THE IMMUNOLOGICAL IMPLICATIONS OF THE HYGIENE HYPOTHESIS

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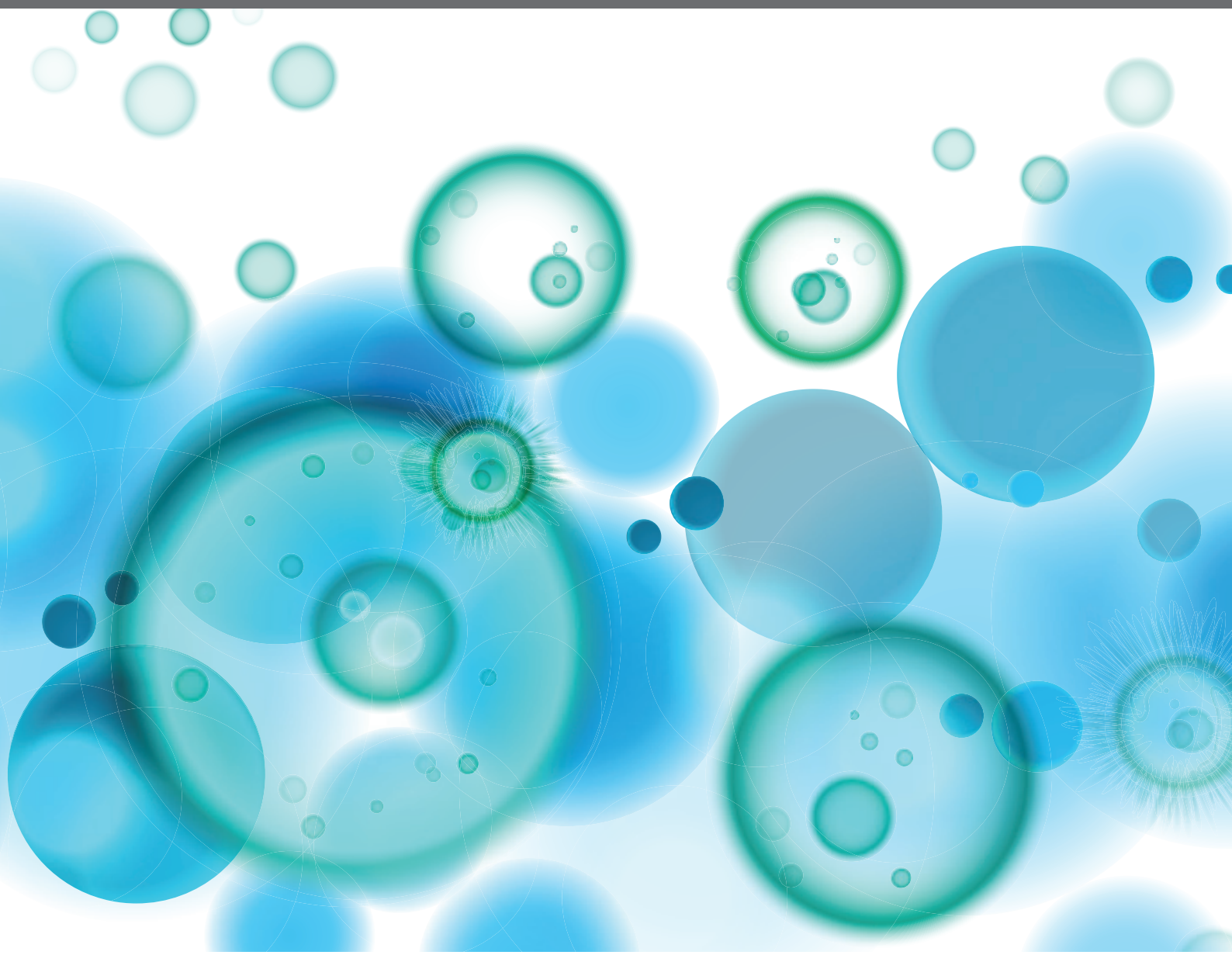

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THE IMMUNOLOGICAL IMPLICATIONS OF THE HYGIENE HYPOTHESIS

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EDITORIAL
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Editorial: The Immunological Implications of the Hygiene Hypothesis

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Keywords: hygiene hypothesis, inflammation, microbiome, bacteria, parasites, environment

Editorial on the Research Topic

The Immunological Implications of the Hygiene Hypothesis

Human beings developed in close vicinity to their natural environment during their long-lasting history. During this time, a stable relationship was established between the highly diverse environmental influences and physiological processes in the human body resulting in an optimal adaptation of humans to the respective ecological niches. Thereby, environmental factors involved both external impacts including frequent (early-life) microbial or parasitic infections and the establishment of an internal microbial community, the microbiome. Due to urbanization and industrialization these relationships were massively disturbed and new interactions developed. Very recently, this was impressively shown again by Wibowo et al., who reconstructed ancient microbial genomes from 1.000 – 2.000 year-old stool samples and showed that these paleofecal microbiomes are similar to those from people currently living in non-industrialized areas of the world, but are significantly different from microbiomes in humans living in highly industrialized countries (1). These rather recent changes are considered to have substantially contributed to the development of modern civilization disorders, which predominantly involve non-communicable diseases (NCDs) such as allergies, autoimmune and neurological diseases, and cancer. Contemplating the potential inverse relationship of early-life exposure to a highly diverse microbial environment and the prevalence of NCDs, the Hygiene Hypothesis was established about 30 years ago. Since then, several studies have added new evidence and refined the postulate justifying this Research Topic on immunological implications of the Hygiene Hypothesis, which now constitutes an impactful collection of review and original articles addressing recent developments and novel findings combined with retrospective and prospective views in this still highly topical field of scientific research.

First, von Mutius, one of the most prominent key opinion leaders in the field provides a concise personal statement discussing the protective effects of traditional farm exposures on the development of childhood asthma and allergies. From her epidemiological point-of-view, (microbial) diversity in the farm environment is key for its allergy-preventive effects (2) and this is supported by the fact that no single component has so far been identified that confers protection.

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However, two key elements of the protective farm effect have been described, exposure to animal sheds, in particular to cowshed dust, and the consumption of unprocessed cow's milk. Moreover, pregnancy has been recognized as an early window of opportunity to prevent immune deviation and allergic outcomes in offspring by traditional farm exposure implying a role for epigenetic imprinting. Finally it has been concluded that a multiplicity of exposures, *via* diverse routes, influence microbiome composition at different mucosal sites and overall these impacts are associated with a lower risk of asthma in farm populations. In a subsequent mini review, we provide a short chronological overview on landmark findings that developed and further shaped the Hygiene Hypothesis over time. We discuss microbial and nutritional aspects with regard to their association to the hypothesis and provide insights into our current understanding on underlying immunological mechanisms (Pfefferle et al.). While the Hygiene Hypothesis was originally established in the context of allergic diseases, Jean-Francois Bach expanded its application to other conditions such as autoimmune diseases in the early 2000s (3). In this Research Topic, he contributes a sophisticated review article revisiting the Hygiene Hypothesis in the context of autoimmune disorders. Similar to allergies, these diseases represent complex multifactorial and polygenic chronic conditions and thus the underlying mechanisms of disease initiation and perpetuation are multiple and complex as well. This review summarizes the current epidemiological evidence for the inverse association of infections and autoimmune disorders and provides insights into the current immunological mechanisms thought to be involved in these interactions (Bach). Expanding the focus towards a more holistic view, Fiuza et al. present their concept of considering humans and their entire associated microbial communities as evolutionarily developed holobionts. They discuss the interactions between the entirety of human-associated microbial communities and the developing immune system during ontogenesis, and how shifts in environmental conditions may impact these well-balanced interrelationships. Further, in line with the Hygiene Hypothesis, the authors describe epigenetic modifications as fundamental mechanisms in gene-environment-interactions and as driving forces for the maturation of the immune system.

A better understanding of the mechanisms underlying the Hygiene Hypothesis may lead to new concepts of allergy prevention. In this context, Sarate et al. demonstrate that exposure of conventional or gnotobiotic mother mice with the probiotic bacterium *Escherichia coli* Nissle 197 may decrease polysensitization to birch and grass pollen allergens and allergic inflammation in the offspring. Interestingly, recombinant variants of the bacterial strain expressing birch and grass pollen allergens did not result in the same extent of allergy prevention. The review by von Mutius already pointed to ß-lactoglobulin (BLG) as an abundant component in raw cow's milk and the ambient air of cowsheds. Based on these findings, Afifi et al. hypothesized that BLG may exert tolerogenic effects thereby contributing to the allergy-preventiving farm effects. Using a mouse model, they were able to show that prophylactic pretreatment with the ligand-filled holoBLG, but not the empty apoBLG, reduced allergen-specific antibody titers, features of antigen presentation, and allergic T cell responses, in allergen-specific (towards apoBLG) as well as an allergen-non-specific (towards birch pollen allergen) manner. In an ambitious latent class analysis (LCA) approach, Hose et al. analyzed daily food consumption patterns in relation to the gut microbiome of children by one year of age and at school age (time point of asthma initiation). The authors found excessive meat consumption to be associated with later asthma development while consumption of milk products compensated for such an effect. Interestingly, microbiome analyses indicated that this association might be related to the "iron battle field" in the gut. Free iron is a limiting nutritional factor for the metabolism of both the host and bacteria. Unbalanced meat consumption fosters accumulation of gut-associated bacterial strains equipped with siderophores that are highly capable of taking up free iron from the gut lumen. Since milk products in general, and unprocessed milk in particular, contain large amounts of iron-binding beta-lactoglobulin, this milk compound might effectively combat the growth of such bacterial iron competitors.

The balance of the local microbiological environment in mucosal tissues is tightly controlled by a variety of innate and adaptive defense systems. Among them, antimicrobial peptides such as the human β-defensin 2 (hBD-2) play a key role and thus disturbances in their production may favor the development of allergic and/or autoimmune disorders. In their manuscript, Borchers et al. clearly demonstrate that genetic variations associated with insufficient hBD-2 production may represent a risk factor for the development of such diseases. Accordingly, prophylactic administration of hBD-2 was able to reduce pulmonary inflammation and improve lung function parameters in a mouse model of allergic airway inflammation. In the context of gene-environment-interactions, genetic predisposition may also represent a decisive factor in shaping early immune mechanisms in the presence of parasitic infection and in asthma development. In a genome-wide association study (GWAS) on *Ascaris lumbricoides*-infected children, Carneiro et al. associate *WSB1* and *IL21R* genetic variants with markers of type-2 immune responses. *WSB1* but not *IL21R* gene expression was suppressed in infected children and increased methylation was concomitantly observed in the *WSB1* promoter region. The WSB1/IL21R pathways may thus represent potential targets for the treatment of type-2-mediated diseases. In the same research area, Korb et al. present an experimental approach that underlines the role of glycosylation of parasite-derived extracts in anti-allergic immunomodulation. Using an ovalbumin-model of allergic airway inflammation, in which sensitized mice were intranasally treated with native *Toxoplasma gondii* lysates, they observed attenuation of experimental asthma parameters. In contrast, application of deglycosylated lysates was shown to exert no protective effect indicating that the carbohydrate modifications are relevant for the strong anti-inflammatory effects provided by *T. gondii*-derived extracts. The potential role of sugars and related receptors in the regulation of immune mechanisms are further highlighted in a mini review provided by Peters and Peters who emphasize the role of carbohydrate lectin receptors (CLRs) in allergic airway inflammation. Diverse allergen glyco-epitopes may

bind to such CLRs, thereby facilitating their uptake and presentation. However, CLRs have also been shown to mediate inhibitory signals and a number of studies have demonstrated anti-inflammatory effects after binding carbohydrate ligands of microbial origin. The authors discuss the involvement of sugar moieties in immune regulation and how CLRs and their ligands may contribute to allergy and asthma protection.

This Research Topic is completed by a forward-looking mini-review by Garn et al. focusing on new developments and insights in immunological and allergy research and how these current perceptions further finetune our understanding on mechanisms underlying the Hygiene Hypothesis. Finally, the application of the Hygiene Hypothesis to explain the increasing incidence of conditions other than allergies and autoimmune disease is also discussed, underscoring the enduring importance of this scientific concept.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Revisiting the Hygiene Hypothesis in the Context of Autoimmunity

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Initially described for allergic diseases, the hygiene hypothesis was extended to autoimmune diseases in the early 2000s. A historical overview allows appreciation of the development of this concept over the last two decades and its discussion in the context of evolution. While the epidemiological data are convergent, with a few exceptions, the underlying mechanisms are multiple and complex. A major question is to determine what is the respective role of pathogens, bacteria, viruses, and parasites, *versus* commensals. The role of the intestinal microbiota has elicited much interest, but is it a cause or a consequence of autoimmune-mediated inflammation? Our hypothesis is that both pathogens and commensals intervene. Another question is to dissect what are the underlying cellular and molecular mechanisms. The role of immunoregulatory cytokines, in particular interleukin-10 and TGF beta is probably essential. An important place should also be given to ligands of innate immunity receptors present in bacteria, viruses or parasites acting independently of their immunogenicity. The role of Toll-Like Receptor (TLR) ligands is well documented including *via* TLR ligand desensitization.

Keywords: hygiene hypothesis, autoimmune diseases, type 1 diabetes, non-obese diabetic mouse, Toll-Like Receptor, gut microbiota, evolution, migrants

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INTRODUCTION

The hygiene hypothesis is a counterintuitive concept. While it is well known that infectious agents are potentially responsible for many diseases beyond infectious diseases, the idea emerged that they could in some cases have a favorable effect on non-infectious and sometimes very serious illnesses. The original report by Strachan in 1989 was based on an observation that might seem anecdotal: hay fever and atopic dermatitis are less frequent in families with many children than in families with only one or two children (1). It was to Strachan's credit that he then proposed the hypothesis that common childhood infections may reduce the frequency of atopic diseases. It was only a little later, in 2000, that he proposed that the increase in the frequency of allergic diseases observed in the three or four preceding decades could be ascribed to the decrease in the frequency of infectious diseases (2). It was also in the early 2000s that the hygiene hypothesis was extended to autoimmune diseases (3). At that time there were already data obtained in experimental models showing that infections, particularly parasitic infections, could prevent the occurrence of autoimmunity (4). Since then, compelling evidence has been gathered to support the hygiene hypothesis. We review it here stressing the importance of causal relationships, since it is not sufficient to show a correlation

between two events to affirm causality. We will highlight the importance of experimental models, particularly those concerning spontaneous diseases, the closest to human diseases.

Like any scientific hypothesis, the hygiene hypothesis has elicited conflicting opinions. By examining several hundred articles devoted to the subject, we find a majority supporting the hypothesis. There are, however, a number of articles expressing reservations or even, more directly, questioning the hypothesis. These challenging reports relate particularly to allergic diseases. Many allergists are more inclined to explain the increase in the frequency of allergic diseases, which nobody denies, by changes in the non-infectious environment, even going so far as to incriminate the increase in the dissemination of pollens. Such claims are difficult to accept when one considers that the increase in the frequency of allergic diseases affects all clinical forms ranging from atopic dermatitis to hay fever and even food allergies. In addition, the parallel evolution of autoimmune diseases does not support the hypothesis of changes in the allergenic environment. Another source of questioning is linked to the fact that all autoimmune diseases are not concerned by the hypothesis, without knowing why some of them are and others not, a subject of very great interest *per se*. Also, it is very difficult to know which infections are involved in the hypothesis. The study of experimental models makes it possible to identify infectious agents, bacteria, viruses and especially parasites, which prevent the occurrence of allergic and autoimmune diseases. Analysis is much more difficult in humans. The lower incidence of allergic and autoimmune diseases in large families mentioned above suggests that common childhood infections play a role. The mirroring chronological course of the decrease in major infectious diseases and the increase in allergic and autoimmune diseases argues for serious infectious diseases being also involved in the hypothesis. The problem is further complicated by the fact that certain infectious agents can cause acute autoimmune diseases as rheumatic fever and Guillain–Barré syndrome.

Autoimmune diseases are multifactorial and polygenic. The predisposing factors for autoimmune diseases are both genetic and environmental (**Figure 1**). Among genetic factors major histocompatibility genes (HLAs) play a major role, variable depending on the disease yet sometimes highly significant as in the case of type 1 diabetes, ankylosing spondylitis and narcolepsy. The role of a very large number of chromosomal regions identified by GWAS is certainly important, although the multitude of regions in question and the very low risk factor associated with each of them makes their priority ranking uncertain. Other genes could be involved in particular rare variants. One must also mention the potential role of epialleles that control epigenetic modifications participating to certain autoimmune diseases. Concerning the environment, a distinction must be made between factors which contribute to the onset of autoimmune diseases and those which prevent them. One must cite the hypothesis, still not proven in humans, according to which viruses could contribute to the triggering of autoimmune diseases such as type 1 diabetes and multiple sclerosis, secondarily to inflammation of the target organ by a local viral infection. The role of the gut microbiota is also relevant, although much remains to be done to demonstrate causality. To all this, we must add the possible implication of stochastic events, the existence of which is well proven in cancer (somatic mutations), still unknown for autoimmune diseases.

At this point it is important to clarify what is intended by "hygiene" when referring to the hygiene hypothesis. When speaking about hygiene, one normally thinks of classic hygiene

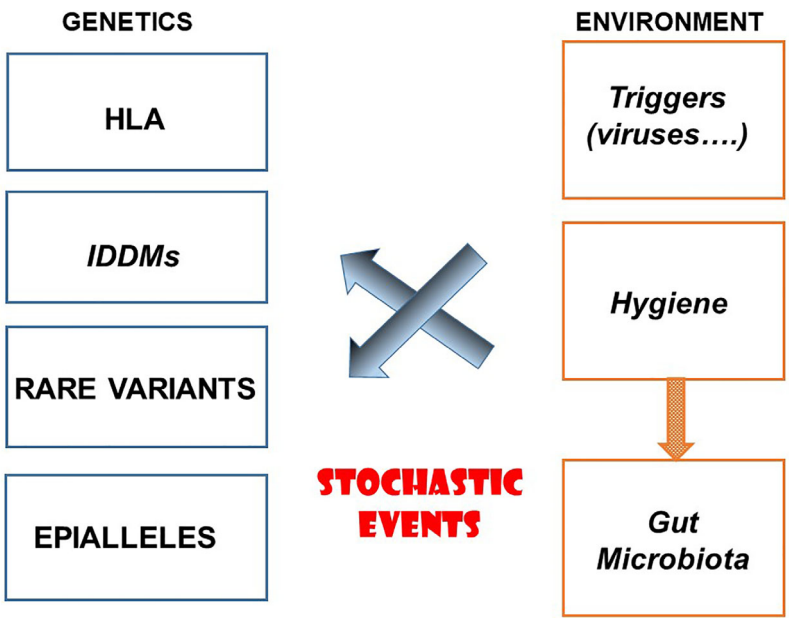

FIGURE 1 | Etiological Factors for Autoimmune Diseases.

as ensured by hand washing or social distancing rules that have reappeared in the foreground during the recent SARS-CoV-2 pandemics. The hygiene hypothesis we are discussing here is something else. It is about the environmental infectious burden which relies more on the socio-economic context specific to each industrialized country, each region or the family social context than to personal hygiene. Most of the factors that contribute to the hygiene hypothesis are collective and not individual. This infectious burden depends, to a large extent, on the quality of the drinking water, respect for the cold chain, the extensive use of antibiotics but also the generalization of vaccines. A development, obviously favorable because it prevents the occurrence of serious infectious diseases, but once again, independently of personal hygiene. The possible solutions to reduce the frequency of allergic and autoimmune diseases will obviously not come from the reintroduction of certain infections but rather from the use of "substitutes" for these infections which will retain their protective benefits.

INTEREST AND LIMITATIONS OF EPIDEMIOLOGY

The hygiene hypothesis in its dynamic aspect is based on the negative correlation observed between the decrease in the frequency of infectious diseases and the increase in that of allergic and autoimmune diseases. The question arises as to whether the trends reported twenty years ago persist today (3). The answer is clearly positive concerning infectious diseases which, under the effect of hygiene, vaccinations and antibiotics, continued to decrease in industrialized countries. However, many common childhood infections persist and new pandemics such as COVID 19 occur. The question is more complex concerning allergic and autoimmune diseases. Unfortunately, there is relatively little recent epidemiological data with no international databases on these diseases as they exist for the major infectious diseases. It can be noted, however, that for T1D, as well as for allergic diseases, the frequency has continued to increase in recent years (5, 6) with, for T1D, affecting very young children (7). The question is more open for other diseases such as multiple sclerosis for which it seems, at least in some countries, that a plateau has been reached.

The main aim of the epidemiological approach to the hygiene hypothesis is to show the existence of a direct relationship between the number of infections and the frequency of allergic and autoimmune diseases. Unfortunately, it is very difficult to count infections because if one usually remembers serious infections it is much more difficult to memorize common infections which, as we have seen, probably play a significant role. Like others, we ourselves have tried to study this problem in the setting of atopic dermatitis and reached the conclusion that reliable enumeration of infections is almost impossible (8). Indirect markers must be used. The most often used concerns the socio-economic environment and family composition, in particular the number of children in families (1, 2). It is very interesting to note, as has been done for decades, that allergic and autoimmune diseases are more common in high socio-economic backgrounds and in families with few children. We find this conclusion in the study of the geographical disparity of allergic and autoimmune diseases on the one hand and infectious diseases on the other (3, 9). Several hypotheses have been put forward to explain this phenomenon (3, 9). Genetic factors do not have a determining role because migrants from countries with a low incidence of allergic or autoimmune diseases to countries with a high incidence develop these diseases with the same frequency as in host countries from the first generation (10–14). It suffices that the migration takes place before the age of 5 years for allergic diseases (14) or fifteen years for multiple sclerosis (12, 13) for the increase in incidence to manifest. This last observation, which suggests that the protective effect of infections develops over a fairly long period of childhood should be taken into consideration when discussing the role of intestinal dysbiosis insofar as the composition of the gut microbiota is fixed very early in life (2 or 3 years of age) (see below). Another interpretation calls for climatic differences. This hypothesis, which could explain the role of parasitic infections that are more frequent in tropical regions, must be considered with caution when we know that the frequency of T1D and allergies is four to six times greater in Finland than in Karelia which differ for socio-economic level while the climate and genetic factors are basically the same in these two contiguous countries (15, 16). Incidentally, one should highlight that the difference of T1D incidence between Finland and Karelia does not apply to islet-cell autoantibodies suggesting that the effect of hygiene applies more to the progression than to the triggering of the autoimmune process (17). In brief, all this suggests that it is the socio-economic factors with all the consequences on health conditions which primarily explain the differences in the frequencies of allergic and autoimmune diseases in the different regions of the world.

It would, however, be interesting to find other indirect markers of infections. This was done by analyzing the prevalence of stigmata of infections by bacteria, viruses or parasites widely distributed in the population. For example, atopy has been shown to be more common in some parts of the world when the rate of seropositivity against hepatitis A virus is low (3, 18). Regarding autoimmune diseases multiple sclerosis is associated with a lower seropositivity for cytomegalovirus (CMV) (19, 20) or Helicobacter pylori (21). The same observation was made for CMV in T1D (22). Also, multiple sclerosis is associated with an abnormally low exposure to *Toxoplasma gondii* (23).

Finally, another extremely original approach results from the analysis of the repertoire of the antigen receptor of T lymphocytes (TCR) in subjects presenting allergy. It has in fact been shown that the diversity of this repertoire was restricted which was interpreted as the reflect of a lesser solicitation of the immune system by infectious agents (24).

CAUSAL RELATIONSHIP

It is not sufficient to observe a negative correlation between the decrease in infections and the increase in allergic and

autoimmune diseases to affirm a cause and effect relationship. It is difficult to prove this in humans, although there are many arguments in favor of such interpretation. The best answer will undoubtedly come from therapeutic trials in which it will be shown directly with statistically interpretable results that the suppression of certain infections increases the frequency of allergic and autoimmune diseases or conversely that the administration of certain infectious agents or parasites, needless to say preferably in the form of extracts (25, 26), prevent their occurrence. Some elements of response have been obtained for allergic diseases, in particular worsening of asthma in patients who have been subjected to antiparasitic treatments (27). We can also mention, although the data are contradictory, the improvement in atopy observed after administration of probiotics (28). Far fewer arguments exist for autoimmune diseases. At most, one can note therapeutic trials of limited size suggesting a favorable effect of the infestation of patients suffering from multiple sclerosis by a live parasite *Tricuris suis* (29, 30). The best arguments come from studying spontaneous experimental models of autoimmunity such as the non-obese diabetic (NOD) mouse and the lupus B/W mouse. It is necessary to set aside the models of induced autoimmune diseases upon administration of autoantigens. These models use adjuvants which are known to themselves induce protection from autoimmunity (31) and therefore complicate the interpretation of a potentially preventive effect by infections. A large number of infectious agents (bacteria, viruses or parasites) prevent autoimmune disease in NOD and BW mice. We refer the reader to a recent review for the NOD mouse (9). With regard to B/W mice, kidney disease and survival can be considerably improved by viral (32) or parasitic (33) infections. It is, in fact, in this model that was published the first convincing observation of the prevention of autoimmune diseases by a parasite, in this case *Plasmodium berghi* (4). Other studies have confirmed this favorable action of parasites (25, 26, 34–36).

As we will see below, the use of these models, in addition to providing the necessary proof of concept for the hygiene hypothesis, have shed light on the underlying mechanisms.

To conclude this data clearly shows that numerous pathogenic infectious agents protect from autoimmune diseases independently of any relationship with the gut microbiota.

HYGIENE HYPOTHESIS AND EVOLUTION

The epidemiological observations on which the hygiene hypothesis is based date back some fifty years. It is obvious that the increase in the frequency of allergic and autoimmune diseases does not have a genetic basis within populations in which changes in the frequency of these diseases have been observed, except in the case of the migrants mentioned above. These phenotypes reflect an adaptation of the organism, more particularly of the immune system, to the environment and more specifically to the infectious environment. It is, however, interesting to note that this deviance implies that the organism has adapted to changes in the environment by creating a phenotype that had not been the object of natural selection during evolution. Other examples of diseases come under the same commentary such as obesity and type 2 diabetes which are linked to overeating in individuals who have been selected to develop energy storage mechanisms (37).

On the other hand, we can ask the question of an interaction between the occurrence of infectious diseases and inflammatory diseases during evolution. This possibility has been considered in particular by L. Quintana-Murci (38, 39). This author highlighted the delicate situation in which the immune system found itself between establishing a strong inflammatory response to fight against pathogens in an environment with a high pathogen load while avoiding the harmful consequences of acute and chronic inflammation, which could lead to inflammatory and/autoimmune diseases (38). Interestingly, Genome Wide Association Studies (GWAS) studies have shown a community of single nucleotide polymorphisms (SNPs) associated with a strong anti-infective immune response and those associated with a predisposition to inflammatory and autoimmune diseases (40–42).

