept referred to as the gut-lung axis [39]. For instance, an elegant mouse study demonstrated that dietary fermentable fiber content changed the composition of gut and lung microbiota and dampened lung allergic inflammation [40]. Gut microbiota metabolized the fiber, thereby increasing the concentration of circulating short-chain fatty

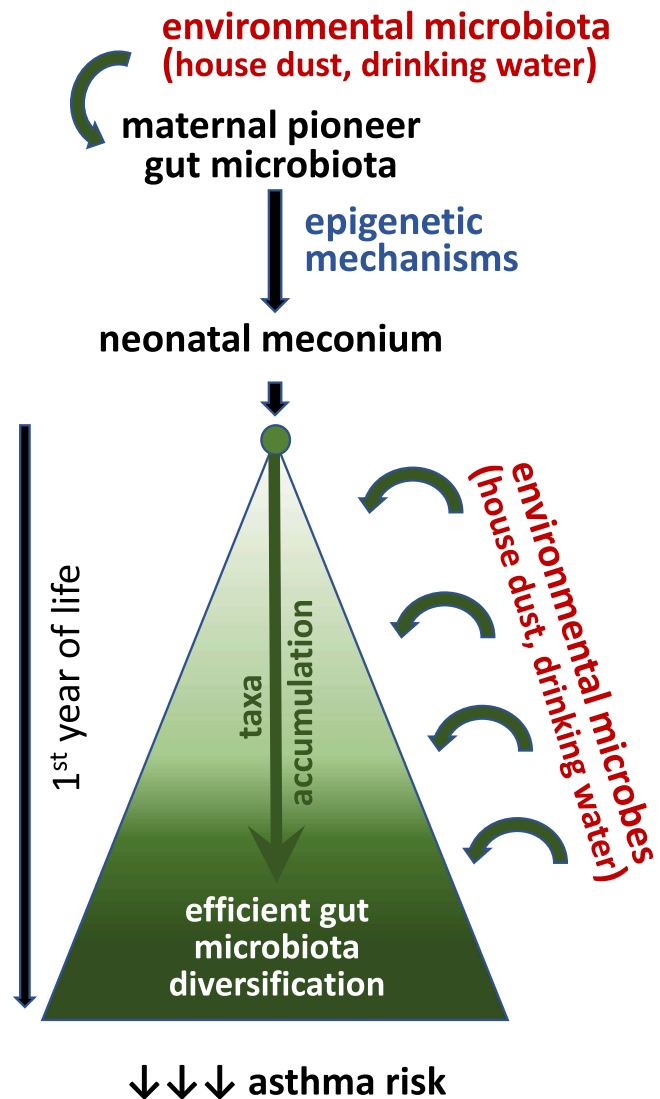


Fig. 1. : Interactions between environmental and personal microbiota influence microbiome development and allergic disease risk. Environmental exposures during pregnancy shape maternal microbiota and immune function, which in turn influence the immune and microbiome profiles of the fetus. Relying on epigenetic mechanisms, these interacting influences train neonatal innate immunity and regulate its ability to interact with the “pioneer microbes” that are vertically transmitted from the mother and initially colonize neonatal body habitats. Depending on their composition and functional properties, these maternal microbes shape immune function, which controls the rate and types of environmental microbes accumulated into these body habitats during the first year of life. The nascent gut microbiome then integrates dietary and antibiotic encounters that decisively affect microbiome membership, development and productivity, and gut microbial products influence immune function both locally and at remote mucosal sites. These processes determine trajectories of microbiota development, innate and adaptive immune development, and ultimately risk of allergic disease.

acids (SCFAs) that protected against allergic inflammation in the lung. Treatment of mice with the SCFA propionate altered bone marrow hematopoiesis and seeded the lungs with dendritic cells (DCs) impaired in their ability to promote type-2 immune cell responses [40].

In humans, the PASTURE study demonstrated that bacterial communities with the potential of producing SCFAs, especially butyrate, contributed to asthma protection. Inverse associations were found between asthma, levels of fecal butyrate, the bacterial taxa that predict butyrate production (*Coprococcus*, *Roseburia*), and the relative abundance of the gene encoding butyryl-coenzyme A (CoA):acetate-CoA-

transferase, a major enzyme in butyrate metabolism. These findings suggest that microbiota may protect from human asthma through the gut-lung metabolic axis [38]. In other studies, higher fecal levels of iso-butyric, iso-valeric and valeric acid at age 3 years (indicative of more rapid maturation of gut microbiota) were found in Swedish farm children compared to rural controls, and high levels of valeric acid were associated with low rate of eczema at 8 years of age [41]. Conversely, the microbiome of infants who developed allergic sensitization during childhood lacked genes encoding key enzymes for carbohydrate breakdown and butyrate production [42].