We can also wonder if the composition of the intestinal microbiota which, as we will discuss below, contributes to the hygiene hypothesis is not also subject to evolution, more precisely to a co-evolution of the immune system and commensal bacteria. One can imagine that the bacteria that resisted evolution did so because they had no pathogenicity and did not endanger people's lives and could even have a favorable influence. A kind of immune tolerance has thus been created, the consequences of which are difficult to perceive and especially the relationship with the role of the microbiota in the hygiene hypothesis.

Asking this question leads us to evoke the selective pressure that has weighed on the human species since the appearance of *Homo-sapiens* (38, 39). In recent years, population genetics work has provided major information thanks to advances in genomics. First, it should be noted that modern *Homo-sapiens* arose from crosses between African humans and Neanderthals. Neanderthals contributed little (about 2%) to the *Homo-sapiens* genome. Nevertheless, it has been repeatedly shown that some of the haplotypes which persist in *Homo-sapiens* and which originate from Neanderthals include genes important for immune responses. It has recently been shown that certain genes predisposing to COVID 19 are derived from Neanderthals (43). Subsequently, over the past 100,000 years, genes involved in immune reactions have evolved through a process of natural selection, positive or negative (purifying). Evolution has taken place under the selective pressure of environmental conditions, first the shift from humans hunters/collectors to humans cultivators, then the migration of humans from Africa to Asia and then to Europe. Numerous studies carried out on this subject, in particular by L. Quintana-Murci, have made it possible to identify the genes in question (38, 39). These are essentially genes controlling innate immunity. We note more particularly the presence of genes encoding toll-like receptors (TLRs). It thus appears, which was intuitive, that the evolution gave rise to an improvement of the immune responses against infectious agents under their pressure.

The case of epidemics illustrates this point. Thus, subjects with a mutation in the NOD-2 gene, associated with inflammatory bowel diseases, seem to be over-represented in populations that have been severely affected by plague epidemics (44).

How, then, can these observations be linked to the hygiene hypothesis? It is important to note that the changes in the genes of immune responses during evolution have been made at a particularly rapid rate. These changes did not span hundreds of thousands of years as is the case for other genes but often only a few thousand years, and sometimes even less in major epidemics or extreme environmental situations. In contrast, the hygiene hypothesis is based on adaptation rather than selection. We thus find ourselves in a situation where the time scale associated with the selective pressure which influenced natural selection intersects with that of the hygiene hypothesis which only applies to the last 50 years. Going further, one can wonder whether the reduction in the infectious burden, on which the hygiene hypothesis is based, will not influence the evolution of the genes controlling the immune responses. Conversely, even if these diseases were serious enough to reduce the number of offsprings in affected subjects, the genes which control them are so numerous and interactive that it is difficult to see how they could influence natural selection, especially since they are rarely deleterious mutations but more often polymorphisms whose isolated presence in healthy subjects has no consequence.

INTESTINAL DYSBIOSIS, CAUSE, CONSEQUENCE, OR MODULATION

The emergence of metagenomics, which has made it possible over the past fifteen years to characterize the composition of the intestinal microbiota, has opened a new page in the hygiene hypothesis. Several arguments, suggest that a decrease in the diversity of the intestinal microbiota could contribute to the occurrence of autoimmune diseases as well as many other pathologies including allergic diseases, type 2 diabetes and obesity.

The mainstay is the existence of a dysbiosis, that is to say an imbalance of the commensal bacteria that make up the intestinal microbiota in the diseases in question. One observes, indeed, in these different diseases a reduction in the diversity of the microbiota, more particularly in certain species, with often a decrease in lactobacilli (45), in particular in type 1 diabetes (9), multiple sclerosis (46–48) and systemic lupus erythematosus (49–51).

At the same time, destruction of the gut microbiota by administration of broad-spectrum oral antibiotics to mothers and newborns has been shown to increase the frequency of T1D in NOD mice (52) and experimental asthma (53).

The link between these observations on the hygiene hypothesis quickly became apparent. We know, in fact, that the composition of the intestinal microbiota is different depending on the level of hygiene. This has been demonstrated in particular by comparing the microbiota of subjects living in Italy or Burkina Faso (54), an observation which is, however, not conclusive because many other elements differ between such countries, in particular diet which influences the composition of the microbiota. A more direct argument is the observation that pigs reared in a clean facility have a different microbiota than those reared in a conventional barn (55).

These observations aroused great enthusiasm. Numerous studies have attempted to characterize commensal bacteria which could be responsible for regulating autoimmunity and whose absence or decrease could contribute to the occurrence of autoimmune diseases. It must be recognized, however, that so far, few conclusive results have been reported.

To these fairly convincing arguments it is necessary to mention other elements which incite more reticence. The main question is that of the causal relationship between dysbiosis and the occurrence of the diseases under consideration. Does said dysbiosis play a role in triggering the disease or in its progression or is it the consequence of the disease. To answer this question, we should not be content to study the composition of the microbiota at the time when the disease is already declared. The microbiota should be studied before the onset of the disease. In fact, this has so far only been done extensively in T1D, where cohorts of subjects with a high inheritance of diabetes have been followed from birth. Dysbiosis has been observed at the time of disease onset (56). However, if we carefully examine the timing of the onset of dysbiosis, we find that it takes place after the development of the autoimmune response against the beta-cells of the islets of Langerhans, suggesting either that it is secondary to the inflammation associated with the onset of diabetes or it contributes to the transformation of respectful insulitis into malignant insulitis which marks the onset of clinical diabetes.

The conclusions from the use of probiotics remain uncertain. The probiotics used were not really calibrated and the number of bacteria administered was very low compared to the number of intestinal bacteria, posing the problem of their mode of action: modification of the composition of the microbiota, but then for how long, pharmacological effect or other mechanisms.

In brief, there are interesting arguments to suggest the role of dysbiosis in the occurrence of autoimmune diseases, but they are fragile. In any case, data suggests in view of the results obtained in animal models that pathogens also play an important role, in particular agents that have no relation to the intestine such as mycobacteria.

MULTIPLE AND COMPLEX UNDERLYING MECHANISMS

Many publications have been devoted to the mechanisms underlying the hygiene hypothesis namely, how to explain that infections can reduce the frequency of allergic or autoimmune diseases. It appears very clearly that no univocal explanation can be presented for all the protective effects of infections. Several major mechanisms appear to operate. The problem is complicated by the fact that the mechanisms can be different depending on the infection. We will briefly discuss the main data available by referring the reader who would like more details and a more documented bibliography to a general review recently published (9).

It has been known for many decades that concomitant immune responses compete, a phenomenon termed antigenic

competition which could well be applied to the hygiene hypothesis by supposing that very strong immune responses against infectious agents could compete with immune responses directed against weak antigens such as allergens or autoantigens due to increased consumption of homeostatic factors. In fact, this mechanism has been studied very little in the case of the hygiene hypothesis. This may be explained by the poor knowledge that we still have today on the molecular basis for antigenic competition, even if converging data seem to give a major role to homeostatic factors, in particular interleukin (IL)-2, IL-7 and IL-15. In any event, as attractive as it is, this hypothesis remains very poorly documented.

Before attempting to present a unitary hypothesis, it is important to mention that certain mechanisms appear to be relatively specific to certain infectious agents. Thus, the protective effect of live mycobacteria or Freund's complete adjuvant involves CD4+CD25 +FOXP3+ regulatory T lymphocytes like other infectious agents but also, more unexpectedly, natural killer or NK cells (57). Lipopolysaccharide (LPS) contained in *Escherichia coli* similarly stimulates regulatory T lymphocytes and it also stimulates a particular subset of IL-10-producing B lymphocytes which play an important immunoregulatory role (58).

The commensal bacteria of the intestinal microbiota also have a protective effect against autoimmune diseases. This has been shown globally with probiotics (9) but also with well-identified commensal bacteria (lactobacilli). In this case too, various mechanisms are involved. As far as probiotics are concerned, a predominant role has been attributed to IL-10. In other situations, such as Clostridium, a major role has been ascribed to CD4+CD25+ FOXP3+ regulatory T cells (59). It should also be noted that many infectious agents modulating autoimmune responses involve the immunoregulatory cytokine TGF beta. This is the case of a gram-positive bacterial extract which protects the NOD mouse from diabetes, an effect which is reversed by the administration of antibodies neutralizing TGF beta but not IL-10 (60). Moreover, a key role of TGF beta has also been shown in the protective mechanisms mediated by various parasites with the exception of schistosomes which appear to act through IL-10 production (61). A role for interferon gamma (IFNγ) has been proposed for both allergy (62) and autoimmunity (63). In brief, multiple mechanisms are involved that mostly rely on immunoregulatory circuits.

At the molecular level, many arguments suggest that both pathogens, bacteria or viruses and also parasites as commensal bacteria exert their protective effects by primarily involving their molecular interactions with TLR receptors. These different infectious agents indeed contain TLR ligands, both pathogens and commensals. Above all, the systemic administration of chemically characterized ligands of the various TLRs reproduces the protective action of the infectious agents mentioned above (64). It should also be noted that it is not necessary for the infectious agents in question to be alive to prevent the onset of autoimmune diseases: they can be substituted with bacterial (60) or parasitic extracts which have the same effect (25, 26). The different TLR ligands could have distinct mechanisms of action depending on the specific receptor involved. For example, TLR4 ligands appear to act through FOXP3+ regulatory T cells, while TLR3 ligands involve invariant NKT cells (64).

It is now well known that the desensitization of macrophages to the pro-inflammatory effect of LPS, also called "endotoxin tolerance", is mediated by a joint action through TLR4 and TLR2 receptors and their common signaling pathways (65). Another example of desensitization is the prevention of type 1 diabetes in NOD mice by TLR2 ligands, called "TLR2 tolerance" (66). In an adoptive transfer model, it was shown that the repeated treatment with the TLR2 agonist Pam3CSK4 of NOD mice receiving diabetogenic T lymphocytes inhibited the development of the disease (66). This same TLR2 desensitization could also be involved in the prevention of experimental allergic encephalomyelitis (EAE) (67). Administration of low doses of two different TLR2 ligands, Pam2CSK4 or Lipid 654 (L654), to naive recipients of encephalitogenic (EAE-inducing) T cells decreased the level of TLR2 signaling at the same time as it attenuated EAE (67, 68). Interestingly, L654 is a TLR2 ligand derived from a commensal of the microbiota which is present in healthy human serum, but whose concentration is significantly reduced in the serum of patients with multiple sclerosis (67, 68). In another mouse model of EAE, repeated administration of a synthetic TLR7 ligand has been reported to significantly decrease the severity of disease as well as the expression of chemokines in the target organ (69). Finally, it is interesting to note that NOD mice genetically deficient in TLR4 and MyD88 show an acceleration in the severity of diabetes and experimental asthma respectively, again suggesting a protective role for TLR signaling (64, 70).

In humans, the role of desensitization by TLRs has been demonstrated in an elegant work by the group of B. Lambrecht concerning children raised on dairy farms (an environment rich in LPS) who present a low incidence of allergy (62, 71). Epithelial cells in the lungs have shown reduced production of cytokines that normally activate dendritic cells to induce TH2-type lymphocyte responses. The TLR4 desensitization induced by LPS which could be responsible for this effect targets the pulmonary epithelium (71).

In conclusion, although very interesting, this data do not suffice to give a complete picture of the mechanisms underlying the hygiene hypothesis. It is therefore necessary to consider the overall response to pathogens or commensals as the result of the integration of positive and negative signals delivered *via* the TLRs. This concept paves the way for a TLR targeted immunopharmacology.

CONCLUSIONS

The hygiene hypothesis, misnamed as it is, teaches us a lot about immunity, immunopathology, epidemiology and evolution. It indicates how flexible the immune system is, under constant control by immunoregulation under the influence of the environment. It sheds light on the mechanisms underlying many immune related diseases, in particular

autoimmune diseases but also probably many other diseases which involve an uncontrolled differentiation of lymphocytes, whether they are allergic diseases or certain malignant lymphoproliferative disorders (72). It opens new perspectives on the etiological factors of autoimmune diseases by distinguishing those that could trigger or on the contrary protect (**Figure 1**). Finally, when it comes to evolution, it provides a particularly bright illustration of the relationships that may exist between natural selection and adaptation under the control of infectious agents.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Pre- and Neonatal Imprinting on Immunological Homeostasis and Epithelial Barrier Integrity by *Escherichia coli* Nissle 1917 Prevents Allergic Poly-Sensitization in Mice

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A steady rise in the number of poly-sensitized patients has increased the demand for effective prophylactic strategies against multi-sensitivities. Probiotic bacteria have been successfully used in clinics and experimental models to prevent allergic mono-sensitization. In the present study, we have investigated whether probiotic bacteria could prevent poly-sensitization by imprinting on the immune system early in life. We used two recombinant variants of probiotic *Escherichia coli* Nissle 1917 (EcN): i) EcN expressing birch and grass pollen, poly-allergen chimera construct (EcN-Chim), and ii) an "empty" EcN without allergen expression (EcN-Ctrl). Conventional mice (CV) were treated with either EcN-Chim or EcN-Ctrl in the last week of the gestation and lactation period. Gnotobiotic mice received one oral dose of either EcN-Chim or EcN-Ctrl before mating. The offspring from both models underwent systemic allergic poly-sensitization and intranasal challenge with recombinant birch and grass pollen allergens (rBet v 1, rPhl p 1, and rPhl p 5). In the CV setting, the colonization of offspring *via* treatment of mothers reduced allergic airway inflammation (AAI) in offspring compared to poly-sensitized controls. Similarly, in a gnotobiotic model, AAI was reduced in EcN-Chim and EcN-Ctrl mono-colonized offspring. However, allergy prevention was more pronounced in the EcN-Ctrl mono-colonized offspring as compared to EcN-Chim. Mono-colonization with EcN-Ctrl was associated with a shift toward mixed Th1/Treg immune responses, increased expression of TLR2 and TLR4 in the lung, and maintained levels of zonulin-1 in lung epithelial cells as compared to GF poly-sensitized and EcN-Chim mono-colonized mice. This study is the first one to establish the model of allergic poly-sensitization in gnotobiotic mice. Using two different settings, gnotobiotic and conventional mice, we demonstrated that an early life intervention with the EcN without expressing an allergen is a powerful strategy to prevent poly-sensitization later in life.

Keywords: allergic poly-sensitization, germ-free, BALB/c, mucosal tolerance, mouse model, *Escherichia coli* Nissle 1917, hygiene hypothesis

INTRODUCTION

The prevalence of allergic poly-sensitization in children has increased significantly over the last decades (1–6). Nowadays, it is approximated that 14 to 45% of children suffer from poly-sensitization (3, 5, 6), which starts as early mono-sensitization to aeroallergens, such as birch and grass pollen allergens (4, 7–11). Despite the availabilities of conventional allergen-specific immunotherapy, based on the injection of increasing doses of allergen mixtures, the exposure to these allergens could pose a risk for the development of new sensitizations. There is a pressing need for the development of novel prophylactic therapies (12–16); therefore, the induction of immune tolerance early in life could be an excellent strategy for preventing poly-sensitization in children.

The priming of the immune system is particularly effective during the early period of life (17). Early life stages such as prenatal, perinatal, and early-postnatal period are crucial for establishing balanced gut microflora, which has been associated with reduced allergy development (18–22). The importance of immunological imprinting has been shown by clinical intervention studies, where early probiotic applications to mothers prevented allergies in their children (23–25). Using a mouse model of mono-sensitization to the major birch pollen allergen Bet v 1, we have previously shown that the perinatal and neonatal colonization with particular wild type probiotic bacteria (21, 26) or with recombinant probiotic bacteria expressing Bet v 1 (22) during the gestation and lactation period, reduced the development of Bet v 1-specific allergy.

Here, we investigated whether *Escherichia coli* Nissle 1917 (EcN), a probiotic Gram-negative bacterial strain with strong immunomodulatory properties (27, 28), can prevent the development of poly-sensitization in mice when applied early in life. Recently, we engineered a recombinant EcN expressing either the major birch (Bet v 1) and grass (Phl p 1 and Phl p 5) (EcN-Chim) pollen allergens or EcN harboring the empty plasmid (EcN-Ctrl) (7, 28, 29) and showed that the intranasal pretreatment with EcN-Chim but not EcN-Ctrl reduced allergic poly-sensitization in adult mice (28).

In the current study, performed in conventional (CV) and monoxenic mice, we had assessed the impact of EcN-Chim and EcN-Ctrl (antigen-specific vs. non-antigen-specific tolerance induction) treatment on the experimental poly-sensitization model when probiotics were applied in the early phase of life. In this phase of life, it was of interest to investigate whether EcN bacteria itself can prevent allergy or if the expression of the respective allergen by EcN is needed for successful allergy prevention.

Therefore, our first aim was to investigate the effects of EcN-Chim and EcN-Ctrl applied orally to mothers during the last week of gestation and lactation on the development of allergy in their offspring in CV conditions. Secondly, we have used the gnotobiotic mouse model to enable mother-to-offspring early bacterial mono-colonization to understand better the EcN-Chim and EcN-Ctrl impact on the prevention of poly-sensitization.

MATERIAL AND METHODS

Bacteria and Growth Conditions

Two recombinant strains EcN previously described (28) were used in the current study: i) EcN-Chim: a clone expressing multi-allergen chimer Phl p 5-Bet v 1-Phl p 1 along with fluorescent mCherry; ii) EcN-Ctrl clone expressing only mCherry without allergen. Both recombinant clones were grown and selected on Luria-Bertani (LB) agar plates with 20 μg ml^{-1} chloramphenicol (CM) at 37°C overnight, as described in Sarate et al., 2019 (28). In liquid medium, recombinant EcN-Chim and EcN-Ctrl strains were cultivated at 30°C on 200 RPM overnight in LB-medium containing 20 μg/ml chloramphenicol (28).

Antigens

Recombinant (r) allergens Bet v 1, Phl p 1, and Phl p 5 were purchased from Biomay GmbH (Vienna, Austria).

Animals and Ethics Statement

For perinatal study, pregnant conventional BALB/c mice in the last week of gestation were purchased from Charles River (Sulzfeld, Germany). The animals were maintained under controlled conventional housing conditions and provided with standard diet and water *ad libitum*. All experiments were approved by the Animal Experimentation Committee of the Medical University of Vienna and by the Federal Ministry of Science and Research (BMWF-66.009/0384-WF/V/3b/2015).

For germ-free (GF) mice studies in neonates, germ-free female BALB/c mice were kept under sterile conditions in Trexler-type plastic isolators, and the absence of aerobic and anaerobic bacteria, molds, and yeast was confirmed every two-weeks by standard microbiological methodology (31, 32). The mice were kept in a room with a 12 h light-dark cycle at 22°C, fed an irradiated sterile diet (Altromin 1414, Altromin, Germany), and provided sterile autoclaved water *ad libitum*. The animal experiments were approved by the Committee for the Protection and Use of Experimental Animals of the Institute of Microbiology of the Czech Academy of Sciences (approval ID: 23/2018).

Mouse Model of Poly-Sensitization Using Recombinant Birch and Grass Pollen Allergens

Poly-sensitization was done as described previously (28). Briefly, female BALB/c mice were sensitized with three intraperitoneal injections (days 10, 24, and 39) of a mixture of 5 μg rBet v 1, 5 μg rPhl p 1, and 5 μg rPhl p 5 adsorbed to aluminum hydroxide ($Al(OH)_3$; Serva, Heidelberg, Germany) (0.68 mg/ml). To induce airway inflammation, one week after the last intraperitoneal immunization, mice were anesthetized by 2% isoflurane in an anesthetic induction chamber and challenged intranasally with 30 μl of a mixture of 5 μg rBet v 1, 5 μg rPhl p 1, and 5 μg rPhl p 5 for 3 consecutive days.

Mice were sacrificed 72 h after the last challenge by exposure to carbon dioxide and blood was collected from the facial vein of

mice at the end of the experiment. Sera were collected and stored at −20°C until used.

The Perinatal Approach in Conventional Mice

Recombinant EcN-Chim and EcN-Ctrl strains were cultivated at 200 RPM, 30°C overnight in LB medium. Bacterial cells were collected and washed with ice-cold PBS. For perinatal application, bacterial cultures were adjusted to 1 x 10^9 CFU/300µl of gavage buffer (0.2 M $NaHCO_3$ buffer containing 1% glucose, pH 8) per mice. Pregnant mice in the last week of gestation were pretreated orally with either EcN-Chim or EcN-Ctrl during the gestation (every day) and lactation (every second day). On day 21, female offspring (n = 6 to 9 mice per group) derived from these mothers were separated and sensitized intraperitoneally with 5 µg rBet v 1, 5 µg rPhl p 1, and 5 µg rPhl p 5 followed by challenge with 5 µg rBet v 1, 5 µg rPhl p 1, and 5 µg rPhl p 5 as described in the poly-sensitization model (21). Mice were sacrificed 72 h after the last challenge, bronchoalveolar lavage (BAL), lung samples and blood were collected for further analysis. After centrifugation, sera were collected and stored at −20°C for further analysis.

The Perinatal Approach in GF Mice

Recombinant EcN-Chim and EcN-Ctrl strains were prepared as described above (in a sterile condition) for the mono-colonization in GF mice. Eight-week-old GF mice were colonized with a single dose (2×10^8 CFU/200 µl PBS), either EcN-Chim or EcN-Ctrl by intragastric gavage and mated 10 days later. During the experiment, drinking water was supplemented with chloramphenicol (200 mg/L) to ensure the long-term stability of the recombinant EcN strains *in vivo*. The stability of colonization was checked by plating of feces on LB agar and counting after aerobic cultivation for 24 h at 37°C. Colonization remained stable throughout the experiment and reached levels of 0.8-1.2 x 10^{10} CFU/g feces (EcN-Chim) and 0.7-1.4 x 10^{10} CFU/g feces (EcN-Ctrl).

On day 21, neonatally colonized female offspring either by EcN-Chim or EcN-Ctrl, as well as germ-free controls, were separated from their mothers and divided into two groups. One group was sensitized intraperitoneally with 5 µg rBet v 1, 5 µg rPhl p 1, and 5 µg rPhl p 5 (Biomay, Austria) emulsified in 100 µl of $Al(OH)_3$ (Serva, Germany) three times at a 14-day intervals. The other group was colonized but not sensitized and challenged. One week after the last i.p. immunization, mice were anesthetized by isoflurane and challenged intranasally with 30 µl of the mixture of 5 µg rBet v1, 5 µg rPhl p 1, and 5 µg rPhl p 5 for three consecutive days as described in the poly-sensitized model. Mice were sacrificed 72 h after the last challenge, and blood, BAL and lung samples were collected for further analysis.

Characterization of Airway Inflammation and Allergic Poly-Sensitization

BAL

To evaluate the allergic airway inflammation (AAI), mice were terminally anesthetized, the tracheas were cannulated, and lungs were lavaged with 2 x 0.5 ml PBS (30, 33). BAL fluids were centrifuged at 300 x g for 5 min at 4°C and cell-free supernatants were stored at −20°C for further analysis. Cell pellets were recovered for cellular analysis. After counting, cytospins were prepared by spinning cells onto microscope slides (Shandon Cytospin®, Shandon Southern Instruments, USA) and staining with H&E (Hemacolor®, Merck, Darmstadt, Germany). Cytospin preparations were differentiated according to standard morphologic criteria by counting 200 cells *via* light microscopy. Collected supernatants were then analyzed for IL-5 and IL-13 response in BAL fluids. Macrophages, lymphocytes, eosinophils, and neutrophils per slide were counted under the light microscope (Nikon Eclipse; 100x magnification) (200 cells per count). Results represent the absolute numbers of cells.

Lung Histology

Small lung tissues were excised and fixed with 7.5% formaldehyde-PBS, followed by paraffin-embedding. Lung sections (5 µm thick) were stained with periodic acid-Schiff (PAS) stain. Lung histological pathology was evaluated using light microscopy. Numbers of PAS-positive, mucus-producing Goblet cells in the bronchial epithelium were counted by an investigator blinded to the experimental setting. Results are given as the mean number of goblet cells per millimeter of the basement membrane (34).

Allergen-Specific Antibodies in Serum

Allergen-specific antibody levels in mouse sera (IgE, IgG1, and IgG2a) were determined by ELISA as previously described (35). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with each of the recombinant allergens Bet v 1, Phl p 1, or Phl p 5 (2 µg/ml) and incubated with mouse sera. Antibody detection was performed using rat anti-mouse IgE, IgG1, IgG2a, followed by peroxidase-conjugated mouse anti-rat IgG. Results show the optical density (OD) values after subtraction of baseline levels from pre-immune sera.

The determination of the allergenic antibody serum activity was performed as previously described (30). Briefly, RBL-2H3 cells were passively sensitized by incubation with serum samples of the respective experimental groups and their degranulation was induced by addition of recombinant allergens rBet v 1, rPhl p 1 and rPhl p 5 (0.3µg/ml) diluted in Thyrode's buffer. Supernatants were analysed for β-hexosaminidase activity. Results are reported as percentages of total β-hexosaminidase release after adding 1% Triton X-100 and are shown after subtraction of baseline release levels obtained with pre-immune sera.

Allergen-Specific Cytokine Detection in BAL

BAL supernatants collected during sacrifice were analyzed for IL-5 and IL-13 levels by using an ELISA specific for murine cytokines (Ready-Set-Go ELISA Kit eBioscience, USA) according to the manufacturer's instructions.

Quantification of mRNA Expression by Real-Time (RT)-PCR

Total RNA was extracted from lung samples of mice from all treatment and controls group at the end of the experiment. RNA

quantification was performed using ND-1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA) and cDNA was obtained using reverse-transcriptase kit (BIO-RAD, Vienna, Austria). Expression of IL-10, Foxp3, and IFNγ mRNA was measured by RT-PCR as described previously (30). The housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (Universal Probe Library probe #64; Roche) were used as a control to standardize the amount of sample cDNA. Data are presented as the ratio of the target genes expression to GAPDH and β-actin expression.

Immunohistochemical Identification of TLR2, TLR4, and Zonulin-1 (ZO-1) in Lung Tissue of Mono-Colonized Mice

For TLR2 and TLR4 analysis, the 3 μm lung sections were deparaffinized and antigens were retrieved in 0.01 M citrate buffer (pH 6) using a microwave vessel for 10 min. After cooling down and washing by PBS, endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 15 min. Non-specific adsorption was eliminated by incubation of the sections in 10% normal rabbit serum in PBS for 1 h. Samples were incubated overnight with polyclonal goat anti-TLR2 (4 μg/ml) or anti-TLR4 (4 μg/ml) (Santa Cruz Biotechnology, Dallas, Texas, USA) at 4°C. After washing in PBS, sections were incubated with rabbit anti-goat IgG conjugated with horseradish peroxidase (1:200 in PBS) (Jackson, ImmunoLabs., West Grove, PA, USA) for 1 h and stained with DAB (3,3′-Diaminobenzidine) (Dako, Carpinteria, CA, USA) for 3 min. The counterstain was carried out with hematoxylin, samples were mounted by the Paramount Aqueous Mounting medium (Dako, Carpinteria, CA, USA) and viewed under an Olympus BX 40 microscope with 40x objective, equipped with and Olympus DP 70 digital camera.

ZO-1 analysis was done as previously described (36). Briefly, the 3 μm lung sections were deparaffinized, antigens were retrieved by protease from *Streptomyces griseus* (1 mg/ml, type XIV, Sigma-Aldrich, St. Luis, MO, USA) at 37°C for 8 min, washed with PBS and endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 15 min. Non-specific adsorption was eliminated by incubation of the sections in 10% normal goat serum in PBS for 1 h. Samples were incubated overnight with polyclonal rabbit anti-ZO-1 (5 μg/ml, Santa Cruz Biotechnology, Dallas, Texas, USA) at 4°C. After washing in PBS, sections were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:200 in PBS) (Jackson, ImmunoLabs., West Grove, PA, USA) for 1 h and stained with DAB (3,3′-Diaminobenzidine) (Dako, Carpinteria, CA, USA) for 1 min.

Quantification of TLR2, TLR4 and ZO-1 immunohistochemistry was done by measuring the optical density (OD). The OD analysis of the TLR4, TLR2 and Zonulin-1 images was evaluated using ImageJ (Fijiv2.0.0; National Institutes of Health, Bethesda, MD). Briefly, 8-bit RGB DAB-stained images were processed through color deconvolution (37), and threshold settings and mean gray value was assessed in the selected area of bronchus/bronchiolar epithelial layer in DAB-extracted images. OD was estimated by the following formula: OD = log (max intensity/mean gray value intensity), where max intensity = 255 (38). OD of background (field without any tissue) was subtracted from OD of epithelial cell layer. Results from two to five bronchi/bronchiole from individual mouse were pooled and five mice per each group were evaluated (37, 38).

Statistical Analysis

Statistical analysis was conducted using GraphPrism, ver. 6. For comparison of more groups the One-Way ANOVA was applied followed by the Bonferroni's Multiple Comparison Test unless otherwise specified. All data are shown as mean ± SEM. Significant differences were considered at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

RESULTS

Conventional Mouse Model

Offspring of Mothers Treated With EcN-Ctrl Exhibited Reduced Allergic Airway Inflammation (AAI)

To assess the effects of perinatal interventions with probiotic bacteria on poly-sensitization, mice were treated orally with either EcN-Chim or EcN-Ctrl during the gestation and lactation period, and their offspring were sensitized and challenged with rBet v 1, rPhl p 1, and rPhl p 5 (**Figure 1A**). The offspring colonized perinatally with EcN-Ctrl exhibited reduction in BAL eosinophils ($P < 0.001$), IL-5 ($P < 0.05$), and IL-13 ($P < 0.01$) levels as compared to poly-sensitized offspring (**Figures 1B–E**). Offspring colonized with EcN-Chim exhibited reduced IL-13 ($P < 0.05$) as well as eosinophil level ($P < 0.05$) in BAL compared to poly-sensitized animals. Lung histology revealed reduced mucus production in the lung of offspring colonized with either EcN-Chim or EcN-Ctrl as compared to poly-sensitized controls (**Figure 1F**). Besides, perinatal colonization with EcN-Ctrl induced a reduction in Bet v 1-specific serum IgE as compared to poly-sensitized controls ($P < 0.01$) (**Figure 1G**).

Gnotobiotic Mouse Model

Perinatal and Neonatal Mono-Colonization With Either EcN-Chim or EcN-Ctrl Prevented AAI

In a gnotobiotic mouse model, perinatal/neonatal mono-colonization of offspring *via* their mothers with either EcN-Chim or EcN-Ctrl (**Figure 2A**) significantly reduced AAI as compared to GF poly-sensitized controls (**Figures 2B–F**). This was reflected by reduced recruitment of eosinophils (EcN-Chim, $P < 0.0001$; EcN-Ctrl, $P < 0.001$), as well levels of IL-5 (EcN-Chim, $P < 0.01$; EcN-Ctrl, $P< 0.05$), and IL-13 (EcN-Chim, $P < 0.0001$; EcN-Ctrl, $P < 0.001$) in BAL samples compared to GF poly-sensitized controls (**Figures 2B–E**). Histological analysis revealed reduced mucus production by airway-lining goblet cells in the lungs of the groups colonized with either EcN-Chim ($P < 0.0001$) or EcN-Ctrl ($P < 0.0001$) compared to GF poly-sensitized control (**Figures 2F, G**).

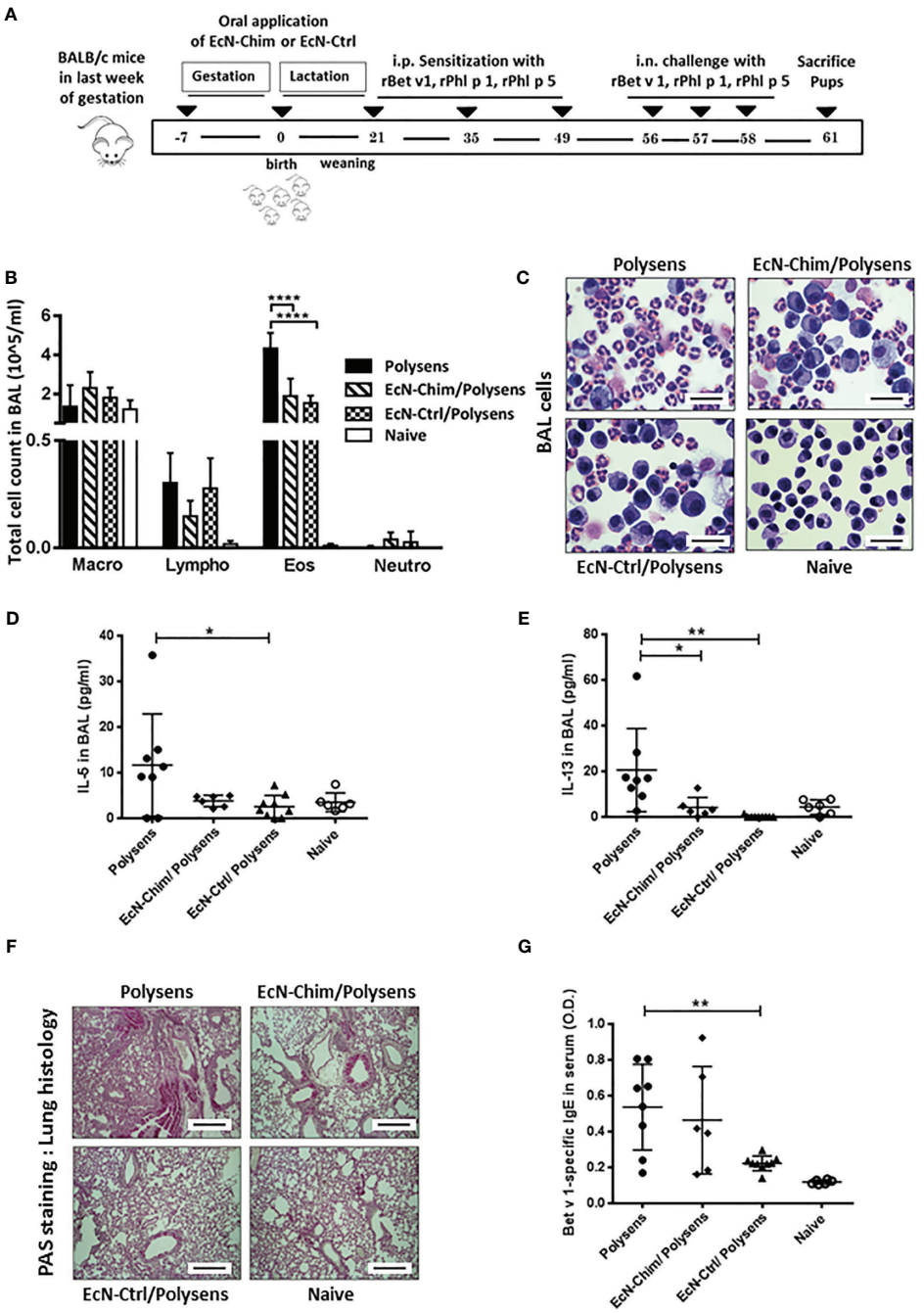

FIGURE 1 | Perinatal application of *Escherichia coli* Nissle 1917 (EcN)-Chim and EcN-Ctrl in conventional mice reduced airway inflammation. **(A)** Schematic representation of the perinatal application of recombinant EcN expressing birch-grass pollen chimera (EcN-Chim) and EcN expressing empty plasmid (EcN-Ctrl) in a conventional mouse model of poly-sensitization. Female BALB/c mice were given either EcN-Chim or EcN-Ctrl orally during the gestation and lactation. On day 21, female offspring derived from these mothers were separated and were then sensitized and challenged with 5 µg Bet v 1, 5 µg Phl p 1, and 5 µg Phl p 5 as described in poly-sensitization model. Mice were sacrificed 72 h after the last challenge. **(B)** Absolute numbers of macrophages, lymphocytes, eosinophils, and neutrophils in bronchoalveolar lavage (BAL) and **(C)** Representative cytospins of BAL of one mouse per group stained with hematoxylin and eosin (H&E; 100x magnification). **(D)** IL-5 and **(E)** IL-13 cytokines in BAL. **(F)** Representative lung tissue sections of one mouse per group stained with Periodic Acid Schiff (PAS) (Red; 10 x magnification; scale bars 100 µm). **(G)** Levels of Bet v 1-specific serum IgE. **(B, D, E, G)** represents the mean ± SEM from two experiments (total n = 6 to 9 mice per group). Error bars show mean ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001 by the One-way ANOVA followed by the Bonferroni's Multiple Comparison Test.

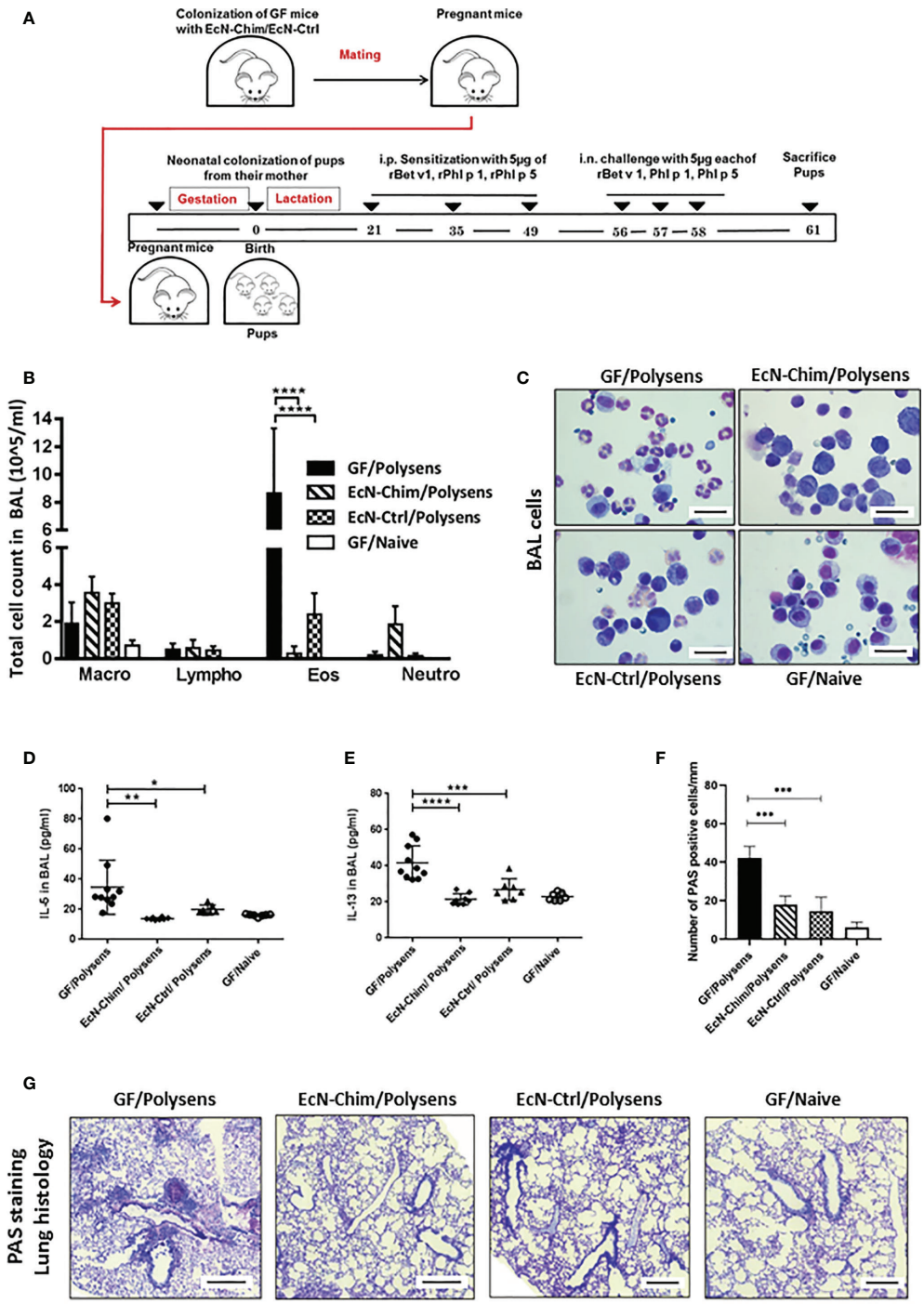

FIGURE 2 | Perinatal and neonatal mono-colonization of *Escherichia coli* Nissle 1917 (EcN)-Chim and EcN-Ctrl in germ-free (GF) mice reduced airway inflammation. **(A)** Schematic representation of the neonatal colonization of GF mice with recombinant EcN expressing EcN-Chim and EcN-Ctrl in a gnotobiotic mouse model of poly-sensitization. Eight-week-old GF mice were colonized with a single dose of either EcN-Chim or EcN-Ctrl by intragastric gavage and mated 10 days later. On day 21, all mono-colonized female offspring were separated from their mothers and divided into two groups. One group was sensitized and challenged with 5 µg rBet v 1, 5 µg rPhl p 1, and 5 µg rPhl p 5 and other not. Mice were sacrificed 72 h after the last challenge **(B)** Absolute numbers of macrophages, lymphocytes, eosinophils, and neutrophils in bronchoalveolar lavage (BAL). **(C)** Representative cytospins of BAL of one mouse per group stained with H&E 100x magnification). **(D)** IL-5 and **(E)** IL-13 cytokines in BAL. **(F)** Quantification of mucus-producing goblet cells. **(G)** Representative lung tissue sections of one mouse per group stained with Periodic Acid Schiff (PAS) (Red; 10x magnification; scale bars 100 µm); arrows indicate cell infiltration. **(B, D–F)** represents mean ± SEM from two experiments (total n = 7 to 10 mice per group). Error bars show mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by the One-way ANOVA followed by the Bonferroni's Multiple Comparison Test.

Perinatal and Neonatal Mono-Colonization With Either EcN-Chim or EcN-Ctrl Inhibits the Development of Th2-Type Allergen-Specific Immune Response

Mono-colonization with EcN-Chim and EcN-Ctrl significantly reduced Phl p 5-specific IgG1 levels (EcN-Chim, $P < 0.05$; EcN-Ctrl, $P < 0.001$) in sera (**Figure 3A**). Perinatal/neonatal mono-colonization with EcN-Ctrl led to a substantial increase of Bet v 1 ($P < 0.01$) and Phl p 5-specific ($P < 0.0001$) IgG2a antibody levels in serum in comparison with GF poly-sensitized control mice (**Figure 3B**). Mono-colonization with EcN-Chim led to increased IgG2a against Phl p 5 ($P < 0.0001$) compared to GF poly-sensitized control (**Figure 3B**). By measuring the IgE-dependent basophil degranulation, we have shown that cells

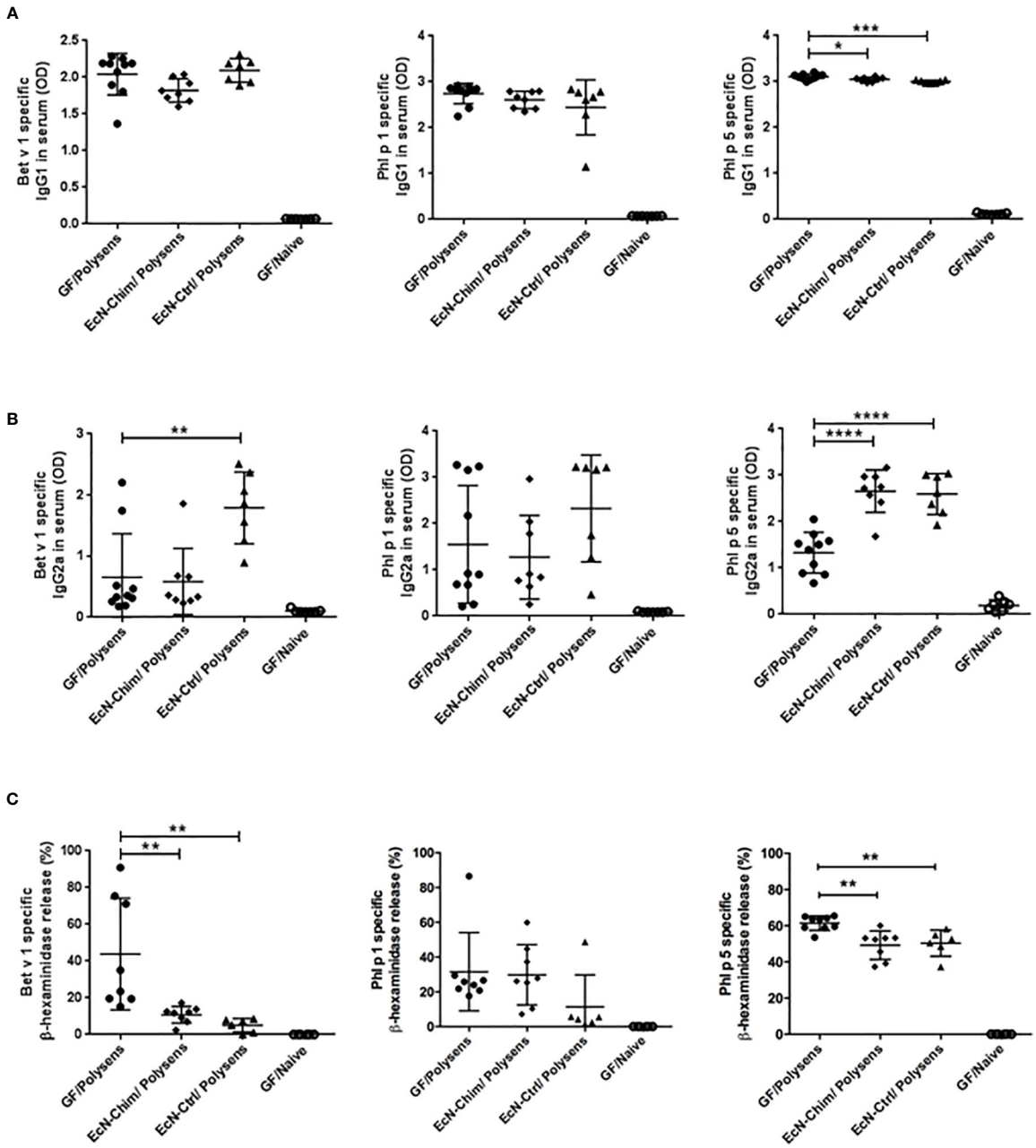

FIGURE 3 | Perinatal and neonatal mono-colonization with *Escherichia coli* Nissle 1917 (EcN)-Chim and EcN-Ctrl inhibits the development of Th2-type allergen-specific immune response. Mice were treated as indicated in **Figure 2A**. Serum samples were obtained from mice on sacrifice day. Allergen-specific antibody levels in mouse sera were determined by ELISA and RBL. The figure represents levels of Bet v 1, Phl p 1, Phl p 5-specific serum **(A)** IgG1 **(B)** IgG2a, and **(C)** IgE. Data represent mean ± SEM from two experiments (total n = 7 to 10 mice per group). Error bars show mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by the One-way ANOVA followed by the Bonferroni's Multiple Comparison Test.

derived from EcN-Chim and EcN-Ctrl-colonized mice and stimulated with rBet v 1 ($P < 0.01$) and rPhl p 5 ($P < 0.01$) exhibited reduced antigen-specific β-hexosaminidase release compared to the sera of GF poly-sensitized controls (**Figure 3C**). No significant difference was observed for Phl p 1-specific IgG2a, IgG1, and β-hexosaminidase release in EcN-Chim and EcN-Ctrl mice in comparison with GF poly-sensitized controls (**Figures 3A–C**).

Perinatal and Neonatal Mono-Colonization With EcN-Ctrl but Not EcN-Chim Induced IL-10, Foxp3, and IFNγ mRNA Expression in the Lung

Expression of IL-10, Foxp3, and IFNγ mRNA in lungs were analyzed with two reference genes GAPDH (**Figures 4A–C**) and β-actin (**Figures 4D–F**). Although mono-colonization with both EcN-Ctrl and EcN-Chim reduced allergy in poly-sensitized mice, only the mono-colonization with EcN-Ctrl triggered the increased expression of IL-10 ($P < 0.01$), Foxp3 ($P < 0.001$), and IFNγ ($P < 0.0001$) mRNA in the lung as compared to GF poly-sensitized control (**Figures 4A–C, E, F**). Mono-colonization with EcN-Chim did not influence the levels of IL-10, Foxp3, and IFNγ mRNA expression in the lung in comparison with GF poly-sensitized mice. EcN-Ctrl showed significantly higher expression of IL-10 ($P < 0.05$), Foxp3 ($P < 0.01$), and IFNγ ($P < 0.01$) compared to EcN-Chim (**Figures 4A–C, F**).

Perinatal and Neonatal Mono-Colonization With EcN-Chim and EcN-Ctrl Increased Expression of TLR2 and TLR4 as Well as Maintained Epithelial Barrier Integrity in the Lung by ZO-1 Expression

Immunochemical staining of the lungs showed increased TLR2 (**Figure 5A**) and TLR4 (**Figure 5B**) expression in the lungs of mono-colonized mice. This increase was confirmed by quantification of TLR2 and TLR4 using ImageJ software. Quantification data showed moderate increase in TLR2 (**Figure 5C**) and strong stimulation of TLR4 (**Figure 5D**) expression in the lung of mice mono-colonized with either EcN-Chim or EcN-Ctrl compared to and poly-sensitized controls.

Lung immunohistochemistry showed maintained levels of ZO-1 in bronchial epithelial cells, in both EcN-Chim and EcN-Ctrl mono-colonized mice as compared to GF polysensitized

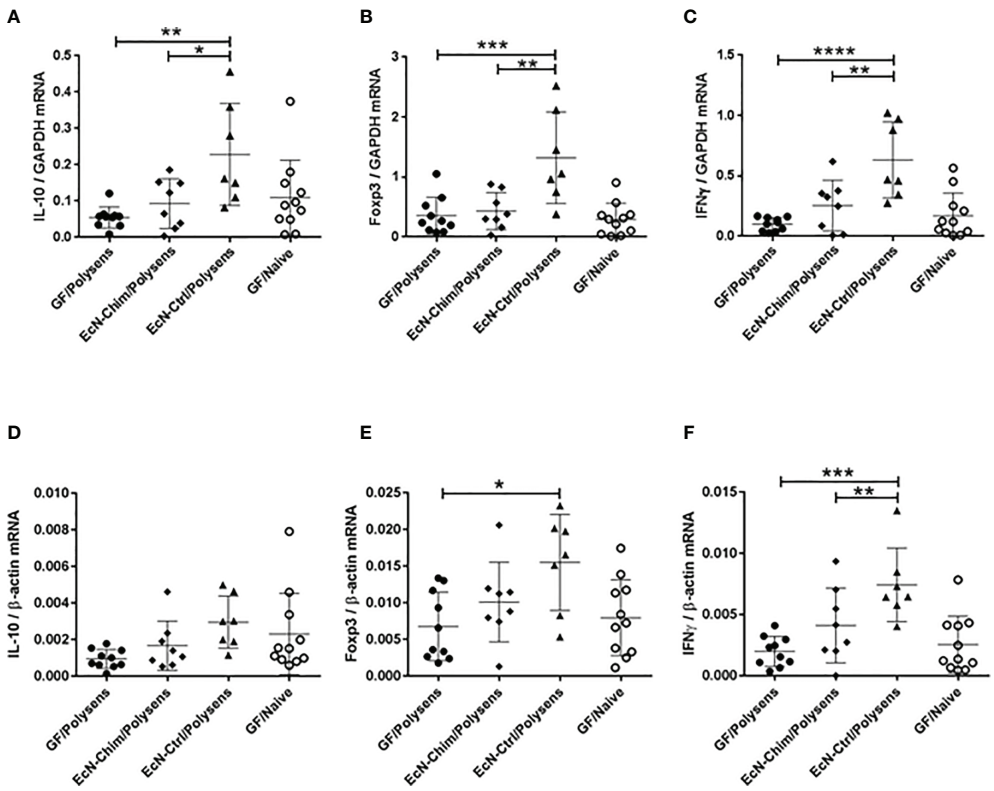

FIGURE 4 | Perinatal and neonatal mono-colonization with *Escherichia coli* Nissle 1917 (EcN)-Ctrl led to increased IL-10, Foxp3, and IFNγ mRNA expression in the lung. Mice were treated as indicated in **Figure 2A**. After sacrifice, lung samples were collected and expression of IL-10, Foxp3 and IFNγ mRNA was measured by real-time (RT)-PCR. The figure represents the ratio of the target genes: **(A)** IL-10, **(B)** Foxp3, and **(C)** IFNγ to the GAPDH reference gene and **(D)** IL-10, **(E)** Foxp3, and **(F)** IFNγ to the β-actin reference gene. Data represents mean ± SEM from two experiments (total n = 7 to 10 mice per group) analyzed by the One-way ANOVA followed by the Bonferroni's Multiple Comparison Test. Error bars show mean ± SEM. Data represents mean ± SEM from two experiments (total n = 7 to 10 mice per group). Error bars show mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by the One-way ANOVA followed by the Bonferroni's Multiple Comparison Test.