Mechanistically, SCFAs bind to G protein-coupled receptor (GPR)–41 and GPR-43, which induce immune-regulation and suppress airway inflammation by promoting the expansion of colonic Treg cells [43] or influencing hematopoiesis and the function of DCs recruited to the lung [40]. For instance, despite an absence of SCFAs in the lungs of mice sensitized and challenged with house dust mite (HDM), systemic levels of propionate enhanced the development of DCs impaired in their ability to activate a type-2 immune response. Recruitment of these DCs to the lung decreased airway inflammation [40]. Trans-placental transport of microbial products including SCFA and other metabolites [44] might explain why the children of mothers that had higher serum acetate levels during pregnancy had a significantly lower rate of wheeze in the first year of life [45]. SCFA binding to GPRs expressed on leukocytes, including neutrophils, eosinophils, and macrophages, was also found to influence myelopoiesis and the migration and function of myeloid cells [46–48].

3. A tale of two US farming populations: Amish and Hutterites

The European farm studies and the variations-on-the-theme they inspired were undoubtedly ground-breaking. The paradigms they proposed, which are collectively known as the Hygiene or Biodiversity Hypothesis and that emphasize protective microbial exposures associated with livestock, water and plants [49,50], were and still are extremely influential (Fig. 2). However, deep gaps in knowledge remained and needed to be filled, especially if this work was to be translated into asthma- and allergy-preventive strategies. Traditional farmers and non-farmers differ in so many aspects of their lifestyles that

dissecting and identifying factors potentially responsible for differences in allergy and asthma prevalence was arduous – in other words, the controls might have been too distant from the test population. Moreover, as discussed in more detail below, some concerns about controls (or lack thereof) also arose about work in animal models.

These issues were addressed in a study in which the differences in asthma and allergy prevalence observed between European farmers and non-farmers were recapitulated and characterized in two unique U.S. farming populations, the Amish of Indiana and the Hutterites of South Dakota (reviewed in [51]). Amish and Hutterites share a complex history. These populations arose in the 16th century during the Anabaptist movement in Switzerland (Amish) and the South Tyrol (Hutterites). Religious persecution forced members of these groups to emigrate to North America. The Amish arrived in the 18th and 19th centuries and settled on single-family farms in Pennsylvania, Ohio and Indiana. The Hutterites emigrated in the 19th century and settled on three communal farms in South Dakota. Both populations grew rapidly thanks to high fertility and strong values for large families. As of 2022, there were almost 350,000 Amish (<https://groups.etown.edu/amishstudies/>) and about 50,000 Hutterites (<https://hutterites.org/>) living across North America.

Both populations have retained traditional lifestyles based on their strict interpretation of the bible. Their primary language is their original Swiss (Amish) or Tyrolean (Hutterites) German dialect. Children learn English only starting in the 1st grade of school and graduate after the 8th grade. In addition to both having large sibship sizes, Amish and Hutterites are very similar with respect to many of the factors known to prevent or promote asthma. For example, they both have diets rich in fat, salt, and raw milk, low rates of childhood obesity, long durations of breast-feeding, minimal exposure to tobacco smoke and air pollution, and taboos against indoor pets, TV, and the internet. However, these two populations differ in one critical respect. The Amish avoid all modern technology and thus practice traditional single-family dairy farming, using horses for fieldwork and transportation. Their farms keep not only cows but also other animals to which all children of both sexes are exposed from an early age. In contrast, the Hutterites live on large communal farms and embrace modern technology. Their industrial-sized farms can house up to 100,000 turkeys, 20,000 hogs, and 600