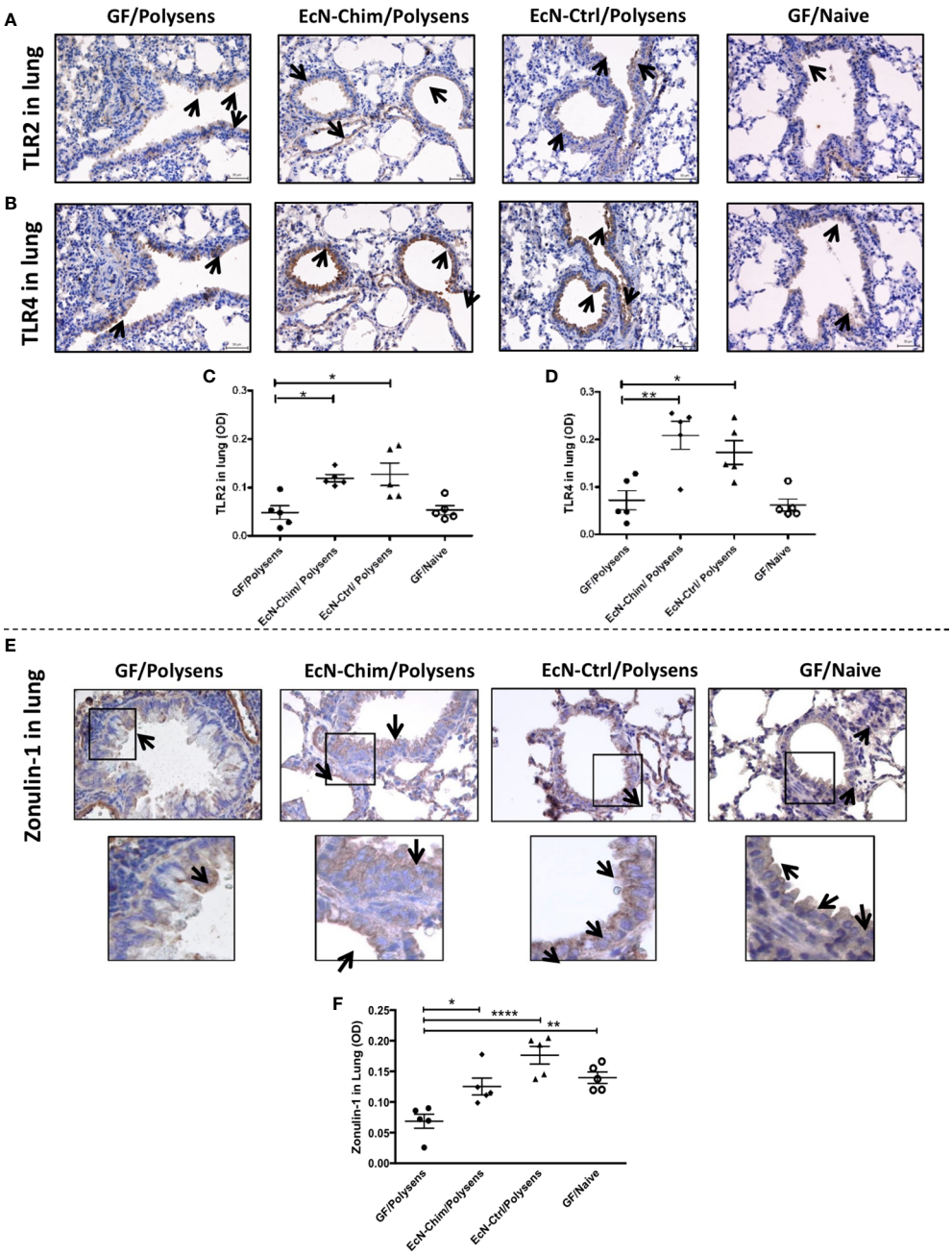

FIGURE 5 | Perinatal and neonatal mono-colonization of either EcN-Chim or EcN-Ctrl activated both TLR2 and TLR4 expression and maintained ZO-1 levels in the lung. Mice were treated as indicated in **Figure 2A**. For immunohistochemistry analysis, lung sections were processed and stained as described in Material and Methods using antibodies against TLR2, TLR4, and ZO-1 followed by 3,3′-Diaminobenzidine (DAB) staining. Samples were analyzed under a light microscope with a 40x objective. Immunohistochemistry staining for TLR2 **(A)**, TLR4 **(B)**, and ZO-1 **(E)** is represented by a brown color (indicated by arrows). Quantification of TLR2 **(C)**, TLR4 **(D)**, and ZO-1 **(F)** expression was performed by optical density analysis (OD) of bronchial epithelial cell layer using ImageJ software. **(C, D, F)** represents mean ± SEM from two experiments (five mice per group were tested). Error bars show mean ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001 by the One-way ANOVA followed by the Bonferroni's Multiple Comparison Test.

mice (**Figure 5E**). Quantification of ZO-1 showed significant decrease in ZO-1 in GF polysensitized mice as compared to both mono-colonized mice (**Figure 5F**). An apparent reduction in epithelial ZO-1 levels was observed in GF poly-sensitized controls as compared to GF naïve group (**Figure 5F**).

DISCUSSION

The constant increase in birch and grass pollen poly-sensitization and the lack of appropriate therapy are raising a considerable demand for a robust prophylactic intervention (5, 6, 39–41). It has

been suggested that the immune response generated during the early phase of life to various environmental factors and antigens may be decisive for the immune response to different allergic diseases later in life (19, 20, 42–44). In this study, we have demonstrated that the perinatal and neonatal mono-colonization of mice with EcN expressing either birch-grass pollen multi-allergen, EcN-Chim, or an empty vector, EcN-Ctrl, prevented the development of poly-sensitization.

Evidence-based studies have revealed that interventions with probiotic bacteria prevent the development of allergy (45, 46). EcN is a probiotic strain with strong immunomodulatory properties and has also been used as an antigen delivery system (47–50). We have previously shown that the EcN-Ctrl was not potent enough to reduce allergic multi-sensitivities in fully-grown mice with established microflora. Therefore, we constructed a recombinant EcN strain, expressing birch and grass pollen allergens chimera, EcN-Chim, which prevented allergen-specific multi-sensitivities in adult mice after intranasal application (28). In the current study, by performing experiments in the perinatal and neonatal phase and taking advantage of early interventions, we have demonstrated that mothers-to-offspring mono-colonization not only with the EcN-Chim but also EcN-Ctrl strain, which does not express the tolerizing allergens, was sufficient to prevent allergy development.

It has been shown that various factors such as reduced infections, delivery by Cesarean section, antibiotic treatment, and microbial dysbiosis during the early age of life can influence the development of the immune system leading to an increased risk of allergic multi-sensitization, particularly in infancy (19, 51–55). The maturation of human immune system occurs early in life in parallel to the establishment of the gut microflora, i.e., approximately from the conception to the end of the second year of childhood (56, 57). Numerous clinical mono-sensitization studies have demonstrated that the early phase of life represents a "window of opportunity" where the immune system can be modulated by the application of certain probiotic bacteria (23–25, 56). In line with other studies, we have previously shown that early intervention with probiotic bacterial strains can prevent birch pollen allergy in mice (21, 22). In the current study, we have demonstrated that the immune system can be modulated by using allergen-specific and allergen-non-specific immunomodulators during the gestation and lactation period to prevent the development of poly-sensitization.

Our group has shown that the route of probiotic application is essential for the outcome of probiotic interventions in animal models of mono- and poly-sensitization (28, 35, 58). In conventional adult mice with fully established gut microflora, we have previously demonstrated that the intranasal route possesses advantages over the oral route of application in reaching the beneficial effects of treatment (28). In the current study in neonates, a single oral application of probiotic bacteria in germ-free mothers was sufficient to induce a local as well as systemic protective immune response against poly-sensitization. This finding has tremendous practical implications since the oral route of application is more convenient and the preferred route for the induction of mucosal tolerance in children against various food- and airborne-triggered allergic diseases (44, 59–61). The oral application to mothers led to the colonization of either EcN-Chim or EcN-Ctrl in offspring (data are not shown). Despite having rich commensal microflora, the EcN strain managed to colonize in the guts of CV offspring during the neonatal period and had a pronounced effect on poly-sensitization. However, it is still unclear how the colonization establishes in offspring. We can assume that this colonization occurs via contact with mothers feces and/or feeding on mothers milk. Several clinical studies demonstrated colonization with probiotic bacteria from mother to offspring *via* breast milk (62, 63).

To investigate the mechanism of tolerance induction by the EcN-Chim and EcN-Ctrl strains against poly-sensitization, we took advantage of a gnotobiotic mouse model. We found that both strains reduced allergic multi-sensitization and AAI. Mice mono-colonized with EcN-Ctrl exhibited increased Th1/Treg responses in the lung. The shift toward Th1/Treg cellular responses in the EcN-Ctrl group was associated with reduced Bet v 1- and Phl p 5-specific IgE measured by rat basophil leukemia (RBL) cells degranulation assay as the level of β-hexaminidase, and increased levels of allergen-specific IgG2a. We did not observe any difference in Phl p 1-specific β-hexaminidase release and IgG2a levels, which might be associated with the high allergenic properties of Phl p 1 (64–66). These results are in line with previous studies suggesting that anti-allergic properties of the EcN are related to the induction of specific Th1/Treg response *via* the induction of Foxp3, IL-10, and IgG2a (28, 50, 67). It is known that Foxp3 and IL-10 play a key role in allergy suppression and maintaining immune tolerance (68–70). Our data indicate that perinatal and neonatal mono-colonization with the EcN-Ctrl precludes poly-sensitization development by polarizing the Th1/Treg response in the lung.

It has been suggested that mucosal TLR2 and TLR4 orchestrate the tolerance in the gastrointestinal and respiratory tract (50, 67, 71). It was further demonstrated that the amelioration of dextran sulfate sodium-induced colitis by EcN is mediated *via* TLR-2- and TLR-4-dependent pathways (72, 73). In our current study, the lung from mice mono-colonized with the EcN-Chim and EcN-Ctrl showed increased expression of both TLR2 and TLR4 compared with GF poly-sensitized and GF naive controls. These findings support our previous *in vitro* data, demonstrating that the EcN can activate both TLR2 and TLR4 expressed in HEK293 cells (28).

Apart from being an important site to initiate the immune response, airway epithelial mucosa plays a vital role in protecting the body from the environment and maintaining the inner homeostasis in the lung (74, 75). Often in allergy, the structure and functions of airway epithelial barriers are markedly impaired by the lung inflammation (76) and this can lead to a more pronounced sensitization (77). To investigate the impact of the EcN-Chim and EcN-Ctrl on barrier functions, we evaluated ZO-1 levels in lung tissue samples. The ZO-1 is the protein expressed in tight junctions that regulates the epithelial barrier function by preventing the entry of different pathogens and antigens (78). The EcN strain has been used previously in premature and full-term infants to enhance postnatal immune competence against necrotizing enterocolitis by improving gut barrier function (79).

In its outer cell membrane, EcN expresses a unique lipopolysaccharide, enabling EcN to exhibit immunomodulating properties without showing immunotoxic effects (27, 80). It was demonstrated that EcN interacts with the intestinal epithelial cells, which fortifies the epithelial cell barrier. It is also suggested that EcN triggers the stimulation of epithelial defensin production, which helps restore the enterocytes' tight junctions (81). Our current study shows that poly-sensitization in the gnotobiotic mouse model led to reduced ZO-1 levels compared to GF naïve mice. However, the neonatal application of both the EcN-Chim and EcN-Ctrl maintained the ZO-1 levels in the airways after poly-sensitization at a similar level as in GF naïve mice.

In conclusion, we provide evidence that interventions with probiotic bacteria early in life could present a promising prophylactic tool to imprint the immune system toward preventing the development of poly-sensitization. Contrary to the adult setting, where the mucosal tolerance was induced only in the presence of tolerizing allergen (EcN-Chim) (28), the interventions during peri- and neonatal period of life led to allergy prevention in mice with the probiotic strain without expressed allergen (EcN-Ctrl). In other words, it was possible to induce tolerance avoiding a potential sensitization with "a foreign" antigen. Therefore, we suggest that the application of wild type probiotic bacteria during the "window of opportunity" is a safe approach. Our work builds the foundation for development of personalized strategies for a prophylactic and therapeutic treatment of poly-sensitization. Further studies in mice and humans are needed to evaluate the full potential of our approach.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experimentation Committee of the Medical University of Vienna and by the Federal Ministry of Science and Research (BMWF-66.009/0384-WF/V/3b/2015) (conventional mice experiments). Gnotobiotic mouse experiments were approved by committee for the Protection and Use of Experimental Animals of the Institute of Microbiology, Academy of Sciences of the Czech Republic (approval ID: 23/2018).

AUTHOR CONTRIBUTIONS

UW received the grant from FWF, and together with HK conceived the study. PS, DS, UW designed the experiments. PS, DS, and NG performed the experiments. PS, NG, DS, AI-K, and UW analyzed the data. MS, AI-K, IS, and UW contributed reagents/materials/analysis tools. PS, AI-K, UW drafted the manuscript. PS, AI-K, IS, and UW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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WSB1 and *IL21R* Genetic Variants Are Involved in Th2 Immune Responses to *Ascaris lumbricoides*

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Genetic and epigenetic factors are considered to be critical for host-parasite interactions. There are limited data on the role of such factors during human infections with *Ascaris lumbricoides*. Here, we describe the potential role of genetic factors as determinants of the Th2 immune response to *A. lumbricoides* in Brazilian children. Stool samples were collected from the children to detect *A. lumbricoides* by microscopy and peripheral blood leukocytes (PBLs) were cultured in whole blood cultures for detection of cytokines (IL-5, IL-10, and IL-13) *in vitro*. Levels of anti-*A. lumbricoides* IgE and IgG4 were measured in plasma. DNA was extracted from PBLs and genotyped using Illumina 2.5 Human Omni Beadchip. Candidate genes associated with *A. lumbricoides* responses were identified and SNVs in these selected genes associated with the Th2 immune response to *A. lumbricoides*. Haplotype, gene expression, and epigenetic analyses were done to identify potential associations with Th2 immune responses. GWAS on samples from 1,189 children identified *WSB1* as a candidate gene, and IL-21R was selected as a biologically relevant linked gene for further analysis. Variants in *WSB1* and *IL21R* were associated with markers of Th2 immune responses: increased *A. lumbricoides*-specific IgE and IL-5/IL-13 by PBLs from infected compared to uninfected individuals. In infected children, *WSB1* but not *IL21R* gene expression was suppressed and increased methylation was observed in the *WSB1* promoter region. This is the first study to show an association between genetic variants in *WSB1* and *IL21R* and Th2 immune responses during *A. lumbricoides* infections in children. *WSB1/IL21R* pathways could provide a potential target for the treatment of Th2-mediated diseases.

Keywords: ***Ascaris lumbricoides*, immunity, polymorphism, *WSB1*, *IL21R***

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INTRODUCTION

A quarter of the world's population is estimated to be infected with soil-transmitted helminth (STH) parasites. The highest prevalence occurs among children living in rural areas of the tropics in conditions of poverty with limited access to treated water and sanitation (1, 2). Among STH infections, *Ascaris lumbricoides* infection is estimated to infect 820 millions causing a significant burden of morbidity and mortality, the latter generally being associated with intestinal obstruction (3–5). Chronic infections in children, particularly among those with high parasite burdens, can impair host nutrition leading to growth stunting and diminished cognitive development (1, 6).

Ascaris lumbricoides infection induces strong Th2-type immune responses in infected humans leading to the production of high circulating levels of total and parasite-specific IgE, generally targeted at larvae that undergo a phase of extra-intestinal migration through the lungs. Th2-induced host protective mechanisms against *A. lumbricoides* parasites include eosinophil-mediated killing of larvae in the tissues, mast-cell degranulation in the tissues and intestinal tract, and increased intestinal mucus production through goblet-cell hyperplasia (7, 8).

WSB1, *IL21,* and *IL21R* genes are important regulators of the IgE response. The *WSB1* gene has a role in the regulation and maturation of the interleukin-21 receptor (IL-21R) (9). The *WSB1* gene was initially described through its relationship with the suppressor-protein-signaling box (SOCS) cytokine family (10, 11). The *IL21R* gene is constitutively expressed on T and B lymphocytes and NK cells (12) and has effects that vary according to the stage of cell differentiation. B cell proliferation and differentiation into plasma cell *in vitro* appear to occur via IL-21 signaling (13) and IL-21R knock-out mice have high levels of IgE and reduced IgG1. In mice, IL-21 inhibits IgE responses through the IL-21 receptor on B cells, triggering IL-4-independent signaling of STAT3 (14). IL21/*IL21R* binding activates STAT-3 and production of interferon-gamma by T cells and NK cells that counteracts the effects of IL-4 on IgE production (13). In contrast, IL-21 activates STAT-3 in human B cells and acts synergistically with IL-4 to increase the secretion of IgE (14). Other studies in humans have shown that IL-21 can suppress IgE synthesis, indicating that effects of the IL-21/IL-12R pathway on IgE production may be affected by host genetics: genetic variants in the *IL21R* gene associated with IgE production have been identified by GWAS (15, 16). IL-21R may have a critical role in the control of allergic responses and helminth infections (17, 18).

The host immune response, during the course of a helminth infection such as *A. lumbricoides*, involves the induction of complex immune responses that include protective Th2-mediated protective mechanisms. Host genetics is likely to play a key role in resistance and susceptibility to *A. lumbricoides* (19). Loci shown to be associated with susceptibility to helminth infection include 5q31-q33, signal transducer and transcriptional activator 6 (STAT6) and ligase 4 (LIG4) (20–22). To date, no genome-wide association studies have addressed the role of host genetics in Th2 responses to *A. lumbricoides* infection and limited candidate-genes studies have been done (22–24). Recently, positive associations between epigenetic alterations of increased histone acetylation and type 2 immune responses including IgE have been observed among individuals infected with *A. lumbricoides* (25).

In the present study, we used a variety of strategies to study genetic determinants of the host Th2 immune response during *A. lumbricoides* infection in children that included gene discovery using a genome-wide approach and a candidate gene approach based on the findings of the former. This was followed by expression quantitative trait loci and epigenetic analyses to explore how genetic variations in candidate genes are linked to host Th2 immune response during *A. lumbricoides* infection.

METHODS

Characterization of the Reference Population

This study was done among children and adolescents in the city of Salvador, Brazil, that has a population of 2.8 millions. The study sample has been described in detail elsewhere (2, 26, 27). Briefly, 1,445 children were recruited in early childhood into a prospective study to measure the impact of a sanitation program in the city of Salvador on child morbidity (28). Data were collected from children born between 1994 and 2001, who lived in sentinel areas of the city. Standardized questionnaires were applied to the legal guardian of each child between 1997 and 2003 (baseline) to collect data on demographic and social variables, as well as on the domestic environment. In 2000, fecal samples were collected for detection of geohelminth parasites by microscopy. The children were surveyed again in 2005 to obtain stool and blood samples for laboratory tests and extraction of genomic DNA.

Ethics

The Brazilian National Research Ethics Committee approved the study protocol and informed written consent was obtained from the legal guardian of each child/adolescent (Resolution Number: 15895).

Blood Collection and Cell Culture

Blood samples were collected in heparinized tubes and peripheral blood leukocytes (PBLs) were cultured in whole blood at a dilution of 1:4 in RPMI medium (Gibco, Auckland, New Zealand), supplemented with 10 mmol/L glutamine (Sigma-Aldrich, St Louis, USA) and 100 μg/ml gentamicin (Sigma-Aldrich, St Louis, USA). PBLs were cultured within 6 h of collection in the presence of *A. lumbricoides* antigen (10 ug/mL, endotoxin-free), pokeweed mitogen (2.5 ug/mL), or no stimulant, in a humidified environment at 37°C with 5% CO_2 for 5 days. Supernatant fluids were harvested for 24 h (IL-10) or 5 days of cultures (IL-5 and IL-13) (2, 29).

IL-10, IL-13, and IL-5 Measurements

Concentrations of IL-5, IL-10, and IL-13 in cell culture supernatant were measured using commercial sandwich ELISAs following the manufacturer's instructions (BD PharMingen, San

Diego, CA, USA). Cytokine concentrations were dichotomized into responders and non-responders using the lowest detection level for each cytokine. Low/high detection limits (in pg/ml) were 15.6/500 for IL-5, 62.5/4,000 for IL-13 and 31.25/500 for IL-10. The number of individuals evaluated for IL-5 and IL-13 production were 67 and 73, respectively.

Parasitological Analysis

Two fecal samples were collected from each child, separated by a 2-week interval, and analyzed for *A. lumbricoides* infection using sedimentation (30) and Kato-Katz methods (31) as described (21). Positive children were defined by the presence of *A. lumbricoides* eggs detected by either method. All positive children were treated with appropriate anthelmintics (26).

IgE and IgG4 Anti-*A. lumbricoides* Antibodies Serum Concentrations

The ImmunoCAP assay (Phadia Diagnostics AB, Uppsala, Sweden) was used for determination of specific IgE serum concentrations against *Ascaris* and positive samples had $\geq$0.35 kU/L of anti–*A. lumbricoides* IgE. Anti–*A. lumbricoides* IgG4 was detected using an indirect ELISA as described previously (32).

Genotyping and Quality Control

Genotyping was performed using the Illumina BeadChip Human Omni2.5-8 Kit (www.illumina.com), by the Consortium EPIGEN-Brazil (https://epigen.grude.ufmg.br/). One individual was excluded due to inconsistency between registered and genetic sex, based on X chromosome SNVs and 61 were removed based on kinship coefficients ($\geq$0.1, to include second-degree relatives) between pairs of individuals (33). SNVs excluded from the analysis were: on X, Y and mitochondrial chromosomes; genotyping call rate <0.98; and deviance in the Hardy-Weinberg equilibrium with a *P*-value $< 10^{-4}$ and Minor Allele Frequency (MAF) <1% (34). After quality control, 1,857,191 autosomal SNVs were included. A total of 636 individuals had detectable values of IgE and/or IgG4 and were included in the analysis. Linear regression was done using ln-transformed ratio of anti-*A. lumbricoides* IgE to IgG4 (35). Through this genome-wide analysis, we selected SNVs in the *WSB1* gene pathway for a candidate gene approach based on biological role in immune response from among the top 20 hits. The closely linked gene pathway for *IL21R* was selected also. Genotype information for these two genes was extracted from the chip at the following regions: *WSB1* from 27294080 to 27315926 (location: NC_000017.11) position at chromosome 17. *IL21R* from 27402162 to 27452043 (location: NC_000016.10) position at chromosome, 16 and a candidate-gene analysis was done for both genes. For quality control, the following filters were applied: genotyping call rate >90%, imbalance of Hardy-Weinberg equilibrium with $P < 0.05$ and the Minor Allele Frequency (MAF) >1% (34). A total of 12 markers on *WSB1* and 35 markers on *IL21R* were analyzed after quality control. These data are deposited in the European Nucleotide Archive [PRJEB9080 (ERP010139) Genomic Epidemiology of Complex Diseases in Population-Based Brazilian Cohorts], Accession No. EGAS00001001245, under EPIGEN Committee Controlled Access mode.

Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

To evaluate the expression levels of *WSB1* and *IL21R* genes, RNA was isolated from PBL cultures using RNeasy Mini Kit (Qiagen, Hamburg, Germany) and 0.3 μg of total RNA from each sample was reverse transcribed into cDNA using 200 U of Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and 500 ng of Oligo (dT) (Life Technologies, Carlsbad, CA, USA), as described previously (36). Pre-synthesized Taqman® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) were used to amplify *WSB1* (Hs00373204_m1), *IL21R* (Hs00222310_m1) and *β-actin* (Hs01060665_g1). cDNA was detected using QuantStudio 12K Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each qRT-PCR assay was performed with 10 ng of cDNA in 10 μL of Taqman-PCR Master mix 2X (Applied Biosystems, Foster City, CA, USA) and 1 μL of primer/probe set and purified using deionized H_2O q.s. 20 μL. Gene expression was normalized to *β-actin* levels. Relative quantification was performed using the comparative threshold cycle (ΔΔCT) method (37–39).

In silico Functional Analysis

RegulomeDB (regulomedb.org) is a database for interpretation of regulatory variants in the human genome. It includes high-throughput, experimental datasets from ENCODE (Encyclopedia of DNA Elements) and other sources. A score ranging from 1 to 6 is attributed for each SNV; the lower the score, the greater the presumed involvement in regulatory processes (40).

DNA Methylation Assessment

We used an epigenetic approach to determine the level of methylation on the promoter region *WSB1* following infection with *A. lumbricoides* using OneStep qMethyl kit (Zymo Research). Primers within the CpG rich (promoter) region of *WSB1* were: forward, 5′-CAG GCC TTT GCA ATG TTT AGG-3′; reverse, 5′-AGC CAG CAG GTT TTA GGA AGG-3′. Methylation percentages were obtained using 20 ng of DNA in duplicate in test and reference reaction mixes. Reactions were done using a QuantStudio 12K Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as follows: 2 h 37°C; 10 min 95°C; 40 cycles 30 s 95°C, 1 min 54°C, 1 min 72°C followed by an dissociation stage to check specificity of PCR products. The Ct values obtained were used to calculate ΔCt values Ct (test) and Ct (reference). Methylation percentages were calculated as the product of 100 × 2−ΔCt.

Statistical Analysis

For GWAS, linear regressions were done to evaluate the association between SNVs and lg (anti-*A. lumbricoides* IgE/anti-*A. lumbricoides* IgG4) using additive models. Power for genetic association analyses depends on effects of individual polymorphisms (depending on both allelic frequency and associated OR/beta), sample size, and type I error. In the context of GWAS, it is common to consider two levels of significance:

TABLE 1 | Baseline characteristics and immunological markers of *A. lumbricoides* infection among 1,189 children, stratified by *A. lumbricoides* infection.