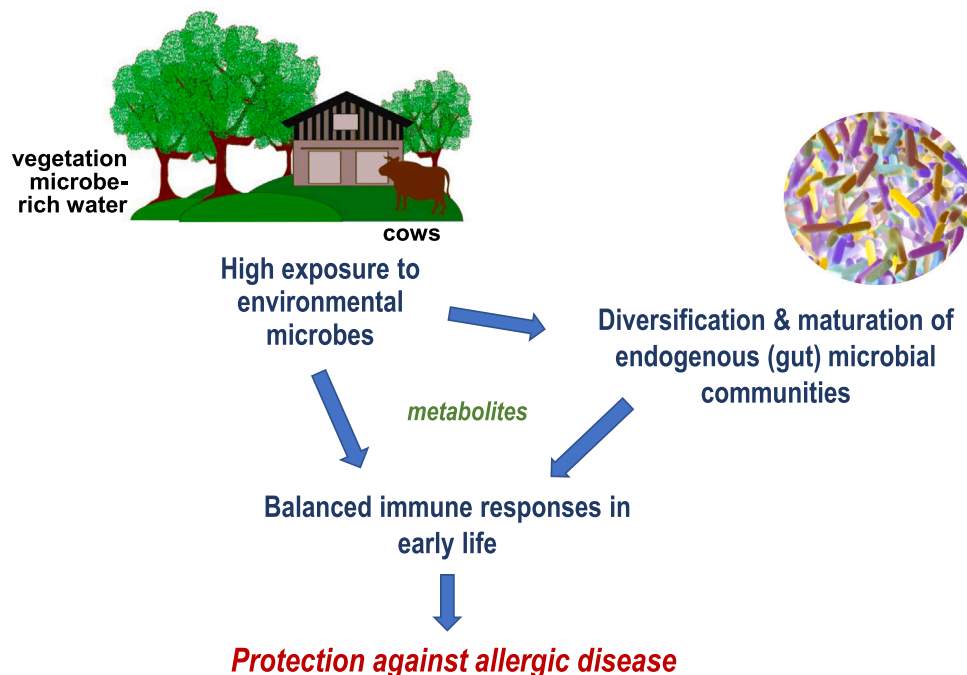


Fig. 2. : Relationships among environmental microbial exposures, endogenous microbial communities, and allergic disease protection, as proposed by the Hygiene and Biodiversity Hypotheses.

cows. Because of the size of the Hutterite barns and their distance from their homes, young Hutterite children are essentially not exposed to farm animals or barns. Moreover, due to their strict division of labor, girls and women have little exposure to the animals and barns throughout their lives. Notably, these differences in farming practices and lifestyle are paralleled by dramatic differences in the prevalence of asthma and allergic sensitization: 5.2% vs. 21.3%, and 7.2% vs. 33.3% in Amish and Hutterite schoolchildren, respectively [52,53].

These observations prompted us to compare immune profiles in Amish and Hutterite schoolchildren. We also obtained airborne house dust from the bedroom and living room in Amish and Hutterite homes [54]. Of note, in preliminary studies to evaluate whether differences could be attributed to population history, we assessed ancestry by principal component analysis of genomic DNA markers and compared allele frequencies using genome-wide single nucleotide polymorphisms. These studies revealed remarkable genetic similarities between Amish and Hutterite children compared to other European populations.

Our findings were strong and often unexpected. Common allergens (cat, dog, house dust mite, cockroach) were detectable in airborne dust from 40% of Amish and 10% of Hutterite homes. In contrast, endotoxin was measurable in airborne dust from all homes, and median levels were 6.7-fold higher in Amish homes compared to Hutterite homes.

Peripheral blood leukocytes (PBLs) from Amish children contained increased proportions of neutrophils, decreased proportions of eosinophils, but similar proportions of monocytes compared to Hutterite children. By flow cytometry, neutrophils from Amish children expressed lower levels of CXCR4, CD11b and CD11c, suggesting that these cells may have recently emigrated from the bone marrow [54]. Although monocyte proportions were similar in Amish and Hutterite children, monocytes from Amish children exhibited an anti-inflammatory phenotype characterized by lower levels of HLA-DR (typically, a consequence of repeated microbial stimulation) and higher levels of the inhibitory molecule ILT3. No difference in percentages of CD3⁺CD4⁺FoxP3⁺CD127⁺ Treg cells was observed between Amish and Hutterite children. The production of a broad panel of cytokines in response to innate stimulation (LPS) was lower in Amish compared to Hutterite children, while no differences between the two groups were detected upon adaptive stimulation (anti-CD3 + anti-CD28 antibodies) [54].

The striking differences in PBL proportions observed between Amish and Hutterites were reflected in the gene expression profiles of these cells: at a false discovery rate of 1%, 1449 genes were upregulated in Amish PBLs as compared with Hutterite PBLs, whereas 1360 genes were upregulated in Hutterite PBLs as compared with Amish PBLs. Whole Genome Co-Expression Network Analysis identified a number of co-expression modules, the most prominent of which was associated with Amish and Hutterite status and therefore also with neutrophil and eosinophil proportions [54]. Eighteen of the genes in this module were overexpressed in Amish PBLs and clustered in a network of innate immune genes that had as hubs TNF and IRF7, two key components of the innate immune response to microbial stimuli [55,56]. IRF7 also regulates type I interferon transcription and is therefore essential for innate airway responses against viruses [57]. Also notable among the genes more expressed in the Amish was *TNFAIP3/A20*, which encodes a ubiquitin-editing enzyme involved in limiting the activity of multiple NF- κ B dependent inflammatory pathways [58]. These findings suggested that in the Amish, intense and sustained exposure to microbes activates innate pathways that shape and calibrate downstream immune responses.

As for T cells, no differences were found in the percentages of CD4⁺, CD8⁺, or double negative T cells, or in the percentage of each T cell subset expressing the effector/memory marker CD45RO. Percentages of CD3⁺CD4⁺FoxP3⁺CD127⁺ Treg cells were also similar in Amish and Hutterite children even though the percentage of CD45RO⁺ICOS⁺ Treg cells was significantly increased in the Amish (36.8 $\pm$ 16.2% vs. 24.2 $\pm$ 11.8%). These markers are characteristic of activated Tregs with more

suppressive function in both humans and mice [59]. However, our most striking findings pertained to CD8⁺ T cells and their phenotypes. The majority of CD8⁺ T cells from Amish children lacked the co-stimulatory molecule CD28 (CD28^{null}), and the proportion of CD28^{null}/CD8⁺ T cells within total CD8⁺ T cells correlated negatively and quite strongly with IgE in Amish but not Hutterite children ($P = 0.025$ and $P = 0.75$ in Amish and Hutterite, respectively).

Moreover, serum IgE levels were correlated to three genes in the innate gene network (*IRF7*, *TNF*, and *TNFAIP3*) that had significantly increased expression in Amish children [54]. *TNF* and *TNFAIP3* also exhibited a positive correlation with the frequency of CD28^{null} CD8⁺ T cells, and all three genes exhibited a negative correlation with serum IgE in Amish children. No significant correlations were found in Hutterite children. Further studies are required to better characterize the CD28^{null} CD8⁺ T cell population selectively found in Amish children and define its functional properties and contribution to asthma and allergy protection.

3.1. Mechanistic studies: Innate immunity is required for asthma protection in the Amish/Hutterite model

Our cell phenotyping results pointed to a potentially profound impact of distinct environmental exposures on the immune profiles of Amish and Hutterite children but did not (and could not) conclusively prove that these profiles were causally related to protection. To create a functional framework to interpret our observations and directly address the protective role of innate immunity in the Amish environment, we turned to mouse models of allergic asthma.

Mouse models had been previously used to characterize some of the agents putatively involved in protection against allergic disease on traditional European farms. For instance, inhalation of cowshed dust extracts from Alpine dairy farms protected mice from ovalbumin (OVA)-induced broncho-alveolar lavage (BAL) eosinophilia and airway hyper-responsiveness (AHR) [13]. Two bacterial species most abundant in cowshed microflora (the Gram-negative *Acinetobacter lwoffii* F78 and the Gram-positive *Lactococcus lactis* G121) reduced allergic inflammation in mice and induced a Th1-polarizing program in DCs [60]. Interestingly, mouse studies in a trans-generational model of OVA-induced experimental asthma showed that maternal intra-nasal exposure to A. lwoffii F78 protected against asthma development in the progeny [61]. This exposure transiently increased lung and serum proinflammatory cytokines and up-regulated lung TLR mRNA. Conversely, suppression of TLRs was observed in placental tissue. Of note, asthma protection was completely abolished in heterozygous offspring born to A. lwoffii F78-treated TLR2/3/4/7/9^{-/-} homozygous mother mice. The mild local and systemic inflammatory response induced by A. lwoffii F78 exposure was also absent in these mothers [61]. These data established a direct relationship between maternal bacterial exposures, functional maternal TLR signaling, and asthma protection in the progeny.