Variables	Subject group				*P*-value*
	Infected (*N*)	%	Uninfected (*N*)	%	
	189	15.9	996	84.1	
Sex					
Male	106	56.1	537	53.7	0.558
Female	83	43.9	459	45.9	
Age					
≤5	60	31.7	379	37.9	0.133
6–7	64	33.9	348	34.8	
≥8	65	34.4	269	26.9	
Anti-*A. lumbricoides*					
IgE	134	70.9	458	45.8	<0.001
IgG4	65	34.4	126	12.6	<0.001
Cytokine production by *A. lumbricoides*-stimulated peripheral blood cells#					
IL-5	36	19.0	95	9.5	<0.001
IL-13	41	21.7	185	18.5	0.589
IL-10	13	6.9	36	3.6	0.082

*P-values were derived using the chi-squared-test.
#Percentage of responders children for each cytokine evaluated. Responders were defined as those children with cytokine concentrations above the lower detection limits for IL-5 (>15.63 pg/mL), IL-13 (>62.5 pg/mL), and IL-10 (>31.25 pg/mL).

a more stringent level such as 5×10^{-8} which may allow a conclusion of statistical significance, and a less stringent level such as 1×10^{-5} to identify potentially suggestive associations. Using an additive model and a type I error of 1×10^{-5}, our sample of 996 uninfected and 189 infected children had a power of 80% to detect a polymorphism with beta of 0.3 and frequency >0.15. The statistical power calculation was done using Quanto software (v1.2.4). Models were controlled for confounding by population stratification by inclusion of the first three components of a principal components analysis (PCA) of ancestry informative markers (AIMs) as described (41). In addition, the genomic inflation factor (λ) was estimated to visualize and avoid inflated test statistics (42). Quantile-quantile (Q-Q) plots were used to evaluate the overall significance of the genome-wide association results (**Supplementary Figure 2**). Associations between polymorphisms in *WSB1* or *IL21R* and *A. lumbricoides* infection, and IL-5, IL-13, and IL-10 cytokine production by PBLs stimulated with *A. lumbricoides* and anti-*A. lumbricoides* IgE and IgG4 were done using logistic regression model in which multivariate models were adjusted for sex, age, and ancestry (first 2 components of PCA analysis of AIMs). Principal components (PC1 and PC2) have categorized individuals according to their ethnic characteristics. Additive models were used in all analyses. Adaptive permutations were also done in adjusted and unadjusted analyses. A computationally intensive procedure based on 1,000,000 permutations was used to estimate the statistical significance of multiple correlation tests in the genetic association analysis (43). Haplotype and genetic risk score analysis were performed using SNPStats program (https://www.snpstats.net/start.htm) (44). Linkage disequilibrium (LD) analysis was done for selected SNVs. Haploview 4.2 software was used to calculate the degree of confidence in the R^2-value. Mann-Whitney or Kruskal-Wallis-tests were used to compare continuous variables and the Chi-squared-test to compare frequencies of categorical variables. Except as specified for GWAS, statistical significance was inferred by $P < 0.05$. Statistical analyses were done using PLINK 1.9 software (www.cog-genomics.org/plink/1.9/), R Statistical Software (Foundation for Statistical Computing, Vienna, Austria), and Prism software version 6 (GraphPad Inc., San Diego, CA).

RESULTS

Characteristics of the Study Population

Of 1,246 children eligible, 61 did not have stool data for *A. lumbricoides* infection and were excluded from the analysis, leaving 1,189 (996 non-infected and 189 *A. lumbricoides* infected) children with complete data. Baseline characteristics of the analysis sample are shown in **Table 1**. Levels of anti-*A. lumbricoides* IgE and IgG4, and levels of IL-5 produced by *A. lumbricoides*-stimulated PBLs were greater among infected than non-infected children ($P < 0.001$).

Genome Wide Association Study for SNVs Linked to Parasite-Specific IgE/IgG4 Responses

The Manhattan plot for the genome wide analysis of SNVs associated with anti-*A. lumbricoides* IgE/IgG4 are shown in **Figure 1** and the top 20 SNVs identified are listed in **Table 2** with results of a mapping analysis for these provided in **Supplementary Table 1**. Among identified SNVs, rs7212516 (Beta: 0.33, CI: 0.18–0.47, $P = 6.675 \times 10^{-06}$), is an intronic variant located in the *WSB1* (WD repeat and SOCS box containing 1) gene and plays an important role in IgE production (9). Further analyses were focused on *WSB1* and the linked gene, *IL21R*. In addition, SNV rs3093406 in *IL21R* was significantly associated with IgE/IgG4 ratio in GWAS ($P = 0.0222$).

Using log-transformed anti-*A. lumbricoides* IgE and IgG4 as continuous variables: IgG4 was associated with immune response genes such as PRKCA (a kinase that participates in macrophage differentiation induced by macrophage colony-stimulating factor) (*P*-values ranging 10^{-5} and 10^{-6}); and continuous IgE was associated with several genes including NKAI2, a sodium-potassium transporter ATPase in T cells (*P*-values ranging 10^{-5} to 10^{-6}).

Associations Between *WSB1* and *IL21R* Variants and Parameters of Host Immune Response to *A. lumbricoides*

The associations between 12 variants in *WSB1* and infection with *A. lumbricoides* and the host immune response to the parasite were studied (**Table 3**). *P*-values refer to the permutational test. With respect to levels of anti-*A. lumbricoides* specific IgE,

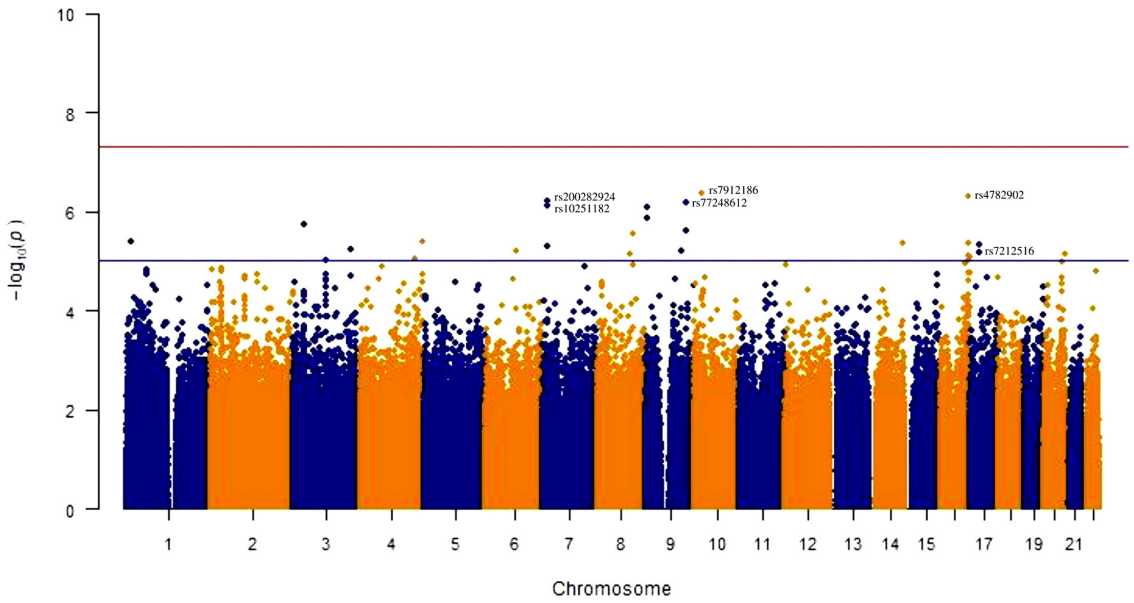

FIGURE 1 | Manhattan plot for genome-wide association with anti-*A. lumbricoide* IgE/IgG4 ratio for 636 subjects. Each single nucleotide variants (SNVs) was tested for association by linear regression using an additive regression model, adjusted for ancestry markers. The red line indicates genome-wide significance ($p = 5 \times 10^{-8}$) and blue line suggestive significance ($P = 1 \times 10^{-5}$).

TABLE 2 | The results for the 20 best hits in the genome wide association with study anti-*A. lumbricoide* IgE/IgG4.

Rank	Chr	SNV	Position (bp)[#]	Risk allele	MAF	Gene	β (CI 95%)	*p*
1	10	rs7912186	25602870	C	0.02	GPR158	0.75 (0.46–1.04)	4.176×10^{-07}
2	16	rs4782902	82449332	C	0.47	Intergenic	0.25 (0.15–0.35)	4.89×10^{-07}
3	7	rs200282924	12171197	C	0.46	Intergenic	0.23 (0.14–0.33)	6.341×10^{-07}
4	9	rs77248612	120566205	A	0.02	LOC105376244	0.80 (0.49–1.12)	6.896×10^{-07}
5	7	rs10251182	12171373	T	0.46	Intergenic	0.23 (0.14–0.33)	7.527×10^{-07}
6	9	rs10081726	2021814	T	0.08	SMARCA2	0.40 (0.24–0.56)	8.095×10^{-07}
7	9	rs12550848	2021655	T	0.08	SMARCA2	0.39 (0.23–0.55)	1.379×10^{-06}
8	3	rs4645161	31977744	T	0.45	OSBPL10	0.23 (0.14–0.33)	1.795×10^{-06}
9	9	rs77772209	120537157	A	0.02	Intergenic	0.73 (0.46–1.12)	2.464×10^{-06}
10	8	rs77284244	108679300	A	0.03	Intergenic	0.71 (0.41–1.00)	2.771×10^{-06}
11	1	rs12738424	1.5E+07	G	0.42	KAZN	0.23 (0.13–0.33)	4.084×10^{-06}
12	4	rs7653904	186607977	T	0.35	SORBS2	0.23 (0.13–0.33)	4.136×10^{-06}
13	16	rs1025065	82451159	T	0.46	Intergenic	0.23 (0.13–0.33)	4.37×10^{-06}
14	14	rs61992474	102532899	C	0.13	Intergenic	0.30 (0.43–0.17)	4.377×10^{-06}
15	17	rs7219758	25597243	G	0.13	Intergenic	0.34 (0.19–0.48)	4.91×10^{-06}
16	7	rs11980827	12171659	C	0.47	Intergenic	0.21 (0.12–0.31)	5.221×10^{-06}
17	3	rs6444926	170133235	T	0.12	Intergenic	0.30 (0.17–0.44)	5.788×10^{-06}
18	6	rs1998219	92382995	T	0.08	CASC6	0.41 (0.23–0.59)	6.252×10^{-06}
19	9	rs7018777	104794048	G	0.02	Intergenic	0.72 (0.40–1.03)	6.493×10^{-06}
20	17	rs7212516	25621797	C	0.13	WSB1	0.33 (0.18–0.47)	6.675×10^{-06}

Analyses were corrected for genetic ancestry.
[#]*Genomic version GRCh37–hg19; Chr, chromosome; SNV, single nucleotide variation; MAF, Minor Allele Frequency; p, P-value.*

SNVs rs7213148, and rs8065359 were positively associated, while rs1060618 and rs9867 were negatively associated. rs9867 was associated with lower production of IL-5 in *A. lumbricoides* antigen-stimulated PBL cultures. With respect to levels of anti-*A. lumbricoides* specific IgG4, rs6505199 and rs9303634 were inversely associated (rs6505199 and rs9303634 are in

TABLE 3 | Significant associations between SNVs on *WSB1* and parameters of the host immune response to *A. lumbricoides* including specific IgE and IgG4, and IL-5 production by *A. lumbricoides* antigen-stimulated PBLs.

SNV	MAF	A1*	Model	OR	CI 95%	*P*-value
Anti-*Ascaris lumbricoides* IgE						
rs7213148	0.02	T	ADD	1.98	1.16–3.36	0.009
rs8065359	0.09	A	ADD	1.47	1.10–1.96	0.016
rs1060618	0.36	G	ADD	0.79	0.66–0.93	0.005
rs9867	0.03	A	ADD	0.67	0.43–0.98	0.027
Anti-*Ascaris lumbricoides* IgG4						
rs6505199	0.43	G	ADD	0.78	0.63–0.97	0.038
rs9303634	0.43	T	ADD	0.78	0.63–0.97	0.038
rs7212516	0.13	T	ADD	1.39	1.01–1.90	0.034
IL-5 production in *Ascaris lumbricoides*-stimulated blood cell cultures						
rs9867	0.03	A	ADD	0.40	0.16–0.99	0.047

**A1, minor allele; SNV, single nucleotide variation; MAF, Minor Allele Frequency; OR, Odds ratio; P-value, permutacional-test.*

TABLE 4 | Significant associations between SNVs on *IL21R* and infection with *A. lumbricoides* and levels of anti-*Ascaris lumbricoides* IgE and IgG4 and parasite antigen induced production of IL-5 and IL-10 by PBLs.

SNV	MAF	A1	Model	OR	CI 95%	*P*-value
Ascaris infection						
rs9938401	0.48	A	ADD	1.34	1.07–1.96	0.012
rs3093406	0.36	T	ADD	1.82	1.12–2.96	0.018
Anti-*Ascaris lumbricoides* IgE						
rs76678990	0.08	T	ADD	0.65	0.48–0.88	0.004
rs58579343	0.21	T	ADD	0.77	0.63–0.95	0.016
rs11074859	0.18	A	ADD	0.77	0.62–0.95	0.017
rs4140673	0.30	T	ADD	0.82	0.69–0.99	0.033
rs115350516	0.23	A	ADD	1.75	1.08–2.83	0.027
Anti-*Ascaris lumbricoides* IgG4						
rs3093412	0.33	T	ADD	0.25	0.10–0.61	0.002
rs179763	0.23	C	ADD	0.73	0.56–0.97	0.029
IL-10 production in *Ascaris lumbricoides*-stimulated blood cell cultures						
rs3091236	0.21	T	ADD	1.78	1.11–2.85	0.017
rs9930086	0.36	C	ADD	0.55	0.34–0.89	0.019
IL-5 production in *Ascaris lumbricoides*-stimulated blood cell cultures						
rs3093319	0.10	G	ADD	1.40	1.05–1.87	0.031
rs115350516	0.03	A	ADD	2.06	1.11–3.83	0.017
rs3093308	0.21	T	ADD	1.40	1.03–1.89	0.025
rs77718993	0.02	T	ADD	1.93	1.01–3.68	0.037

A1, minor allele; SNV, single nucleotide variation; MAF, Minor Allele Frequency; OD, Odds ratio; P-value, Permutacional-test.

total LD- see **Supplementary Figure 1A**) and rs7212516 was positively associated.

Because *WSB1* is functionally related to IL-21R activation, we scanned the *IL21R* gene for SNVs associated to *A. lumbricoides*. **Table 4** shows significant associations between *IL21R* polymorphisms with the presence of measured parameters of infection and immune response to *A. lumbricoides*. The SNVs rs9938401 and rs3093406 were positively associated with a presence of active *A. lumbricoides* infection. SNVs rs3093412 and rs179763 were inversely associated with levels of anti-*A. lumbricoides* IgG4. Four SNVs were inversely associated with levels of anti *A. lumbricoides* IgE (T allele rs76678990; T allele rs58579343; A allele rs11074859; and T allele rs4140673), while two SNVs were positively associated (T allele rs3093308; A allele, rs115350516). These latter two SNVs were associated also with higher IL-5 production by PBLs stimulated with parasite antigens (T allele, rs3093308; A allele, rs115350516) as were rs3093319 and rs77718993. The SNV rs3091236 was positively associated with IL-10 production while rs9930086 was negatively associated. A high degree of linkage disequilibrium was seen between rs58579343 and rs11074859 (see **Supplementary Figure 1B**).

TABLE 5 | Associations between haplotypes for rs115350516 and rs3093308 in the *IL21R* gene and levels of anti-*A. lumbricoides* IgE and IL-5 produced by *A. lumbricoides*-stimulated PBLs.

Haplotype	rs115350516	rs3093308	Frequency	OR[a] (95% CI)	*P*-value
IL-5 production in *A. lumbricoides*-stimulated PBLs					
Reference	G	C	0.74	1	—
Haplotype1	G	T	0.23	1.73 (1.19–2.52)	0.004
Haplotype2	A	C	0.03	3.31 (1.60–6.88)	0.001
Anti-*A. lumbricoides* IgE					
Haplotype1	A	C	0.03	2.08 (1.14–3.80)	0.018

[a]*Adjusted for gender, age, and ancestry markers; OD, Odds ratio.*

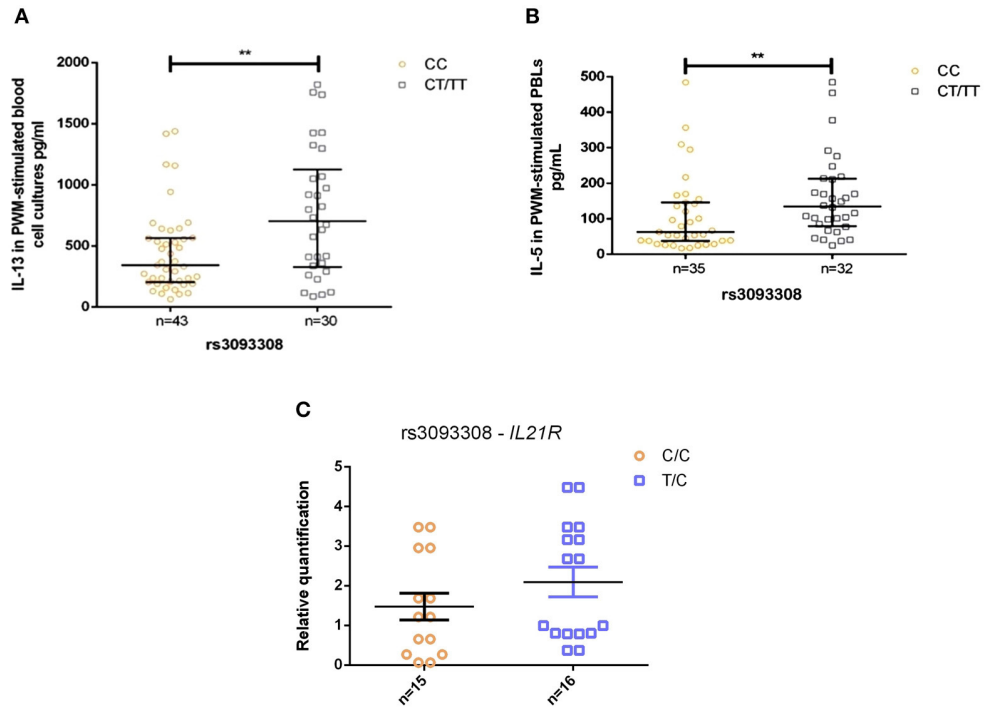

FIGURE 2 | Levels (pg/ml) of IL-5 and IL-13 in *A. lumbricoides* infected subjects produced by parasite antigen stimulated PBLs, by allele for SNV rs3093308 in *IL21R*: **(A)** IL-13, **(B)** IL-5, and **(C)** gene expression analysis for *IL21R* in PBLs (non-significant). CC, genotype CC; CT/TT, genotype CT/TT. $^{**}p < 0.01$.

Haplotype analysis for *IL21R* SNVs rs115350516 and rs3093308 showed that PBLs from individuals with haplotypes GT and AC produced greater levels of IL-5 when stimulated with parasite antigen (**Table 5**). Individuals with haplotype AC produced greater levels of anti-*A. lumbricoides* IgE.

SNV rs3093308 in *IL21R* Is Associated With Type 2 Cytokine Production

The T allele of SNV rs3093308 was associated with elevated levels of anti-*A. lumbricoides* IgE and parasite antigen induced IL-5 production, both indicators of a strong Th2 response and potential resistance to *A. lumbricoides* infection (45). The presence of one T allele of rs3093308 was associated with higher levels of Th2 cytokines (IL-5 and IL-13, **Figures 2A,B**) by mitogen-stimulated PBLs among infected individuals ($P <$ 0.01). None of the other SNVs studied were associated with alterations in *in vitro* cytokine production by infection status (data not shown). However, *IL21R* gene expression by PBLs was not significantly different between the two alleles (**Figure 2C**).

Expression of *WSB1* and *IL21R* in *Ascaris*-Infected and Uninfected Individuals

Figure 3 shows the expression levels of the *WSB1* and *IL21R* genes in *A. lumbricoides*-infected and uninfected individuals. Expression of *WSB1* was lower in infected ($N = 15$) subjects compared to uninfected ($N = 16$) subjects ($P = 0.0207$; **Figure 3A**). No difference was observed between the two groups for *IL21R* gene expression (**Figure 3B**).

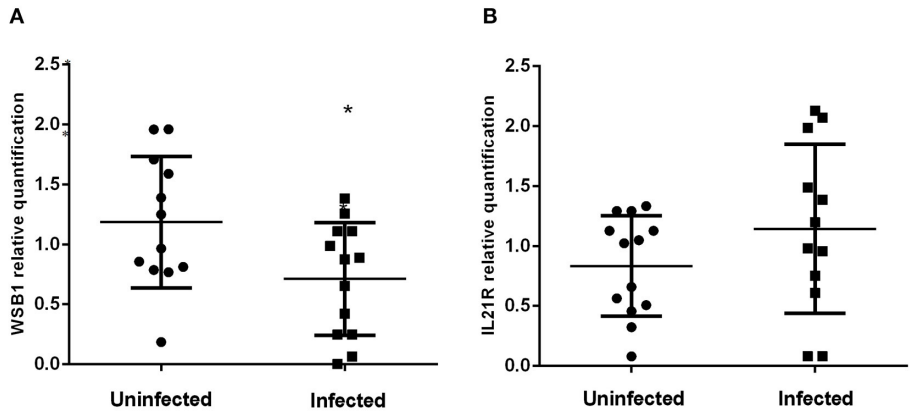

FIGURE 3 | Levels of *WSB1* **(A)** and *IL21R* **(B)** gene expression in *Ascaris lumbricoides*- infected ($N = 15$) and uninfected ($N = 16$) subjects in peripheral blood leukocytes. $^*P \leq 0.05$.

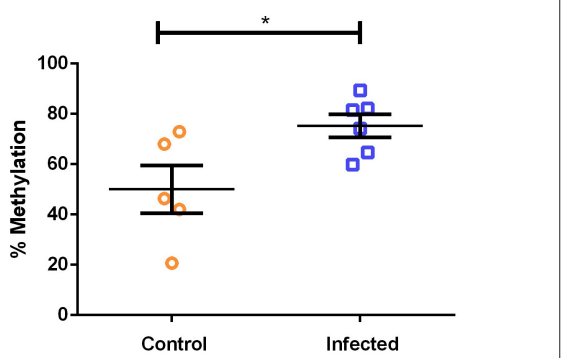

FIGURE 4 | Percentage of DNA methylation in the WSB1 promoter region in *Ascaris lumbricoides*-infected ($N = 9$) and uninfected ($N = 8$) individuals ($^*P = 0.031$, Mann-Whitney-test).

Methylation of *WSB1* Promoter Region

Figure 4 shows the percent methylation of *WSB1* gene in PBLs from *A. lumbricoides*-infected ($N = 9$) and uninfected ($N = 8$) individuals. Infection with *A. lumbricoides* was associated with increased *WSB1* methylation ($P = 0.031$). rs7212516 is in the first intron (position 692 bp). The region included in the methylation analysis is in the promoter region (position −771 bp to −443 bp) containing 14 CpG sites and 4 restriction sites (according to NCBI, Gene ID: 26118). The other SNVs are in the position above 6,000 bp. The amplified region for DNA methylation was analyzed in the reference populations of the 1,000 genome project and found 13 SNVs in that region, two of them with a frequency >1% in Africans and African Americans (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/?assm=GCF_000001405.25). These two SNVs do not alter or create restriction sites for the enzymes present in the OneStep qMethyl kit (Zymo Research). In addition, none of the 13 SNVs identified in the 1000 genomes project belong to Illumina BeadChip Human Omni2-8-8 Kit, used here for genotyping as well.

DISCUSSION

Previous studies suggest that the balance between helminth specific IgE and IgG4 might determine resistance or susceptibility to *helminth* infections, showing that levels of specific IgE have been correlated with resistance to infection, whereas levels of IgG4 have been associated with susceptibility (34–36). In this manuscript, we have conducted two distinct approaches to determine genetic markers associated with *A. lumbricoides* infection. First, we conducted a GWAS for anti-*A. lumbricoides* IgE/IgG4 ratio for *A. lumbricoides* infection in a cohort of an admixture population to determine if there are common genetic variants contributing to susceptibility to *A. lumbricoides* infection and as a second phase, based on the GWAS pieces of evidence, we focused our attention to WSB1/*IL21R* pathway, which revealed associations with markers of exposure and cytokine responses to *A. lumbricoides*. For the best of our knowledge, these associations have never been reported before.

We did not identify any novel SNVs meeting genome-wide significance but did identify several SNVs below the genome-wide threshold as being of potential interest: (1) rs7912186 in the *GPR158* gene, described as being linked to plasma membrane scaffold protein in retinal bipolar neurons, contributing to the pathophysiology of steroid-induced ocular hypertension and glaucoma, (46, 47) and also involved in the regulation of the prefrontal cortex with a potential role chronic stress and depression (48); (2) rs10081726 and rs12550848, located in SMARCA2, that plays a role in the development of lung cancer, hepatocellular carcinoma and esophageal adenocarcinoma (49, 50); and (3) rs7212516, located in the *WSB1* gene on chromosome 17 that is known to be involved in IgE regulation (9).

Previous epidemiological studies have shown inverse associations between levels of anti-*Ascaris* IgE and parasite burden with *A. lumbricoides* indicating a potential role for IgE in resistance to infection (51, 52). WSB-1, a IL-21 receptor binding

molecule, enhances the maturation of IL-21 receptor. *WSB1* gene plays an important role in the regulation and maturation of the *IL21R*, and both genes are important for IgE production (9). For this reason, we included the *IL21R* as a gene of biological relevance in our candidate gene analysis. There are several lines of evidence showing that IL-21/IL-21R signaling plays a clear role modulating Type 2 cytokines production (9, 11). Mice deficient for *IL21R* had reduced airways eosinophilia in a model of mite-induced asthma (18), and knock-out mice for *IL21R* expressed higher levels of IgE and lower levels of IgG1 than normal mice after mite antigen exposure (17).

We explored if genetic variants in *WSB1/IL21R* might influence Th2-associated immune responses during *A. lumbricoides* infection using immunological markers of susceptibility and resistance to infection including production of Th2 cytokines *in vitro*. Our results show that variants in these two genes are associated with such markers of the host Th2 response during this helminth infection. SNVs in *WSB1* (rs7213148 and rs8065359) and *IL21R* [rs115350516 (A allele) and rs3093308 (T allele)] were associated with increased production of *A. lumbricoides*-specific IgE (**Tables 4**, **5**) and could be potentially linked to greater resistance to infection. The same two SNVs in *IL21R* SNVs were associated with greater parasite antigen-induced IL-5 production that has been linked to resistance to geohelminth infections (45). These SNVs have not been linked previously to helminth infection or Th2-driven inflammatory conditions.

The T allele of rs3093308 in *IL21R* was associated also with increased production of Th2 cytokines (IL-5 and IL-13) by mitogen-induced PBLs among infected compared to uninfected children (**Figures 2A,B**), and the same SNV tended to increase *IL21R* gene expression (**Figure 2C**). These findings could be indicative of a stronger protective immune response against *A. lumbricoides* infection. No previous studies have reported a role for this SNV. In a study evaluating IL21/*IL21R* signaling in murine model of intestinal inflammation, Th2 responses (IL-4 and IL-5 by CD4+ T cells) were markedly suppressed in *IL21R* deficient compared to wild-type mice (53).

Our data can explain, at least in part, findings from previous studies showing elevated *Ascaris*-specific IgE levels to be associated with decreased worm burden and increased resistance to infections with this helminth (51, 52). Other studies have shown significant associations between *locus* 13q33 that includes the genes, LIG4, ABHD13, and TNFSF13B, with *Ascaris*-specific IgE levels (22, 54, 55). Thus, consistent with our findings, genetic regulation of IgE production may play an essential role in susceptibility to *Ascaris* infection.

In our population, the G and T alleles of SNVs rs6505199 and rs9303634, respectively, in *WSB1* (see **Table 3**), were associated with reduced *Ascaris*-specific IgG4 levels. These results favor increased production of IgE relative to IgG4, the latter known to be a marker of susceptibility to infection (51, 56). Both SNVs were in high linkage disequilibrium ($r^2 = 1.00$), (see **Supplementary Figure 1A**). Conversely, the T allele of SNV rs7212516 was positively associated with *Ascaris*-specific IgG4 and perhaps greater susceptibility to the infection.

We also did haplotype analyses in *WSB1* and *IL21R* genes for anti-*A. lumbricoides* IL-5 and IgE production. Two SNVs *IL21R* (rs115350516 and rs3093308) and their haplotypes, especially the AC haplotype, were associated with increased production of IL-5 by *Ascaris*-stimulated PBLs. This same haplotype showed a positive association with anti-*A. lumbricoides* IgE levels. Interestingly, in regression analyses these same SNVs were associated with increased anti-*A. lumbricoides* IgE and IL-5 by *Ascaris*-stimulated PBLs which could be linked to a more effective protective immune response against the parasite. Previous studies have analyzed levels of *WSB1* expression in the brain, spleen, kidney and placenta, primarily with research focusing on cancer development (9, 57). There is no previous study describing the role of *WSB1* in helminth infections or any other Th2-driven condition.

In our gene expression assay, the *WSB1* had lower expression levels in infected subjects when compared with non-infected subjects (**Figure 3A**). This result allows us to hypothesize that low levels of *WSB1* expression in infected subjects may be related to epigenetic regulation as we have demonstrated increased methylation of the *WSB1* promoter region in infected individuals (**Figure 4**). Although cell populations within whole blood cultures may differ between cases and controls [infected subjects had greater total leukocyte and eosinophil counts than uninfected subjects (data not shown)], our findings indicated that infection was associated with greater methylation but lower gene expression. However, further studies are required to support a potential effect of *A. lumbricoides* infections on *WSB1* gene hypermethylation. Epigenetic events, such as post-transcriptional modifications of DNA at CPG sites, regulate gene transcription activity, thereby determining the kinetics and final expression (58, 59). On the other hand, there was no statistical difference in gene expression levels for the *IL21R* (**Figure 3B**). Persistent helminth infections appear to induce changes in DNA methylation in CD4+ cells from helminth-infected individuals. Other epigenetic mechanisms may also be involved in the expression of key genes in the type 2 immune response. A study evaluated histone acetylation in individuals exposed to *A. lumbricoides* found that histone acetylation levels in IL-4 and IL-13 genes were altered by infection (25).

This study has a number of potential limitations including: a relatively small sample size limiting power using the GWAS genome strategy; we were unable to do a replication analysis because of a lack of previous studies collecting data on the same variables (e.g., anti-*A. lumbricoides* IgE or IgG4); and use of whole blood cultures rather than more homogeneous lymphocyte populations due to logistical issues inherent to a population-based study such as ours (26).

Our results, therefore, provide novel mechanistic insights into how helminth infections that affect immune response regulation may modulate also epigenetic processes. Further studies are needed to improve our understanding on how such regulation may occur and the consequences for Th2-driven inflammatory conditions.

DATA AVAILABILITY STATEMENT

The genetic data sets generated in this study can be found online upon request at https://cidacs.bahia.fiocruz.br/en/platform/epigen-genomic-epidemiology-of-brazilian-cohorts/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute of Collective Health, Federal University of Bahia, Salvador, Brazil. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

VC, HS, GQ, RV, PO, NC, AP, and RS have conducted the field work, managed the database, and performed the genetic experiments. FS, EB, CM, and RC were responsible for the supervision of the field work and/or laboratory experiments. VC and HS were responsible for data analysis and writing of the manuscript. MB, NA-N, and PC drafting the paper and revising it critically. CF substantial contributions to research design, or the acquisition, analysis or interpretation of data. All authors have actively participated of the analysis of research results and approve the version that is being submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.622051/full#supplementary-material

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Human β-Defensin 2 Mutations Are Associated With Asthma and Atopy in Children and Its Application Prevents Atopic Asthma in a Mouse Model

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Asthma and allergies are complex, chronic inflammatory diseases in which genetic and environmental factors are crucial. Protection against asthma and allergy development in the context of farming environment is established by early animal contact, unpasteurized milk consumption and gut microbiota maturation. The human β-defensin 2 (hBD-2) is a host defense peptide present almost exclusively in epithelial tissues, with pronounced immunomodulatory properties, which has recently been shown to ameliorate asthma and IBD in animal models. We hypothesized that adequate hBD-2 secretion plays a role in the protection against asthma and allergy development and that genetic variations in the complex gene locus coding for hBD-2 may be a risk factor for developing these diseases, if as a consequence, hBD-2 is insufficiently produced. We used MALDI-TOF MS genotyping, sequencing and a RFLP assay to study the genetic variation including mutations, polymorphisms and copy number variations in the locus harboring both genes coding for hBD-2 (*DEFB4A* and *DEFB4B)*. We administered hBD-2 orally in a mouse model of house dust mite (HDM)-asthma before allergy challenge to explore its prophylactic potential, thereby mimicking a protective farm effect. Despite the high complexity of the region harboring *DEFB4A* and *DEFB4B* we identified numerous genetic variants to be associated with asthma and allergy in the GABRIELA Ulm population of 1,238 children living in rural areas, including rare mutations, polymorphisms and a lack of the *DEFB4A*. Furthermore, we found that prophylactic oral administration of hBD-2 significantly curbed lung resistance and pulmonary inflammation in our HDM mouse model. These data indicate that inadequate genetic capacity for hBD-2 is associated with increased asthma and allergy risk while adequate and early hBD-2 administration (in a mouse model) prevents atopic asthma. This suggests that hBD-2 could be involved in the

protective farm effect and may be an excellent candidate to confer protection against asthma development.

Keywords: hBD-2, asthma, atopy, prevention, defensin

INTRODUCTION

Exposure to a diversity of microbes is crucial for the development and maturation of the human immune system (1–3). Children born and growing up in a traditional farm environment are protected against chronic inflammatory diseases such as asthma, allergy and inflammatory bowel diseases (IBD) (4–6), in which epithelial barrier dysfunction plays a central role (7–10). While the mechanism of protection is still elusive, associations with animal contact (11), drinking of unpasteurized milk (12) and maturation of the gut microbiota (13) were described.

Contact of microorganisms with human surfaces induce epithelial barrier defense mechanisms and the release of host defense peptides (HDPs), such as β-defensins (14–16). These are capable of eliminating a broad range of microorganisms and fine-tune the elicited immune response in multiple diseases, including IBD, cystic fibrosis, and rhinovirus infections (17–19). Genetic and/or environmental disturbances in the secretory capacity of functional HDPs may facilitate bacterial translocation (20) and simultaneously disrupt microbial homeostasis (21), collectively fueling systemic inflammation. Both aberrant inflammation and early airway colonization with microbial pathogens may predispose for childhood asthma (22). The immunomodulatory nature of many β-defensins, combined with their effective regulation of microbial colonization, positions these HDPs at center stage for a causal role in the development of inflammatory diseases and at the same time make them promising candidates for prevention and treatment of these diseases. Human β-defensin 2 (hBD-2), which is almost non-existent in the unprovoked airways and gut but profoundly abundant in inflamed epithelial tissues in response to environmental stimuli (23–26), may be of particular interest. Indeed, hBD-2 has recently been shown to ameliorate asthma and IBD in animal models (17–27).

Diverse microbial exposure in early life as well as the individual's personal capacity to mount adequate immunological responses seem to be crucial to avoid asthma and allergy development (28). A significant part of the genetic susceptibility for asthma and allergy may be driven by variation in genes contributing to immunological processes, depending also on differences in environmental exposure (28). While recent studies on the subject have used genome-wide association study (GWAS) data, crucial regions of the genome are not covered in this approach: the genetic locus containing multiple defensin genes including hBD-2 on chromosome 8 is one of them. Due to its complex genetic architecture (29, 30) it is one of the few regions in the human genome still not fully deciphered. A further obstacle in defensin research has been the technical difficulty to produce purified defensin for experimental studies. As hBD-2 protein only became available in sufficient quantities recently, only a handful of studies have investigated the role and potential of the molecule so far (17, 27, 31, 32).

Here, we now investigated genetic variability in the extremely complex gene locus of hBD-2; performed association studies with some focus on farming environment exposure; and performed mouse experiments to explore if hBD-2 could play a role in preventing asthma development, as described in the hygiene hypothesis.

MATERIALS AND METHODS

Human Studies

Study Cohort GABRIELA Ulm

To allow for the assessment of the effects of genetic variations in *DEFB4A* and *DEFB4B* on asthma and atopy in the context of farm environment exposure, we performed genetic association analyses in the GABRIEL Advanced Studies (GABRIELA) from Ulm, comprising 1,238 participants recruited in and around the city of Ulm, Germany. The primary aim of the study was to identify mechanisms of the protective farming effects on the development of asthma and atopic sensitization in primary school children from approx. 6 to 10 years of age with baseline assessment in 2006 (33, 34). Yearly follow-up assessments were conducted from 2010 to 2016 (35). A description of demographic factors is given in **Supplementary Table 1**. Asthma was defined as either reported wheeze in the past 12 months or ever inhaler use for asthma or a reported doctor's diagnosis of asthma at least once or wheezy bronchitis at least twice throughout the lifetime. Atopy was defined as specific IgE antibodies of at least 0.35 kU/L against *Dermatophagoides pteronyssinus*, cat dander, common silver birch, or grass mix (34). The distribution of the cohort according to presence of asthma and/or atopy and farm exposure is given in **Supplementary Table 2**.

Single Nucleotide Polymorphism (SNP) Selection and Genotyping

To study the region on chromosome 8p23.1 containing the two almost identical genes coding for hBD-2, *DEFB4A* and *DEFB4B*, in detail and to retrieve information for already described/existing gene variants, we used publicly available online tools: Ensembl[1], NCBI resources[2], and the UCSC genome browser[3]. To compare the transcription factor binding sites upstream of both hBD-2 genes, we used the online tool PROMO 3.0 provided by the Algorithmics and Genetics Group (ALGGEN) in the Computer Science Department of the Universitat Politècnica

[1] www.ensembl.org
[2] https://www.ncbi.nlm.nih.gov/
[3] https://genome.ucsc.edu/

de Catalunya (Barcelona, Spain). To investigate the association between SNPs in or nearby the *DEFB4A* and *DEFB4B* genes (10 kb downstream each copy and the 477kb section between them) and the development of asthma and atopy, we searched for all annotated SNPs with a minor allele frequency (MAF) ≥ 0.01 in CEU population of the 1000 Genomes project [Utah Residents (CEPH) with Northern and Western European Ancestry], which were either present in a putative regulatory (e.g. promoter region) or coding regions (or close vicinity) of the *DEFB4A* and *DEFB4B* genes. Out of 458 reported SNPs identified in the 1000 Genomes project in this region, 40 fulfilled the described criteria. For genotyping design, we used parameters (i.e. modified flank size and amplicon length), which slightly deviated from the standard ones to increase the chances of finding primers in the highly repetitive area (Agena Assay Design Suite v2.0, Agena Bioscience, USA). In total, only 13 out of 40 targeted SNPs passed quality control from the *in silico* assay design and were successfully genotyped in all 1,238 participants from the GABRIELA Ulm cohort (36). Genotyping was performed using the Sequenom MALDI-TOF mass spectrometry system in collaboration with the Institute of Clinical Molecular Biology, Kiel University.

Distinguishing Between *DEFB4A* and *DEFB4B* and Copy Number Variant Analysis

To determine the presence of one or the other gene copy and to distinguish between *DEFB4A* and *DEFB4B*, we selected 200 gender- and age-matched subjects from GABRIELA Ulm as an exploratory cohort equally distributed between different exposure and outcome groups (**Supplementary Table 2**). We developed RFLP (Restriction Fragment Length Polymorphism)-based assay to discriminate between *DEFB4A* and *DEFB4B*. First, we retrieved the sequences of *DEFB4A* and *DEFB4B* from the online genome browser Ensembl[4] (37) (GRCh38.p12 primary assembly) and aligned them using the Basic Local Alignment Search Tool (BLAST, NCBI; U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda MD, 20894 USA). We used commercially available restriction enzymes to digest only one of the copies after targeted PCR fragment amplification. We used the free online tool NEBcutter V2.0 (38) to identify suitable enzymes. We designed a primer pair framing a specific single nucleotide difference between the two gene copies: at this position, *DEFB4B* is cut by a specific restriction enzyme (Alu I, New England Biolabs, Germany) while *DEFB4A* remains uncut. As an internal positive control of the experiment, the primer pair also included two cutting sites in both gene copies. A standard PCR was carried out for each sample using a final DNA concentration of 1.3 ng/μL and annealing temperature of 61°C. The primer sequences were 5′-*TGTAATGAGCATTGCACC CAATAC*-3′ (forward) and 5′-*TCACAGTATAGGCTGGGC CTTA*-3′ (reverse). Digestion was conducted at 37°C for 10 h followed by enzyme-inactivation at 80°C for 1 h. After digestion, fragments from the two different gene copies were separated by agarose gel-electrophoresis (**Supplementary Figure 1A**). As a control for the accuracy of the RFLP method, we purified (ReliaPrep™ DNA Clean-Up and Concentration System, Promega, USA) and sequenced (Sanger sequencing, ThermoFisher Scientific, Germany) the 667bp (*DEFB4A*) and 445bp (*DEFB4B*) fragments from DNA of 43 healthy controls from an independent explorative study cohort called EXACT (39). Fragment purification from agarose gels was done by using the protocol for DNA purification from gel slices as provided by the manufacturer (Promega, USA). Sequencing revealed an accurate overlap of the fragments with the respective references, including the single nucleotide differences between the gene copies. The only discrepancy was detected in the area of the 9 bp insertion of *DEFB4A*, which appeared on the gel as a double band at the height of the 667 bp fragment. Therefore, the double band may indicate that the 9 bp insertion of *DEFB4A* is not present in both alleles (**Supplementary Figure 1A**).

Statistical Analysis

Deviation from Hardy-Weinberg equilibrium (HWE) was analyzed by chi-square test in the control group using PLINK version 1.0 (40). All markers were in HWE ($p>0.0001$). We generated Linkage Disequilibrium (LD) plots with Haploview (41) and performed all further analyses with R software (Version 3.0.1). Normally distributed data are presented as mean with standard deviation (SD) or 95% confidence interval (CI); non-parametric data as median and interquartile range (IQR). Differences between two groups were analysed using unpaired Wilcoxon or Student's t-test depending on their Gaussian distribution. We evaluated associations of binary traits primarily by logistic regression, stratified for farmer status as previously described (33), using the *survey*-package in R statistics[5]. Odds ratios (OR), 95% CI and *p*-values are reported for association analyses. All *p*-values <0.05 were considered statistically significant as the analyses were hypothesis-driven. Only SNPs with a minimum of three minor allele carriers within the cohort were analyzed. To investigate whether the presence or absence of *DEFB4A* and *DEFB4B* was associated with health status, we applied chi-square tests.

Murine Asthma Model Experiments

Mice

To assess preventive effects of hBD-2 in a model of inflammatory airway disease, we used female BALB/c mice between 7 and 8 weeks of age (Charles River, Italy), which received food and water *ad libitum* during the experiments. Animal-related research followed the 2010/63/EU and National legislation regulating the use of laboratory animals (Official Gazette 55/13) and the Institutional Committee on Animal Research Ethics (CARE-Zg). All animal experiments were performed according to the specifications of the senior investigators (MK, JW, PN) by Fidelta Ltd., Croatia.

Sensitization Procedure

For sensitization of the animals, a solution with 1 mg house dust mite (HDM) protein/ml saline was prepared according to the manufacturer's instructions (33 mg HDM/vial; lot no 305469;

[4] www.ensembl.org

[5] https://www.jstatsoft.org/article/view/v009i08

Greer, USA). This solution was combined with equal amount (1:1 ratio) of complete Freund's adjuvant (CFA) dissolved in PBS. The animals were distributed according to sensitization and treatment into three groups of 12 mice each (results section, **Figure 3**). Mice from group 1 received subcutaneous sensitization with saline containing CFA while groups 2 and 3 were sensitized subcutaneously with 100 µg HDM in 0.2 ml saline and CFA at day 0. Group 1 did not receive any treatment, while mice from group 2 received oral treatment with vehicle (0.5% carboxymethylcellulose) at days 12, 13, and 14, and mice from group 3 received oral hBD-2 at the same days. Sensitization on day 0 was applied with a 100 µl Hamilton glass syringe and a 16G cannula. According to the sensitization and treatment received, the groups were also called: Saline (1), vehicle/HDM (2) or hBD-2/HDM (3).

Preparation and Dosing of the Test Compound Human Beta-Defensin 2 (hBD-2)

Oral treatment with 1.2 mg/kg/day (0.4 mg/kg, 3 times a day) hBD-2 (Novozymes, Denmark) started at day 12, 2 days prior to the challenge with saline or HDM (day 14). The first daily dose was given at approximately 8 a.m. each day, followed by two additional administrations with 6-h intervals. The last dose was administered orally on day 14, 1 h prior to the challenge, at the volume of 10 ml/kg. The solutions for each dosing group had a concentration of 0.04 mg/ml. Concentration of the used hBD-2 ampules was confirmed with a CV% < 0.5 (triplicates) and purity was further verified to be 98.6% (UPLC). Endotoxins were below 0.004 EU/mg.

Asthma Induction and Prophylactic hBD-2 Administration

To induce allergic asthma, mice from groups 2 and 3 were immunized subcutaneously on day zero with 100 µg HDM in 0.2 mL saline per animal; group 1 received 100 µL of CFA dissolved in saline instead. On days 12 and 13, the mice received either vehicle (group 2) or hBD-2, 0.4 mg/kg, orally, three times a day (group 3). On day 14, only two doses of vehicle or hBD-2 were administered, respectively. One hour after the last dose, mice from groups 2 and 3 were intranasally challenged with 25 µg of HDM in 50 µL of saline. No additional treatments following challenge were carried out.

Airway Hyper-reactivity Measurements

At day 16, approximately 48 h after HDM application, 6 mice from each group were challenged with methacholine. Immediately after the challenge, lung resistance (cm H_2O/mL/second) and dynamic lung compliance (mL/cm H_2O) were automatically measured by a DSI's Buxco® FinePointe™ RC system (DSI, USA), and the results were computed by the RC system's software. Each mouse was anesthetized (0.2% Xylazine plus 5 mg/mL Narkamon dissolved in saline) and tracheostomized after approximately 10 min for direct measurement of the respiratory flow and lung pressure. Mice were then loaded into a plethysmograph in a supine position, and a water or ethanol filled tube was placed two-thirds down the esophagus. For artificial ventilation, tracheal tubes were placed and fixed with tied suture, (stroke volume: body weight/100, rate: 120 breaths/min). Additionally, 0.1 mL of diluted ketamine (10 mg/mL) per 10 g body weight was added intraperitoneal to each mouse. For airway hyper-reactivity measurements, mice were initially exposed to aerosolized PBS for the baseline value, followed by increasing concentrations of 5 µL methacholine (3.125, 6.25, 12.5 and 25 mg/mL), nebulized for 3 min before lung function was recorded. The automated data acquisition software Finepointe™ (DSI's Buxco) was used to continuously record various basic parameters, automatically generating resistance and dynamic compliance, and performing statistical analysis. The data were calculated at each time point, and lung resistance and compliance values were shown as a curve for each group.

Bronchoalveolar Lavage Fluid and Lung Cell Collection and Processing

Bronchoalveolar lavage fluid (BALF) was collected from all the animals (n=36) 48 h after HDM challenge. In brief, the trachea of each animal was cannulated, the lungs were washed with 3 volumes of cold PBS (0.4, 0.3 and 0.3 mL; 1 mL in total) and the collected BALF was placed into an Eppendorf test tube for each mouse. The tubes were centrifuged at 3,500 rpm and 4°C for 5 min, and cell pellets were re-suspended in 600 µL PBS by vortexing. Total and differential cell counts in BALF were determined on an automated haematological analyzer (Sysmex XT-2000iV).

After the bronchoalveolar lavage, the lungs of all animals were exposed and excised by opening the thorax, cutting down either side of the sternum and ribs, and trimming back. The lungs were removed from the thorax, snap frozen in liquid nitrogen and stored at −80°C until preparation of the homogenates for cytokine measurements. Frozen lungs were placed into PBS (1 volume tissue to 5 volumes PBS) containing a protease inhibitor cocktail (Roche, Switzerland) and homogenized using an IKA Turbo XT laboratory homogenizer (IKA, Germany). The tubes were subsequently centrifuged for 10 min at 12,000 rpm and 4°C. The clear supernatant was transferred to a new tube and stored at −80°C until further analysis. The concentration of seven asthma-related inflammatory cytokines, namely Interleukin (IL)-4, IL-5, IL-6, IL-9, IL-13, IL-33, and tumor necrosis factor (TNF)-α, was determined in lung homogenates of all mice using commercially available ELISA kits, following the manufacturer's instructions (R&D systems, USA).

Statistical Analysis

For comparison of lung resistance and compliance, cell counts and cytokine levels from the different groups, we used one-way ANOVA with post-hoc t-test (Bonferroni corrected). Homogeneity of variances was asserted using Levene's Test, which showed that equal variances could be assumed (all p-values > 0.24). Statistical analyses were performed using SPSS Statistics (IBM, USA). All *p*-values <0.05 were considered statistically significant.

RESULTS

Mutations, Polymorphisms and Copy Number Variants in the hBD-2 Region Are Associated With Asthma and Atopy in Children

The genes *DEFB4A* and *DEFB4B*, both coding for hBD-2, are located on chromosome 8 (chr8p23.1) in close vicinity to five other β-defensins (*DEFB103*, *DEFB104*, *DEFB105*, *DEFB106*, *DEFB107*), all of which are aligned in a peculiar, mirror-like cluster with their respective gene copies (**Figure 1A**). In the middle of this mirrored region resides a stretch of approximately 50 kb of DNA, which evaded all sequencing attempts so far due to massive repetition and duplication (42). *DEFB4B* locates approximately 477 kb upstream of *DEFB4A* in opposite reading directions, a general characteristic of β-defensins in this region. The structure and sequence of both *DEFB4A* and *DEFB4B* is highly similar (**Figure 1B**). The putative promoter region of *DEFB4A* reaches 1,612 bp upstream from the transcription start site and that of *DEFB4B* up to 1,874 bp. Overall, 61 out of 81 (75.3%) identified transcription factor binding sites of the region 2,000 bp upstream are identical. Exonic sequences are identical in 194 of 195 base pairs, leading to identical amino acid sequences, as the single nucleotide difference in exon 1 is a silent mutation. Intronic regions are 97.4% identical (1,629 out of 1,672 bp). The 3′ UTR and the first 48bp of the 5′ UTR from both genes match by 100%. However, the 5′ UTR of *DEFB4A* (84 bp) is 48bp longer than of the *DEFB4B* (36 bp).

Of the 458 SNPs annotated in the 500-kb region of interest in public databases (43), 40 were of interest for genotyping within 10 kb downstream of both genes, including the 477 kb between the genes, and followed the inclusion criteria described in detail in the methods section. From those, 17 were located in or very close to *DEFB4A*, 19 were described in or around *DEFB4B*, and four additional SNPs locate in different regulatory regions between the genes (**Figure 1**, **Supplementary Table 3**). Out of the 40 genetic variants [7 in high LD (LD>0.8)], we were able to genotype 13 and verify the existence of only 7 in our population with the applied settings. This low genotyping success rate was specific for this region, and thus contrasted with the high success rate in several other chromosomal regions genotyped at the same time, in the same cohort using identical design parameters (information available upon request). The complex nature of the region and the close-to-identical DNA sequence of the two genes could not be overcome by alternative genotyping methods applied. In case of a very low minor allele frequency (i.e., < 3 minor alleles) statistical analysis may lead to spurious results and thus, ORs and CIs were not calculated.

For the seven genetic variants we could confirm, minor allele frequencies and their association with asthma and allergy in our study population are shown in **Table 1**. Overall, MAF was magnitudes lower than expected when compared to CEU data. Four out of seven present genetic variants were extremely rare yet provided here for completeness. Indeed, all rare variants and two out of three more common polymorphisms showed associations with the outcomes under investigation (asthma and allergy, and the combination thereof), hence exceeding stochastic expectations. Due to the very low MAF, we could not assess the influence of farm exposure on these associations in stratified analyses as planned. *In silico* functional analyses showed that two associated variants (rs6651513 and rs543538872) have putative functional relevance: rs6651513 resides in an enhancer approximately 2kb upstream of *DEFB4B* and correlates with *DEFB4A* and *DEFB4B* expression, particularly in lung, skin, stomach, and esophagus

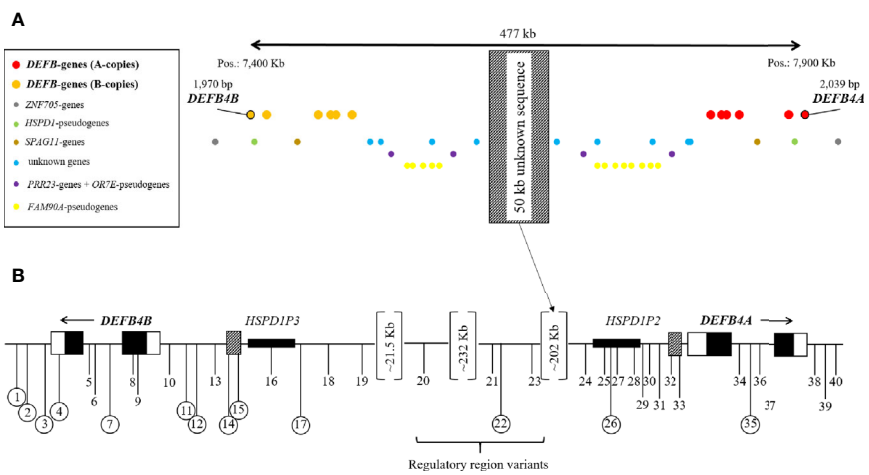

FIGURE 1 | *DEFB4* locus on chromosome 8p23.1 with surrounding genes and pseudogenes **(A)** and location of the 40 selected SNPs for genotyping in GABRIELA Ulm **(B)**. **(A)** Red dots: A-copies of the β-defensin genes; orange dots: B-copies of the β-defensin genes; red and orange dots with black circle: *DEFB4* genes. Blue dots: unknown genes; yellow dots: *FAM90A*-pseudogenes; purple dots: *PRR23*-genes and *OR7E*-pseudogenes; brown dots: *SPAG11*-genes; green dots: *HSPD1*-pseudogenes; grey dots: *ZNF705*-genes. The hatched rectangle in the middle represents a not yet sequenced 50 Kb area, which presents like a mirror in this cluster. **(B)** positions of the selected SNPs for genotyping are shown as black vertical lines with numbers in relation to the position of *DEFB4A* and *DEFB4B*. Circled numbers represent SNPs that were successfully genotyped. Black boxes refer to exonic regions, white boxes to UTRs, hatched boxes to putative promoter regions.