In other studies, intra-nasal administration of *Bacillus licheniformis* from animal sheds [62] or farm-derived Gram-positive *Staphylococcus sciuri* W620 [63] also protected against allergen-induced type-2 lung inflammation. More recently, in an HDM-dependent model of asthma, epithelial cell expression of *A20/TNFAIP3*, one the innate immunity genes most overexpressed by PBLs of Amish children [54], was required to protect mice chronically exposed to low-dose endotoxin or farm dust [64]. Moreover, expression of *A20/TNFAIP3* mRNA and protein was significantly reduced in air-liquid interface-cultured bronchial epithelial cells from mild and severe asthmatics compared with healthy subjects [64].

The results from animal studies mentioned above were interesting because they consistently supported the asthma-protective role of farm-derived microbial agents. However, these results lacked specificity because mice were exposed exclusively to microbes or farm products epidemiologically associated with protection – in other words, appropriate negative controls were missing. In contrast, relying on the unique

characteristics of the Amish/Hutterite model, we could design experiments in which extracts of dust collected in Hutterite farm homes lacking asthma-protective activity served as controls for identically prepared extracts of dust collected from asthma-protective Amish farm homes. In this rigorously controlled setting, intra-nasal administration of Amish house dust extracts was sufficient to strongly protect OVA-treated mice from experimental asthma, as evidenced by decreases in AHR, lung eosinophilia, IL-13 and IL-5 secretion in BAL, and OVA-specific serum IgE [54]. In contrast, mice that received Hutterite house dust extracts (either at the same concentration as the Amish ones, or in amounts that reflected the total dust load in the relevant homes) were not protected from experimental asthma [65]. The inhibitory effects of these extracts required innate immunity because protection was completely abrogated in mice lacking both MyD88 and Trif, molecules critical for multiple innate immune signaling pathways. These results made two essential points: exposure to natural products from a traditional farm environment is *sufficient* to induce protection from allergic asthma, and innate immunity is *necessary* for this protection.

Interestingly, in a subsequent study we could show that intra-nasal administration of *Hutterite barn* dust extracts inhibited HDM- and OVA-induced AHR and BAL eosinophilia as effectively as Amish barn dust extracts [66]. These results demonstrated that an environment with strong asthma-protective properties exists not only on Amish but also on Hutterite farms. However, unlike Amish children, young Hutterite children typically do not spend time near farm animals or in the barns, which are much larger and located at much greater distances from their homes than are Amish barns, and thus do not benefit from this asthma-protective exposure.

4. From farm microbes to microbial lysates

The substantial body of evidence discussed above shows that allergic disease pathogenesis is critically influenced by alterations in the environmental microbial exposures with which humans evolved. Studies in humans and germ-free mice suggest that live gut microbiota may also protect against asthma [36,37]. These data have heightened interest in the development of translational approaches to disease prevention based on microbial interventions [67]. However, the manufacturing, standardization and regulation of such interventions remains a daunting task. Moreover, while epidemiological data consistently point to the central role that diversity of exposures plays in allergic disease protection, individual components able to confer protection have not been identified, at least so far. This is hardly surprising: asthma- and allergy-protective cowsheds and unprocessed cow's milk contain "soups" of multiple potentially relevant environmental elements [50]. In fact, protection from food allergy, atopic dermatitis and asthma was associated with increased diversity of food introduction [68] and the diversity of farm animal exposure during pregnancy was associated with lower risk of atopic dermatitis [69]. Finally, the diversity of the environmental microbiome was associated with a 14–38% reduction in the risk of asthma in two distinct farming population studies [10].

These considerations motivate current efforts to decrease asthma burden by using standardized products that derive from multiple microbial (typically, bacterial) strains and modulate immune maturation and function. The most widely adopted strategy seeks to avert the development of childhood asthma by enhancing resistance against lower respiratory tract infections, common harbingers of the disease [70,71]. Among the microbial products currently most advanced in terms of clinical testing and mechanistic characterization, some (e.g., OM-85) are bacterial lysates while others (e.g., MV130) are whole-cell heat-inactivated bacterial mixtures.