TABLE 1 | Genetic associations of SNPs in or nearby *DEFB4A/B* with asthma and atopy in the GABRIELA Ulm cohort (n=1,238).

			MAF		Asthma		Atopy		Atopic asthma		Non-atopic asthma	
No.	**SNP**	**Minor allele**	**CEU**	**GABRIELA Ulm**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**
17	rs533344477	C	0.005	0	–	–	–	–	–	–	–	–
12	rs538653319	C	0.01	0	–	–	–	–	–	–	–	–
3	**rs538901702**	T	0.005	0.0004	**1.44^{-26}**	NA	**5.67^{-33}**	NA	**8.68^{-30}**	NA	**4.59^{-23}**	NA
11	**rs543538872**	T	0.015	0.002	**2.42^{-90}**	NA	0.169	4.03 (0.55–29.28)	**9.14^{-82}**	NA	**6.47^{-91}**	NA
2	rs546946128	A	0.005	0	–	–	–	–	–	–	–	–
26	rs558364144	T	0.005	0	–	–	–	–	–	–	–	–
35	rs558912368	A	0.005	0	–	–	–	–	–	–	–	–
4	rs562192342	A	0.005	0	–	–	–	–	–	–	–	–
1	**rs562864847**	G	0.01	0.008	0.067	3.5 (0.91–13.41)	0.732	1.27 (0.33–4.87)	**0.0273**	4.79 (1.19–19.20)	**3.92^{-181}**	NA
7	**rs567390989**	T	0.015	0.0008	0.077	12.19 (0.77–194.01)	0.344	4.2487 (0.21–84.70)	0.050	17.37 (0.99–304.04)	**4.38^{-23}**	NA
22	**rs62640720**	G	0.328	0.0004	**1.3^{-21}**	NA	**5.22^{-28}**	NA	**1.57^{-24}**	NA	**1.15^{-18}**	NA
15	**rs6651513**	A	0.116	0.046	0.155	0.56 (0.25–1.24)	0.229	1.3935 (0.81–2.39)	0.4755	0.74 (0.32–1.69)	**0.001**	**0.08 (0.02–0.35)**
14	rs73199779	C	0.056	0.028	0.839	0.92 (0.39–2.14)	0.281	0.6916 (0.35–1.35)	0.446	0.66 (0.23–1.92)	0.940	1.71 (0.50–5.92)

SNP, single nucleotide polymorphism; CI, confidence interval; MAF, minor allele frequency; NA, not applicable; OR, odds ratio.
Bold values represent significant association of the SNP with the respective disease.

mucosa (eQTL data provided by GTEx Portal[6]). Variant rs543538872 locates within a DNaseI Hypersensitivity Cluster 901 bp upstream of *DEFB4B* (44) (UCSC genome browser[7]), indicating that it could be part of the *DEFB4B* promoter region.

Sequence alignment of the *DEFB4A* and *DEFB4B* showed a 98% sequence overlap with very few single nucleotide differences as mentioned above and depicted in alignment (**Supplementary Figure 1B**). To distinguish between the two almost identical genes, we used one single nucleotide difference in the intronic region (**Supplementary Figure 1C**). In total, 155 out of 200 subjects (77.5%) selected from our strata of interest from the GABRIELA Ulm cohort as described above (method section), have a *DEFB4A* gene. *DEFB4A* was found significantly less frequently in atopic individuals (p=0.012) and the same, but non-significant trend, was found in asthmatics (**Figure 2**).

Prophylactic Oral Treatment With hBD-2 Reduces Effects of HDM Challenge in a Murine Asthma Model

As multiple associations between genetic variations in the *DEFB4A/DEFB4B* gene cluster and asthma and atopy in children suggest a role of these genes in asthma and allergy development, we investigated if hBD-2, due to its known functional entities, could help to explain protection against asthma and allergy by farm exposure and consumption of unprocessed milk on these farms. In an established HDM asthma mouse model, we studied the impact of orally administered hBD-2 (or vehicle only) prior to HDM challenge in 36 mice sensitized with either saline or HDM and assessed their lung hyper-reactivity and inflammatory response (**Figure 3**).

We assessed lung resistance and dynamic compliance between vehicle-treated (vehicle/HDM, group 2) and hBD-2-treated mice (hBD-2/HDM, group 3) after HDM challenge. Six animals per group initially received nebulization with PBS and subsequently with four different concentrations of methacholine (3.125, 6.25, 12.5 and 25 mg/mL). Vehicle-treated, HDM-challenged mice exhibited a dose-dependent increase in lung resistance paralleled by reduced lung compliance when compared with both unchallenged, and hBD-2 treated, HDM challenged mice (**Figure 4**). At the highest methacholine concentrations, the vehicle/HDM group showed significantly increased lung resistance compared to the hBD-2/HDM group (p=0.02 at 12.5 mg/ml and p=0.025 at 25 mg/ml). Notably, mice treated prophylactically with orally administered hBD-2 were fully protected against HDM-induced lung resistance, despite diminished lung compliance at the highest methacholine doses (**Figure 4**).

Total and differential cell count in BAL fluid was conducted 48 h after HDM challenge. The number of total and specific immune cells (eosinophils, neutrophils, macrophages and lymphocytes) was significantly increased after HDM challenge compared to saline-challenged mice, but no significant differences were observed between vehicle-treated and hBD-2-treated groups (**Figure 5A**). Thus, prophylactic oral treatment of HDM-sensitized mice with hBD-2 did not influence cell counts in BAL fluid after HDM challenge.

We next assessed hallmark asthma cytokines in lung homogenates to evaluate if oral hBD-2 treatment mitigated the pulmonary inflammation processes, hence explaining the relative protection against deteriorated lung function as described above. Vehicle-treated, HDM-challenged mice exhibited significantly enhanced tissue concentrations of all tested cytokines (IL-4, IL-5, IL-6, IL-9, IL-13, IL-33, and TNFα) compared to their PBS challenged counterparts (**Figure 5B**). Again, prophylactic oral administration of hBD-2 fully protected from HDM-induced cytokine release (with IL-33 being the sole exception) and thus significantly dampened pulmonary inflammation.

[6] https://www.gtexportal.org/
[7] https://www.genome.ucsc.edu/

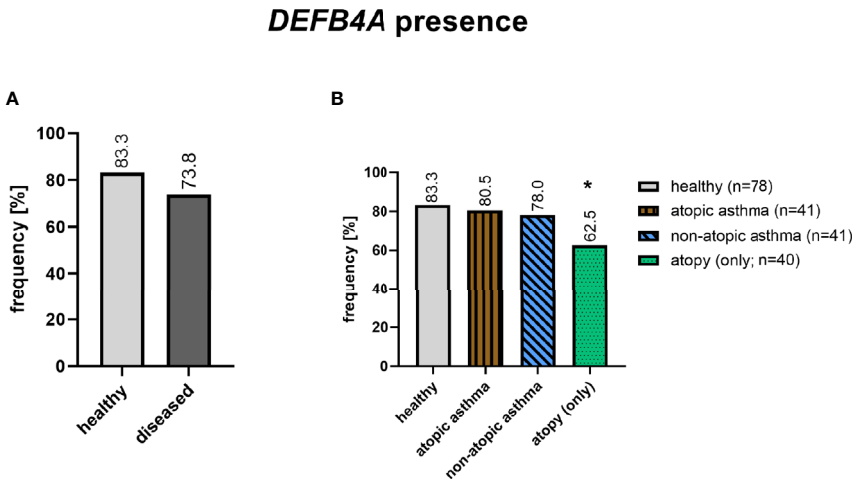

FIGURE 2 | Presence of *DEFB4A* in healthy (n=78) and diseased (n=122) subjects after RFLP assay for *DEFB4A* and *DEFB4B*. The diseased group include 41 non-atopic asthmatics, 41 atopic asthmatics and 40 atopics. *p=0.012 (chi-square test).

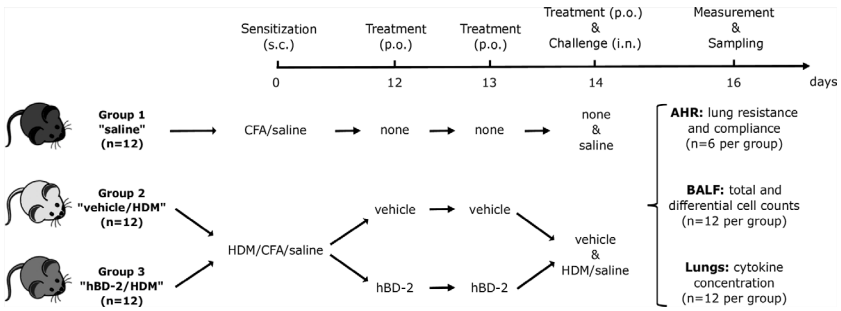

FIGURE 3 | Prophylactic administration of hBD-2 in BALB/c mice and grouping according to sensitization and treatment. Challenge was carried out 1 h after the last dose of saline or vehicle on day 14. AHR, airway hyper-reactivity; BALF, bronchoalveolar lavage fluid; CFA, Complete Freund's Adjuvant; hBD-2, human beta-defensin 2; HDM, house dust mite; *i.n.*, intra-nasal; *p.o.*, per oral; *s.c.*, subcutaneous.

DISCUSSION

Genetic variations such as rare mutations, polymorphisms and copy number variations in the *DEFB4A*/*DEFB4B* genetic cluster associate with asthma and allergy in children. Furthermore, we provide evidence that hBD-2 could contribute to protective farm effects such as those mediated by the consumption of unprocessed cow's milk (12) when prophylactic administration of oral hBD-2 mitigates pulmonary resistance and inflammation in a mouse model of HDM-induced asthma.

Investigating the locus harboring *DEFB4A* and *DEFB4B* specifically, we could confirm the high complexity of the region, which is truly at the frontier of the technical resolution currently possible in genetics. Numerous variants in the region evaded genotyping by different techniques and others showed dramatically lower MAF than predicted in the CEU population. These differences may only in part be explained by population characteristics but rather, are an expression of the high genetic complexity of the locus. As described in more detail below, the association with genetic variants in the hBD-2 locus for very comprehensible reasons did not allow to specifically perform an analysis stratified by farming exposure as originally planned.

The existence of two identical genes transcribing hBD-2 suggests a potential biological advantage in the capacity to mount a strong hBD-2 response if needed. This situation resembles the so called "cytokine gene cluster" on chromosome 5, containing the IL-4 and IL-13 coding genes, which are thought to have arisen from a gene duplication and also share a locus control region (45). Interestingly, IL-4 and IL-13 are both necessary and complementary for a type-2 immune response (46), as their functions have diverged over time, unlike that of the two (almost) identical genes coding for hBD-2. Preservation of genetic integrity in a coding sequence of a gene is always an active effort, which hints at the biological importance of the gene product. A similar situation exists for LL-37, an additional defensin (own unpublished data).

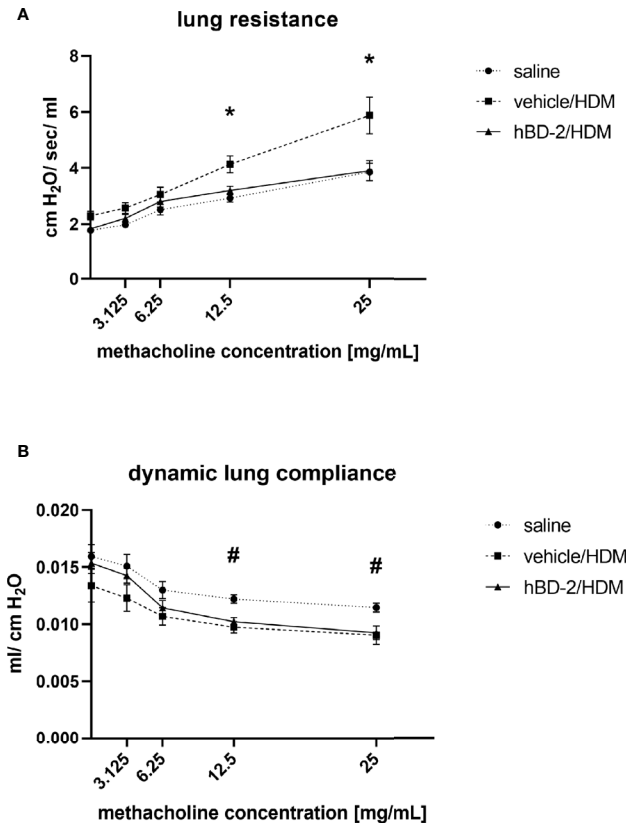

FIGURE 4 | Lung resistance **(A)** and compliance **(B)** in 6 mice per group 48 h after saline or HDM challenge. Saline: Mice without treatment and challenged with saline only. Vehicle/HDM: Mice prophylactically treated with vehicle and challenged with HDM. hBD-2/HDM: Mice prophylactically treated with hBD-2 and challenged with HDM. *significant difference (ANOVA with post-hoc t-test; $p<0.05$) between vehicle treated and hBD-2–treated mice; #significant difference (ANOVA with post-hoc t-test; $p<0.05$) between saline-challenged and HDM-challenged mice. hBD-2, human beta-defensin 2; HDM, house dust mite.

Subjects with atopy (with and without asthma) are more likely to lack *DEFB4A* in our study population. While all copy number analyses in previous studies did not discriminate between *DEFB4A* and *DEFB4B*, our approach explores for the first time the two copies separately. Despite copy segregation obtained by only a single nucleotide difference in the RFLP-like experiments, subsequent sequencing confirmed specificity for the respective copy. Still, it remains possible that *DEFB4A* is present in a mutated, hitherto unannotated form, and thus not identified in our analysis. In addition, some individuals in our study cohort may not simply miss *DEFB4A*, but have duplicated *DEFB4B*, similar to a homozygous state of a certain genotype (here: *BB* instead of *AB*). Further mutations and polymorphisms in the *DEFB4A*/*DEFB4B* cluster are also associated with a high likelihood to influence the regulation of gene expression according to *in silico* eQTL analyses. rs6651513 is located in an enhancer region 2 kb upstream of *DEFB4B* and it seems to negatively influence gene expression in the lung and skin and positively influence the expression in stomach, esophagus, and vagina, according to the online tool ensembl[8]. rs62640720 is also located in a regulatory region (CTCF binding site, in promoter) and positively influences the expression in vagina and skin, while the expression in stomach, esophagus, and lung are negatively affected. Assuming that increased expression of hBD-2 in the lungs protects against asthma and allergies, these two mutations seem to promote the development of asthma and allergies by decreasing hBD-2 expression.

Yet, our genetic data on mutations have to be interpreted with caution since some of the genotyped variants are extremely rare, not allowing to calculate odds ratios. In the context of all the evidence, including copy number variance and polymorphisms, the sum of all the effects is rather suggestive if not convincing. Taken together, these findings point toward the possibility that a lack, or inadequate production, of hBD-2 could lead to an increased susceptibility to asthma and allergy development, under the premise of a potentially protective effect of hBD-2 in these diseases.

Differences in copy numbers of the hBD-2 coding genes have been reported to correlate with other chronic inflammatory diseases. In the IBD, Morbus Crohn's Disease, a decrease of hBD-2 gene copy numbers has also been found (47), whereas in psoriasis hBD-2 gene copy numbers seem to be increased (48). This corresponds well with the more recent observation that asthma and Crohn's disease are comorbidities (49) and also share genetic traits (50) while such overlaps are not common with psoriasis. Interestingly, in comparison with psoriatic skin lesions, hBD-2 is reduced in the skin of patients with atopic dermatitis (51).

In addition to their shared lack of gene copies for the hBD-2 production, both asthma and Crohn's Disease are influenced by the same environments: early exposure to farm environment is protective against the development of both (4–6, 10). We used the GABRIELA Ulm cohort, which is based on children living in rural areas, some with and some without farming contact, to explore the connection between mutations in the hBD-2 coding genes and asthma and allergy in a protective farm environment for the development of inflammatory diseases. Our previous studies have already suggested that genetic effects may be modified by environmental exposures and in combination have significant effects on the microbiome, explaining some of the protective farm effects (13, 33). However, in these studies the *DEFB4A* and *DEFB4B* genes were consistently neglected, as the genotyping chips used did not cover this genomic region. Unfortunately, the unexpectedly low MAF of all investigated genetic variants in the locus did not allow for further stratified analysis in our population due to its very low frequency, this kind of analysis is even beyond the resolution of much larger datasets. It would have been of interest to investigate gene by environment effects in individuals exposed and non-exposed to farming environments, but the absence of these analyses does not hamper the overall conclusion, that genetic alterations in a gene coding for a potential key player in the protective farming effect, is associated with asthma and allergy development in the

[8] www.ensembl.org

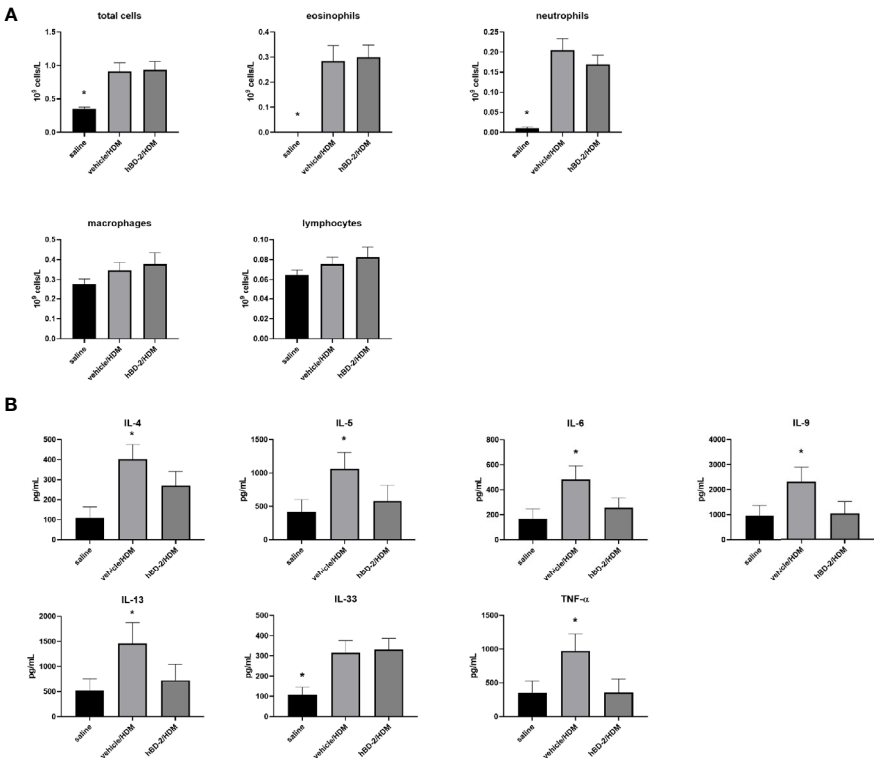

FIGURE 5 | Total and differential cell counts (eosinophils, neutrophils, macrophages, and lymphocytes) in bronchoalveolar lavage fluid **(A)** and concentration of inflammatory cytokines (TNFα, IL-4, IL-5, IL-6, IL-9, IL-13, and IL-33) in lung homogenates of the 3 treatment groups (n=12 per group) **(B)**. Saline: mice sensitized with saline + CFA only (group 1). vehicle/HDM: mice sensitized with HDM at day 0, prophylactic treatment with vehicle at days 12 to 14, and challenge with HDM at day 14 (group 2). hBD-2/HDM: mice sensitized with HDM at day 0, prophylactic treatment with hBD-2 at days 12 to 14, and challenge with HDM at day 14 (group 3). *$p < 0.05$ when compared to other groups (ANOVA with post-hoc t-test,). hBD-2, human beta-defensin 2; HDM, house dust mite.

general population. In contrast, this suggests that alterations of hBD-2 expression are associated with asthma development irrespective of protective environments.

As data from farm studies in asthma and allergy suggested an independent protective effect of unprocessed farm milk consumption, we hypothesized that if hBD-2 plays a role in this mechanism, hBD-2 production cannot only occur in the airways but also in the gut and based on the genetic data it should be increased to exert its protective function. As farm effects are only protective if they occur early in life, we further hypothesized that such increased hBD-2 production should occur prior to asthma development. As our initial goal was to investigate the potential protective effect of hBD-2 against asthma, we applied hBD-2 after sensitization and before challenge, which is also in line with standard protocols for asthma prevention experiments.

Our proof-of-concept mouse experiments support biological relevance of prophylactic hBD-2.

Specifically, prophylactic oral hBD-2 treatment of HDM-sensitized mice lowered the production of an array of classical asthma associated cytokines, namely TNF-α, IL-4, IL-5, IL-6, IL-9, and IL-13, in lung tissue of exposed mice subsequent to HDM challenge and concomitantly reduced lung resistance after methacholine-challenge. Since IL-4, IL-5, and IL-13 promote airway eosinophilia, mucus overproduction and bronchial hyper-responsiveness, it is likely that hBD-2 improves lung resistance indirectly by lowering those cytokines in the lung (52). Interestingly, hBD-2 had no effect on the cell counts in BALF, suggesting at least in this model, that its immune regulatory properties reflected *in situ* manipulation of pulmonary cytokine release rather than affecting immune cell influx. These data align well with a recent publication elucidating the immunomodulatory potential of hBD-2 in experimental colitis as well as in lipopolysaccharide-challenged mice and human peripheral blood mononuclear cells (17). Unaffected cell counts in BALF of hBD-2-treated mice seem exclusive to the oral administration route described here for the first time. However, one could speculate that the observed effect is due to possible impurities in the production process of the protein, but our analyses showed a high degree of purity. To completely rule out this possibility, an additional control group, treated with a peptide of similar but random amino acid composition like hBD-2, could be included in future experiments. While intranasal administration of hBD-2 even in a therapeutic setting lessens pulmonary inflammation in models of both steroid sensitive and steroid refractory asthma, treatment efficacy in those models was mirrored by diminished cell influx (27). Notably, intranasal administration of another antimicrobial peptide, mCRAMP (murine orthologue of LL-37),

triggered asthma exacerbation in an allergic asthma mouse model (53), hence suggesting a unique role of hBD-2 in this setting.

Obviously, one cannot directly translate these findings from experimental mouse models to humans, and additional research is still necessary to enhance our understanding of how hBD-2 may affect asthma development in humans. Considering the respiratory challenges of asthmatic patients, it is nevertheless of significant relevance that oral administration of hBD-2 effectively mitigated pulmonary inflammation. Another future approach could be taken by applying hBD-2 even before sensitization to investigate a potential protective effect of hBD-2 against allergy development.

Future studies are needed to define the pertinent crosstalk between gut and lung in this setting and to establish if and how lung and gut microbiome signatures may affect disease trajectories. To this end, it was first shown in 2006 that colonic bacterial dysbiosis can alter the immune response of the lung after pulmonary infection in mice (54). It has further been demonstrated that constitutively expressed hBD-1 retains antimicrobial activity after proteolytic degradation by gastrointestinal proteases (55), while other defensins, e.g. HD5 and HNP4, not only preserve their antimicrobial activity upon degradation, but even enhance antimicrobial potency and specificity (56, 57). If this situation occurs in the context of orally administered hBD-2, and the extent to which such phenomenon would enhance transport of biologically active fragments from the gut to the lung, remains to be described.

It is also possible that the potent immunomodulatory nature of hBD-2 imprints systemic immunity to curb lung inflammation. Indeed, a recent report with detailed immune assessment of 514 infants 18 months of age corroborate exaggerated systemic immunity, in particular blood neutrophils and IL-13 producing T cells, at the time of enrollment to predict both transient and persistent childhood asthma (22). Early changes in the microbiome of children developing asthma and allergy have also been described recently (13). It is thus plausible that an aberrant and weak hBD-2 response either due to genetically determined limitations or a missing signal from the environment may play a role in both an abnormal development of immunity and the microbiome during childhood.

In conclusion, we demonstrate that hBD-2 and its coding genes, *DEFB4A* and *DEFB4B*, play an important yet underestimated role in the onset of asthma and atopy. Genetic alterations in the gene locus or absence of *DEFB4A* significantly associate with the prevalence of asthma and atopy in children while our mouse experiments clearly indicate that hBD-2 can have a prophylactic role in preventing features of allergic asthma. If and how these mechanisms could be used to intervene in the development of childhood asthma and allergy, still needs to be determined.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee of Ulm University Germany (104/06, 69/10, and 137/14). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Study design: NB, AT, PN, BJ, JW, JG, and MK. Data collection: NB, AT, PN, BJ, JG, AF, and MK. Analysis and data interpretation: NB, ES-V, VG, AT, BJ, and MK. Manuscript writing: NB, ES-V, AT, BJ, and MK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.636061/full#supplementary-material

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REVIEW
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Understanding Asthma and Allergies by the Lens of Biodiversity and Epigenetic Changes

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Exposure to different organisms (bacteria, mold, virus, protozoan, helminths, among others) can induce epigenetic changes affecting the modulation of immune responses and consequently increasing the susceptibility to inflammatory diseases. Epigenomic regulatory features are highly affected during embryonic development and are responsible for the expression or repression of different genes associated with cell development and targeting/conducting immune responses. The well-known, "window of opportunity" that includes maternal and post-natal environmental exposures, which include maternal infections, microbiota, diet, drugs, and pollutant exposures are of fundamental importance to immune modulation and these events are almost always accompanied by epigenetic changes. Recently, it has been shown that these alterations could be involved in both risk and protection of allergic diseases through mechanisms, such as DNA methylation and histone modifications, which can enhance Th2 responses and maintain memory Th2 cells or decrease Treg cells differentiation. In addition, epigenetic changes may differ according to the microbial agent involved and may even influence different asthma or allergy phenotypes. In this review, we discuss how exposure to different organisms, including bacteria, viruses, and helminths can lead to epigenetic modulations and how this correlates with allergic diseases considering different genetic backgrounds of several ancestral populations.

Keywords: asthma, allergies, holobiont, microbiome, epigenetics

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INTRODUCTION

Asthma and allergy are the most common chronic inflammatory diseases, especially in children (1). The prevalence of asthma is elevated in economically developed countries in Western and Eastern Europe and higher in the United States compared to other countries (2, 3). A progressive increase in the prevalence of asthma in low-income countries has also been observed (4, 5), which makes asthma prevalent worldwide. According to the World Health Organization over 80% of asthma-related deaths occur in low- and low-middle-income countries, and difficulties in accessing treatment and management are also related to that (6). On the other hand, the prevalence of eczema, allergic rhinitis, and food allergies in childhood is distributed differently

between tropical countries and temperate zones (7–14). Geographic differences in the prevalence of allergies between and within populations may reflect both exposure to common environmental factors and a host genetic background, which can either increase or decrease risk (15). In terms of genetics, large genome-wide association studies (GWAS) initiatives were unable to completely explain such high and still increasing prevalence of allergic disorders as well as their phenotypic heterogeneity (1). Among the top pathways linked to asthma in such initiatives, include those related to epithelial barrier dysfunction and reduction of immune tolerance (16). In addition, studies have found not only shared but also distinct genetic components between asthma subtypes, indicating that heterogeneity is related to individual genotype (17, 18) but still do not completely explain everything.