4.1. OM-85, a bacterial lysate

OM-85 is a standardized, low-endotoxin alkaline lysate of 21 bacterial strains from five genera (*Moraxella*, *Hemophilus*, *Klebsiella*,

Staphylococcus and *Streptococcus*) found in the human airways [72]. OM-85 has been used empirically by over 100 million individuals for the prophylaxis of early life recurrent upper respiratory infections and its safety profile is impeccable [73]. In studies performed in Europe and Australia, OM-85 reduced the rate and duration of wheezing attacks in pre-school children with acute respiratory infections [74] and increased the time to severe lower respiratory illnesses in at-risk infants [75]. An NIH-sponsored trial (NCT02148796) is currently testing whether OM-85 can delay the occurrence of the first episode of wheezing lower respiratory tract illness in high-risk U.S. infants.

It is noteworthy that although childhood asthma is often ushered in by viral infections that target the lower respiratory tract and induce wheezing [71], it is not obvious that agents able to suppress virus-induced wheezing will automatically suppress the development of childhood asthma. Asthma and wheezing are related but distinct phenotypes that can and should be modeled in dedicated mechanistic studies. OM-85 has been studied mechanistically for its ability to inhibit infection by respiratory viruses such as respiratory syncytial virus [76], rhinovirus [77] or influenza [78], but what sets OM-85 apart from all other bacterial preparations currently studied for their asthma-protective potential is the extent and detail to which this agent has been characterized in models of asthma and allergic lung inflammation.

For instance, mechanistic studies in allergen-sensitized rodents treated orally with OM-85 showed inhibition of airway inflammation accompanied by increased proportions of activated Treg cells and attenuated DC responses in the airways, especially the trachea, upon allergen challenge [79,80]. Adoptive transfer of OM-85-treated unfractionated tracheal CD4⁺ T cells protected allergen-sensitized animals from airway inflammation, and these effects were reduced upon depletion of T cells bearing gut-homing receptors [79]. A trans-placental immunomodulation study later described dampened allergic airway inflammation in the offspring of mothers treated orally with OM-85 during pregnancy, which was paralleled by altered numbers and phenotypes of conventional DCs in lungs and lymph nodes [81] and transcriptomic signatures of accelerated bone marrow myelopoiesis [82].

So far, in both human and animal studies OM-85 has been administered orally - a choice shaped by decades of clinical practice [83] and the notion that immune cells programmed in the gut may modulate distant mucosal responses [40]. On the other hand, our studies in the Amish model demonstrated that microbial products extracted from traditional farm dust abrogate allergic asthma and support the integrity of the airway epithelial barrier when administered intra-nasally, a route that grants direct access to the regulatory networks residing in the airway mucosa ([54,66] and dos Santos, unpublished results). Therefore, we investigated whether direct administration of OM-85 to the airway compartment of mice protects against experimental allergic asthma and we characterized the cellular and molecular mechanisms underpinning protection through this natural route.

Our analyses combined hypothesis-generating and hypothesis-driven approaches, and relied on distinct allergens (OVA, *Alternaria alternata*), different mouse strains (Balb/c and C57BL/6), and a variety of models in which OM-85 was administered concurrently with, or before and during, allergen sensitization. Our results consistently and conclusively showed that OM-85 administration to the airway compartment strongly protected against allergic asthma in mice. Multiple components of the innate and adaptive immune response were affected by this bacterial lysate: the epithelium//IL-33/ILC2 axis that initiates type-2 inflammation in the airway mucosa, allergen-induced type-2 responses that promote asthma-related respiratory phenotypes, and *Myd88/Trif*-expressing DCs whose OM-85-induced tolerogenic reprogramming (evidenced by the expression of CD103 and CD207) was sufficient to adoptively transfer protection from allergic asthma [84] (Fig. 3). It is noteworthy that intra-nasal OM-85 administration dampened allergen-induced type-2 airway responses rather than promoting type-1 immune deviation, thus inducing immune profiles reminiscent of those

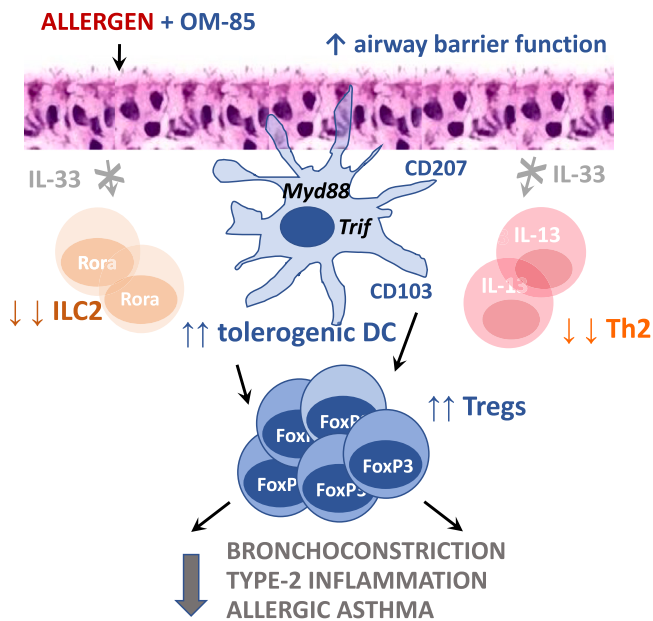


Fig. 3. : Mechanisms of action and targets of the OM-85 bacterial lysate in the airways. OM-85 administration to the airway compartment protects against experimental allergic asthma by affecting multiple components of the innate and adaptive immune response: the epithelium//IL-33/ILC2 axis that initiates type-2 inflammation in the airway mucosa, allergen-induced type-2 responses that promote asthma-related respiratory phenotypes, and Myd88/Trif-expressing DCs whose OM-85-induced tolerogenic reprogramming (evidenced by the expression of CD103 and CD207) is sufficient to adoptively transfer protection from allergic asthma. DC: dendritic cell; FoxP3: forkhead box P3; ILC2: innate type-2 lymphoid cell; Rora: receptor-related orphan receptor-alpha; Tregs: T regulatory cells.

found in both asthma-protected Amish farm children and asthma-protected mice exposed to Amish farm dust through the airway compartment [54,85].

This work provided the first demonstration that a defined cell type (OM-85-reprogrammed *Myd88/Trif*-expressing DCs with enhanced tolerogenic properties) is sufficient to transfer microbe-induced asthma protection to allergen-sensitized mice. The critical protective role played by these cells was highlighted not only by adoptive transfer experiments, but also by studies that linked OM-85-dependent phenotypic and functional changes in DC populations to enhanced induction of Treg cells and increased Treg/Th2 cell ratios in the lung [84]. On the other hand, our extensive phenotyping and transcriptomic analyses showed that the effects of OM-85 likely involved additional immune and structural lung cells and pathways. Indeed, airway OM-85 administration fully suppressed cardinal asthma phenotypes (AHR, BAL eosinophilia, lung Th2 cytokine expression) induced by *Alternaria* in a model that is driven exclusively by i.n. allergen exposure and thus carries high physiological relevance. Interestingly, OM-85 was also able to block early *Alternaria*-induced innate events (IL-33 secretion in BAL and ILC2 recruitment to the lung), and this ability appeared to reflect airway epithelial barrier stabilization through profound, time-dependent effects on the epithelial transcriptome. In line with this notion, prophylactic but not concurrent i.n. treatment with OM-85 protected against innate responses acutely elicited by *Alternaria*-induced mucosal damage [84].

Finally, it is also noteworthy that OM-85 administration through the nasal route achieved significant, often complete asthma suppression at a cumulative dose 27–46-fold lower than the one reportedly used through the oral route (8–14 vs. 375 mg/mouse, respectively) [79]. We surmise that the high efficacy of airway-administered OM-85 may reflect both the diverse microbial stimulation this lysate provides and its direct access to the cellular and molecular networks that reside in the airway mucosa and that regulate its response to allergens. Because previous

evidence from oral treatment models could not identify cell-intrinsic effects sufficient for OM-85-dependent suppression of allergic asthma, at this time it is unclear whether oral and intra-nasal OM-85 administration trigger similar or distinct protective mechanisms. Regardless, the intense, multi-pronged effects of intra-nasal OM-85 on allergen-induced innate and adaptive mouse airway responses warrant further studies to determine whether administration of this standardized, safe bacterial lysate to the airway compartment can protect humans from the development and/or exacerbation of allergic asthma.

4.2. MV130

Unlike OM-85, which is a bacterial lysate, MV130 is a whole-cell, heat-inactivated preparation containing 90% of Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*) and 10% of Gram-negative bacteria (*Klebsiella pneumoniae*, *Branhamella catarrhalis* and *Haemophilus influenzae*), all of which are frequently found in the respiratory tract [86]. MV130 is receiving attention in the asthma field because a phase 3 randomized, double-blind, placebo-controlled, parallel-group trial recently demonstrated that sublingual administration of this agent prevented wheezing attacks typically associated with viral respiratory infections in non-atopic young children (<3 years old) who had had at least 3 wheezing attacks during the previous year. Compared to placebo controls, MV130-treated subjects had a significantly lower number of wheezing attacks [3.0 vs. 5.0 ($P < 0.001$)] as well as a decrease in the duration, symptoms and medication scores of these attacks [87].

So far, mechanistic studies on MV130 have only addressed its immune and anti-viral effects. *Ex vivo* and *in vitro* work in human DCs demonstrated that MV130 promotes IL10-regulated Th1/Th17 responses via RIPK2- and MyD88-mediated signaling. Potent systemic Th1/Th17 and IL-10 responses were also detected in Balb/c mice immunized sublingually with MV130 [88]. Additional studies, also in mice, showed that intra-nasal prophylaxis with MV130 provides long-term protection against respiratory viral infections (influenza or vaccinia virus) and systemic candidiasis through innate immune pathways. Bone marrow progenitor cells from MV130-treated mice and human monocytes exposed to MV130 *in vitro* exhibited enhanced chromatin accessibility in genomic regions associated with inflammatory responses and TNF production, increased TNF secretion, and a metabolic shift towards increased glycolysis [89]. In combination, these immunologic, metabolic and epigenetic profiles suggest that MV130 might train innate immune responses [90]. However, to this date no mechanistic connection links MV130-induced immune effects directly to asthma protection in humans and no mechanistic studies with MV130 have been reported in animal models of asthma and/or allergic lung inflammation.

5. Conclusions

Looking back at the initial call to action issued by Jean-François Bach in 2002, the studies of the environmental agents and mechanisms that protect from allergic diseases have come a long way. Exposures consistently associated with protection, such as those found on traditional farms, have been analyzed and dissected both epidemiologically and immunologically, and microbial products derived from animals (and likely, plants) have been identified as a major source of protective cues. The role of endogenous microbiota, first and foremost those in the gut, is also under scrutiny, together with the role environmental microbes have in shaping the endogenous microbiome. The emphasis on microbes and their products has motivated a renewed interest in pharmacological-grade microbial agents that are amenable to standardization and safe utilization. A critical finding now emerging from a mechanistic comparison of environmental (farm-derived) microbial products and lysates of microbes found in the human body is that these agents share fundamental properties associated with allergic disease

protection in vivo. Indeed, both Amish farm dust extracts and microbial lysates suppress allergen-induced AHR and BAL eosinophilia, boost airway epithelial barrier function, and require Myd88/Trif-mediated innate immune signaling to induce protection, even though it is currently unclear to what extent sharing of molecular pathways and/or phenotypic canalization contribute to these patterns.

Despite these advances, many questions still need to be answered: for instance, which substances and pathways are responsible for the protective effects of environmental farm products? Which gut microbes are primarily involved in protection, and which suites of metabolites do they rely on to induce their effects? Is there a core set of environmental and/or endogenous microbes and metabolites that are necessary and sufficient for protection, or is the process relatively redundant? Can we identify immunological pillars of protection in naturally exposed Amish children and use them to guide immunological manipulations in unexposed individuals? Will it be possible to administer candidate microbes and microbial products during pregnancy, and if protection against pathogenic type-2 responses is achieved in the child, how long will it last? Answering these questions, and the many others that will inevitably arise as this research progresses, will require multidisciplinary teams well-versed in immunology, biochemistry, conventional and germ-free mouse models, microbiome work and of course, allergic disease phenotyping. However, the epidemiology of the farm effect throughout the world tells us that allergic disease protection is possible in real life. Therefore, if the functional convergence between natural microbial products and standardized, pharmacological-grade bacterial lysates holds and extends to other relevant phenotypes such as virus-induced disease exacerbations, atopic dermatitis and food allergy, we might have an unprecedented opportunity to translate our novel understanding of allergic disease-protective exposures into strategies that can be effectively implemented.

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