Thus, the knowledge about the interactions between the genetic pool and the environment is increasing with several lines of evidence explaining those trends (19–21). In this context, some hypotheses explain the links between environmental changes that occurred in recent decades with the prevalence of allergies across the globe, such as urbanization, housing condition, diet, and fewer exposures to organisms such as bacteria, virus and helminths (21, 22). In fact, there is a link between the higher incidence of allergic diseases and reduced infections/exposure to organisms in Western countries and across the globe and this has been studied for several years now and appears to reflect the economy and sanitation in each territory. Additionally, the degree of industrialization and consequent changes in the habits and lifestyle of the population imply that limited exposure to several environmental factors for reducing biodiversity may contribute to an increased risk of developing or exacerbating asthma and allergies (23). David Strachan observed in 1989 that infections transmitted in early childhood, through contact between older siblings, could restrict the development of allergies (22, 24). Urbanization and improvements in hygiene, better housing conditions, and reduced chances of cross-infection in younger members of the family are the basis for what we know as the "hygiene hypothesis" (24). The initial mechanistic explanation of the hygiene hypothesis emphasized the role of Th1 cells in regulating Th2 responses. Later, the role of regulatory T cells was emphasized in the regulation of both Th1 and Th2-induced inflammatory responses through mechanisms that include the production of regulatory cytokines (25). The mechanistic pathways of the hygiene hypothesis were described extensively in the literature, other theories amplified the initial concept such as the "old friends" hypothesis (26) and, afterwards, the biodiversity hypothesis, proposed by The Karelia Allergy Study from 1998 (27). Both theories attempt to explain the impact of modifications in human living conditions and habits on the prevalence of immune-mediated diseases (28, 29).

Abbreviations: AAI, allergic airway inflammation; BCG, Bacillus Calmette–Guérin; CpG, cytosine-phosphate-guanine; ERK, Extracellular signal-regulated kinases; ES, excretory/secretory antigens; EWAS, epigenome-wide association studies; GWAS, genome-wide association studies; HAT, histone acetyltransferase; HAV, Hepatitis A virus; HDAC, histone deacetylase; ICS, inhaled corticosteroids; MAPK, mitogen-activated protein kinase; miRNAs, MicroRNA; SOCS, suppressor of cytokine signaling; STH, soil-transmitted helminths; VNN1, Vanin-1.

Studies show that early exposure to antibiotics during childhood increases the risk of developing allergic diseases (30) and also regular anthelmintic use (31). Numerous epidemiologic studies reinforce that the increase in allergic diseases, eczema, and food allergies is inversely related to parasitic infections (32–36). Soil biodiversity and climatic characteristics of a country are also determinants in the types of environmental exposures and consequent development of infectious diseases and allergic sensitization. The climate and biodiversity of the tropics (fauna and flora) favor intestinal helminth infections and the dissemination of human infectious diseases transmitted by vectors like insects (37–42). According to (6), Soil-transmitted helminth infections are distributed in tropical and subtropical areas, with the highest incidence in sub-Saharan Africa, the Americas, China, and East Asia.

The tropics are also marked by sharp economic and social inequalities that reflect health and sanitary conditions and an increased risk of spreading fecal-oral transmission diseases (toxoplasmosis, giardiasis, hepatitis A, worms). In addition, the relationship between helminths and allergies is complex and is influenced by the parasite burden, chronicity, first infection or reinfection, coinfections, and parasite species present in the environment (33). In contrast, allergic sensitization to house dust mite species such as *Dermatophagoides pteronyssinus*, *D. farina,* and *Blomia tropicalis* is prevalent in the tropics, markedly in individuals living in better sanitary conditions and urban areas (43–45).

The importance of environmental exposures does not underestimate the fundamental participation of the family history of atopy and/or asthma and genetic background. Thus, we still have an enormous challenge to explain the occurrence of allergies and asthma. Increasing attention has been given to epigenetic modifications, i.e., modifications in DNA without sequence changes, triggered by individual exposure to environmental factors, for instance, by products of combustion, drugs, diet, and infections. Epigenetic mechanisms, such as DNA methylation and histone modifications, can modulate gene expression upon exposure to a specific environmental agent (46). Such biochemical alterations can alter different targets within the body, leading to the risk or protection of several conditions. In this review, we present the concept of holobiont and discuss how exposure to different organisms, including bacteria, viruses, and helminths, can lead to epigenetic modulations and how this modulation correlates with allergic diseases, taking into account different genetic backgrounds of several ancestral populations.

THE CONTEXT OF MICROBIAL EXPOSURE, THE CONCEPT OF HOLOBIONT, AND THE MECHANISMS INVOLVED IN IMMUNE MODULATION

Holobiont Concept

Microbes are the most ancient, abundant and arguably the greatest successful form of life on Earth, contributing to the evolution and function of all more complex multicellular organisms (47). Since the early days of life, microbes interacted

and established intrinsic symbiotic relationships which could evolve as a unit. The term holobiont was first coined by Lynn Margulis (48) and consisted of a simple and elegant way to explain how a host and its symbiont would evolve (49). This concept has been expanded, and it is well-accepted that a holobiont consists of a set comprised of the host and its associated microbial communities, i.e., the microbiota composed of the three domains of life, and viruses (50). According to this concept, the host (i.e., plant or animal) is subject to ecological and evolutionary pressures, so the entire community would evolve according to natural selection (51–53). This concept has been widely adopted, especially in the coral and human microbiome literature (54, 55), and it is relevant to understand its implications on human health. Understanding the relationships and interactions between microorganisms and parasites, such as helminths and protozoans, with host cells and tissues within a holistic approach is of paramount importance (49) and may provide practical solutions for challenging problems such as antibiotic resistance, allergies and asthma (56). This concept is tightly linked with the One Health framework, which is a multidisciplinary collaborative effort to achieve most appropriate health for people, animals and environment (50, 57).

The advances in DNA sequencing technologies and computational tools enabled us to explore in great detail the microbial communities and their ecological relationships on several times and space scales (58). This is a flourishing time for microbiome studies and a robust body of literature has already elucidated how environmental drivers shape free-living and host-associated microbial communities (58). Several lines of evidence show that human health is tightly linked with the equilibrium of the commensal microbial community, ultimately holobiont homeostasis. The microbial biodiversity and the relationships and interactions among microbes lead to functional outcomes. Reducing diversity, usually by a dominant microorganism, promotes a more variable and less resilient microbiota, a phenomenon known as dysbiosis, which can alter the ecosystem services provided by the microbiota, leading to a disease state.

More specifically, for the scope of the present review, the mammalian gastrointestinal tract harbors a wide diversity of microorganisms. It is estimated that *Homo sapiens* DNA makes up only a small percentage of the overall DNA on and within the human body—far greater genetic contributions are derived from bacteria, fungi, viruses, archaea, and other microorganisms as part of a vast (and individually distinct) residential community collectively known as the human microbiome (48). Additionally, more than 100 trillion microorganisms, colonize the oral–gastrointestinal tract (59). The microbiota interacts and stimulates the host immune system by activating bacterial metabolism through biochemical pathways (60), mediated by diet, host and microbiota metabolites, and antimicrobial compounds (60). The commensal microbiota is essential not only for the use of nutrients through good digestion and resistance to infections by pathogens but also supports the regulation of the host immune system, influencing innate, and adaptive immune responses (61). Dysbiosis can lead to a disruption on immune homeostasis and, consequently, to diseases such as allergy, asthma, neurodegenerative disorders, autoimmune, cardiovascular, and metabolic diseases (60, 62).

Host-Bacterial Interactions

The presence of organisms/microbes in the human body is important to induce a proper immune response, including a regulatory mechanism that could even have a bystander effect of inflammatory conditions (63). The immune system is regulated by immune organs and cells, soluble cytokines, and cell receptors (64). The gut-associated lymphoid tissue is composed of three different lymphoid structures of the mucosa: immune cells present in the compartments of the intestinal epithelium, lamina propria, and Peyer's patches of the small intestine (61, 64). Commensal human host bacteria modulate the immune system through a bridge between epithelial cells and lymphoid structures (65). It has been previously described that microbiota can induce both Th17 and T regulatory (Treg) immune responses (66). The interaction with epithelial cells induces Th17 cell polarization and a positive regulation of antimicrobial proteins. Th17 cells are vital for protective host immunity and have been implicated in autoimmune disease development by producing the pro-inflammatory cytokines IL-17A, IL-17F, and IL-22 (59, 66).

Clostridia, segmented filamentous bacteria, *Bacteroides fragilis,* and other microorganisms can induce the development and/or activation of Treg cells by stimulating intestinal epithelial cells, lamina propria dendritic cells (DCs) and macrophages (59). However, it is unclear which molecular mechanisms commensal microbiota induce Treg cells in the gut (67, 68). Treg cells control autoimmune reactivity, suppress inflammatory responses, and maintain homeostasis of the microbiota (69). According to Kamada et al. (59), the reduction of Treg cells can increase the expansion of CD4+ Th cells expressing commensal bacteria-specific T cell receptors (TCRs), leading to intestinal inflammation.

In fact, the mechanisms whereby commensal microbiota can modulate immune response is an area of increasing interest. In this context, the immune cells in the Peyer's patches are responsible for the surveillance of the intestinal lumen (70). Peyer's patches contribute to the generation of B cells, which, once activated, produce intestinal secretory IgA (sIgA) (64). IgA is the most abundant class of immunoglobulin produced in mucosal tissues, mostly the gut (59, 71). sIgA is essential for the neutralization of toxins and response to pathogens. It promotes intestinal barrier function and supports maintaining host–commensal mutualism. In addition, IgA is involved in determining the diversity and regulating the composition and function of the gut microbiota (59, 70). Innate lymphoid cells (ILCs), categorized into three subsets (groups 1, 2, and 3), help also with the homeostasis, control the composition of the microbiota, contribute to the resistance to pathogens and heal the gut (59, 64). ILC1s promote homeostasis through the production of IFN-γ, while ILC2s are activated by IL-25 (induced by commensal microbiota) to release amphiregulin (Areg), which is responsible for tissue repair, and IL-5/6, which has a role in the production of IgA by B cells (72). IL-22 induces the production of ILC3s, leading to mucus production, the release of the antimicrobial peptide, fucosylation (a type of glycosylation)

of the proteins from the lumina and lipids that offer energy for the microbiota (72).

Some commensal bacteria, such as *Clostridia* strains, have been shown to suppress the immune response by promoting the differentiation of Tregs and IL-10 production in the gut (65, 73). The induction of colonic Tregs can depend on *Clostridium* cluster IV and XIV and the production of metabolites, such as short-chain fatty acids (SCFAs), which have immune and metabolic functions involved in the regulation of cellular processes (74, 75). SCFAs are metabolites synthesized by bacterial fermentation of indigestible carbohydrates, in the colon, and decomposition of dietary fibers (61, 76). Propionate, butyrate, and acetate are the most predominant SCFAs in the gut and enable Treg production (73, 75). Butyrate is involved in Treg differentiation by binding G-protein-coupled receptor 43 (GPR43), a receptor of SCFAs present in colonic T cells (76). Butyrate has also been shown to induce Treg cell differentiation via dendritic cells dependent on GPR109a (77). This metabolite also can regulate central steps of the eosinophil lifecycle and function (78), inhibit ILC2 proliferation and cytokine production likely through inhibition of GATA3 expression (79), inhibit nuclear factor- κB (NF-κB) signaling via protein acetylation by a HDAC inhibitor (80) and limit the production of TNF by lipopolysaccharide (LPS)-stimulated neutrophils (81) and peripheral blood mononuclear cells (66) (**Table 1**).

Escherichia coli trytophanase produces indole from tryptophan (94). This metabolite activate aryl hydrocarbon receptor, a transcription factor that induces expression of genes such as CYP4501A1, which cleans chemicals and toxins (95). Indole has an immunomodulatory function by maintaining the integrity of the enteral mucosa and promoting the epithelial barrier defense against pathogens by stimulating the production of anti-microbial peptides, mucins, and proliferation of intestinal goblet cells (62) (**Table 1**).

Polysaccharide produced by *Bacteroides fragilis,* a species of gut microbiota, was described to conduct systemic immunological maturity and could restore the balance between Th1 and Th2 cells and CD4+ T cell deficiency in germ-free mice (65, 66, 74). *B. fragilis* triggers toll-like receptors to create a symbiosis between the host and microbiota and affects the differentiation and development of T cells (74). *Lactobacillus reuteri* is a Gram-positive facultative anaerobic bacterium that also resides in the gut microbiota. This microorganism has many benefits as a probiotic, such as reducing infection, influencing the integrity of gut mucosa, and modulating the host's immune responses (96). *L. reuteri* has a role in protecting lung infections, stimulating the production of gut granulocyte-macrophage colony-stimulating factor, which promotes clearance of pathogens by alveolar macrophages (74, 96).

Host-Fungus and Viruses Interactions

Although bacteria are a main component of the human microbiota, there are other organisms also composing the holobiont such as fungi, viruses, and multicellular parasites that are also important for a good balance, with potential effects on human health. The most-reported fungi in the intestines of mice and humans include *Saccharomycetes* (*Candida* and *Saccharomyces* spp.), *Eurotiomycetes* (*Aspergillus* and *Penicillium* spp.), *Tremellomycetes* (*Cryptococcus* and *Trichosporon* spp.) along with *Cladosporium*, *Wallemia*, and *Malassezia* spp. (97).

Candida albicans interacts with intestinal epithelial cells through some events, including adhesion, invasion, damage, and apoptosis (98). This interaction can lead to superficial overgrowth and epithelial invasion, followed by disease and immune activation (82). The Candidalysin, a cytolytic peptide toxin released by *C. albicans*, induces proinflammatory cytokines, chemokines and antimicrobial peptides of epithelial cells that are necessary for the recruitment of immune cells, via MAPK signaling, specifically the p38 pathway, resulting in the activation of the AP-1 transcription factor c-Fos, and the ERK1/2 pathway, leading to the activation of MKP1 (MAPK phosphatase 1), which regulates the immune response (82).

Aspergillus fumigatus produces a variety of precursors of toxins such as gliotoxin, which represses IFN-γ responses and induces neutrophil apoptosis through inhibition of NF-κB, a transcriptional regulator of the host proinflammatory response (99); and fumigaclavine C that down-regulates Th1 cytokines, by binding to IFN-ɤ receptor 1 (IFN-ɤ R1) (100) and induces host cell apoptosis via caspases-3,−8, and−9 (83, 101).

In addition to bacteria and fungi, the intestinal virome is composed of DNA and RNA viruses and includes eukaryotic viruses, endogenous retroviruses and bacterial viruses (102). According to (84), eukaryotic viruses and bacteriophages can stimulate changes in the immune response. Eukaryotic virus by altering the hematopoiesis or immune activation, improving a secondary infection. Bacteriophages by stimulating the production of inflammatory cytokines and type I interferon. These changes in immune responses can contribute to inflammatory diseases. In this review, we will focus in unicellular and multicellular organisms leading to immune modulation.

Host-Helminths Interactions

Moreover, the different life cycle stages of helminths and protozoa challenge host immune responses to recognize and respond to different antigens. Distinct pattern recognition receptors members participate in the recognition of these parasites and are responsible for driving the TCD4 + cells polarization. Many molecules secreted by adult intestinal worms known as "excretory/secretory antigens" (ES) can stimulate different effects on the host's immune cells. The helminth ES products activate basophils, eosinophils, mast cells, innate lymphocyte T cells 2 (ILC2) and TCD4 + cells and drive the production of innate and adaptive cytokines. Different classes of lipids extracted from schistosome eggs and adult worms have been able to stimulate the production of several inflammatory cytokines (IL-6, IL-8, IL-10, IL-12, TNF-α). Schistosomal lysophosphatidylserine through TLR2 stimulates activation of dendritic cells with subsequent development of IL-10 producing Treg cells (85) and *Ascaris lumbricoides* derived phosphatidylserine containing preparations in the presence of interaction between TLR4 and LPS induced TLR2 with activation of TH2 response (91).

Schistosomal-Derived Lysophosphatidylcholine *in vivo* was able to induce cytokine production and eosinophil

TABLE 1 | Summary of the main products (molecules) from holobionts with immunomodulatory potential and biological activities in the host.

Microbial	Molecules	Biological activities	References
Clostridium	SCFAs	• Anti-inflammatory activities • Regulate Treg production • Inhibit nuclear factor- κB (NF-κB) signaling • Limit the production of TNF in neutrophils and peripheral blood mononuclear cells	(60, 77, 80, 81)
Escherichia coli	Indoles	• Immunomodulatory function • Integrity of the enteral mucosa • Promotes epithelial cell barrier function	(62)
Bacteroides fragilis	Polysaccharide A	• Influences T cells fate through its • Interaction with the toll-like receptor 2.	(66)
Candida albicans	Candidalysin	• Induces proinflammatory cytokines, chemokines, and antimicrobial peptides	(82)
Aspergillus fumigatus	Gliotoxin and Fumigaclavine C	• Suppresses interferon (IFN)-γ • Downregulates Th1 cytokines • apoptosis	(83)
Eukaryotic Virus		Alteration in hematopoiese or immune activation	(84)
Bacteriophages		Production of inflammatory cytokines and type I interferon	(84)
Schistosoma mansoni	Schistosomal-Derived Lysophosphatidylcholine; The soluble extract of eggs (SEA) and lacto-N-fucopentose III; Schistosomula tegument (Smteg) Sm22·6, PIII, and Sm29 antigens Schistosomula tegument (Smteg) Sm22·6, PIII, and Sm29 antigens	TLR2 activation • IL-10 producing Treg cells • Eosinophil recruitment • DC2 maturation • Polarization of the Th2 response. • Phosphorylation of ERK • Up-regulation of CD40 and CD86 expression • IL-12 and TNF-α production • Reduction of eosinophils in the BAL • Reduction of specific IgE • Increase in IL-10 (Sm22·6) • Reduction in IL-4 and IL-5 levels in the BAL. (PIII and Sm29)	(85–90)
Ascaris lumbricoides	Phosphatidylserine containing preparations (PS)	• TLR2 activation • Polarization of the Th2 response.	(91)
Schistosoma ssp. A. lumbricoides	Glutathione transferases	• Stimulate specific IgE antibodies	(43, 92)
Leishmania spp., *Toxoplasma gondii*	The glycosylphosphatidylinositol (GPI) anchors	• TLR2 and TLR4 activation induce of TNF-α	(93)

recruitment potentially through TLR2 recognition (86). Lysophosphatidylcholine participates in the recruitment of eosinophils (85) IL-5 and IL-3 stimulate eosinophilia, and recruitment is mediated mainly by chemoattractant CCL11 and CCL26 (eotaxins). Activation of eosinophils results in degranulation of chemical mediators such as Matrix metalloproteinases, cysteinyl leukotrienes, major basic protein and others (103). It has been shown that patients with *Schistosoma* infection exhibit a higher concentration of CCL3, CCL5, and CCL11 in plasma compared to uninfected individuals. These chemokines favor granulocyte recruitment, granulomatous response against egg antigens (104, 105).

Antigens from *Schistosoma mansoni*, Sm22·6 (soluble protein from the tegument of *S. mansoni*), PIII (multivalent antigen from the *S. mansoni* adult worm) and Sm29 (a membrane-bound glycoprotein from the adult worm tegument) were tested in a murine model of induced airway inflammation and showed immunomodulatory ability. These antigens induced a reduction in the number of eosinophils in bronchoalveolar lavage (BAL) and lower levels of specific IgE. In addition, Sm22·6 was associated with an increase in IL-10 while PIII and Sm29 showed a reduction in IL-4 and IL-5 levels in the BAL (90).

The soluble extract of *Schistosoma mansoni* eggs and lacto-N-fucopentose III (carbohydrates group in *S. mansoni*) has been associated with DC2 maturation and induction of the Th2 response dependent on recognition by TLR4, as well as induces phosphorylation of ERK (87, 88). In addition, schistosomula tegument (Smteg) can induce up-regulation of CD40 and CD86 expression and production of proinflammatory cytokines, such as IL-12 and TNF-α, and such activation is TLR4-dependent (89).

The glutathione transferases from helminths (*Schistosoma ssp.* and *A. lumbricoides*) stimulate specific IgE antibodies (92, 106) The glycosylphosphatidylinositol anchors from protozoan (*Leishmania* spp., *Toxoplasma gondii*) is involved in the activation of cells of lymphoid and myeloid lineage, such molecules are recognized by TLR2 and TLR4 with activation of NF-κB and subsequent induction of TNF-α in murine macrophage cells (93).

The secretion of ES products from hookworms induces activation of ILC2s and tolerogenic dendritic cells, followed by increased expression of molecules associated with tolerance and

reduced expression of co-stimulatory molecules with expansion of Treg cell numbers in the gut and suppresses Th17 cell, this implies a decrease in inflammation and proliferative capacity of the parasite (107, 108). Interestingly, the Howkworms' tolerance ability was demonstrated in experimental hookworm infection in patients with celiac disease, *Necator americanus* infection suppressed gluten-induced IFNγ, IL-17, and IL-23 expression and increased the expression of IL-10, TGFβ, and IL-22 in the gut (107, 109).

It has been shown that infection with geohelminths (*A. lumbricoides, Trichuris trichiura,* hookworm) induces IL-10 and a higher mRNA expression of the Foxp3, PD-1, and regulatory molecules suppressor of cytokine signaling (SOCS) (−3) (110), reinforcing the immunomodulatory capacity of geohelminths. In addition, many of the ES components have pleiotropic immunomodulatory properties.

Taken together, it is possible to see that a balanced holobiont is necessary to maintain homeostasis. Any alteration in this environment can lead to dysregulation of the immune system and metabolism. Further studies are needed to exactly describe how holobionts changes regulate the host immune system, and which changes in its composition is associated with specific diseases.

THE RELATIONSHIP BETWEEN THE SHIFTS IN HOLOBIONT COMMUNITY'S COMPOSITION WITH ASTHMA AND ALLERGIES

Exposures during the peri- and post-natal periods are critical for the host's immune homeostasis, reflecting immune maturation, the development of immune tolerance mechanisms, and susceptibility to disease, also known as the first "window of opportunity" (111). This exposure includes fetal environment conditioned to the individual to the mother's lifestyle, type of delivery, diet, use of antibiotics, exposure to other children and animals, and contact with parasites and environmental microbes (112). Studies have reported that exposure to specific immunostimulatory molecules (from helminths and bacteria mainly) in childhood could reduce or block allergic disease development or progression (113). In embryonic development the immunological regulation of pregnancy is complex and an increased production of Th2 cytokines is observed, along with decreased production of Th1 cytokines. In addition, TGF-β1 appears to be involved in the differentiation of the trophoblast being an important inducer of regulatory T cells (CD4 + CD25 +) and Th17 cells, this seems to be essential for avoiding fetal allorejection (114, 115). Microbial exposures in childhood determine factors in modulation and gradual replacement for T cells and cytokines other than Th2 (116).

Moreover, universal initiatives seeking to improve the population's health conditions, such as immunization in children, improved hygiene and sanitation, access to clean water, indiscriminate use of antibiotics and anti-parasitic drugs, have been implied in reducing opportunities of microorganism's exposure/infections in early childhood with decreased Th1 responses and or decreasing Treg activation and polarizing the immune response to the Th2 profile, breaking homeostasis. Changes in the exposure of antigen patterns, including proteins released from environmental particles or infections in childhood, can impact the diversity of commensal microorganisms that make up the microbiota (117).

The use of antibiotics by mothers during pregnancy is associated with a child's asthma risk, promoting an imbalance between commensal, and pathogenic bacteria (118). Changes in the colonization of the lung microbiota of neonatal mice have (119, 120) been associated with decreased aeroallergen responsiveness induced by Helios– regulatory T cells (Helios–Treg cells) activated depending on interaction with programmed death-ligand 1 early in life, widely known as a regulator of allergic responses. Imbalance in the formation of these cells implies increased susceptibility to atopy in adulthood (119). Likewise, the altered composition of the airway microbiota is often found in asthmatic patients (120, 121). This could be explained partially by differences in environment.

Rural vs. Urban

For instance, the prevalence and severity of asthma differ between urban and rural areas. An agricultural environment has been associated as a protective factor against the development of asthma, hay fever, and atopic sensitization in children (12, 122). An explanation would be associated with concentrations of endotoxin significantly higher in rural homes than in urban centers (123). Exposure to higher levels of endotoxin and other bacterial components in early childhood can play a protective role against allergies and asthma (123). The endotoxin constitutes the membrane of gram-negative bacteria, inducing the Th1 response by stimulating cytokines such as IL-12 and IFN-ɤ (12). In addition to that, helminth infections caused by *Ascaris lumbricoides*, *Trichuris trichiura*, are more prevalent among children living in areas of the rural tropics in poverty and poor access to clean water and sanitation (124).

Helminths vs. Asthma/Allergies

Helminths and allergic asthma induce similar immune responses, including elevated serum IgE, systemic eosinophilia, and cytokines such as IL-4, IL-5, IL-9, and IL-13, the hallmark of an immune Th2 response (125). Additionally, basophils, mast cells, neutrophils and innate lymphoid cells are involved (126). Interestingly, infections by parasite species such as *A. lumbricoides*, *Schistosoma mansoni*, *Strongyloides stercoralis*, and *T. trichiura,* have been associated with a reduction in airway allergic inflammation (34) with decreased Th1 responses (**Table 2**). The immunomodulatory ability of geohelminths to reduce susceptibility to allergies in humans has been recognized, and it is related to the immune-regulatory network, including helminth-derived products. Recombinant proteins of *S. mansoni* were associated with an increase in IL-10 and TGF-β, an increased frequency of regulatory T and B cells, and a reduction in the frequency of activated T lymphocytes that produce IL-4 and IL-13 in individuals with severe asthma and animal models (142, 143). In addition, *T. trichiura* infection appears to modulate the immune response among asthmatics, with some studies

TABLE 2 | Summary of the immunomodulatory effects of some halobiont's organisms on asthma and allergy.

Holobiont	Immunomodulatory effects	Consequences
Schistosoma mansoni	↑TNF-a and IFN-g in acute phase	Prevent against the development of allergies and asthma (32, 34)
	↑ IL-10 in chronic phase	Down-modulate the inflammatory response in murine model of ovalbumin (OVA)-induced airway inflammation (90)
Ascaris lumbricoides,	↑IL-4, IL-5, and IL-10	*Ascaris lumbricoides* eggs was associated with an increased prevalence of asthma (124) Reduced risk of wheeze (127) Anti-A lumbricoides IgE antibodies were associated with risk of wheezing in atopic children and atopia (36)
Trichuris trichiuria	Modulation of pro and anti-inflammatory cytokine (35) ↓TNF-α and IL-6 levels among asthmatics infected ↑IL-10	↓allergen skin test reactivity (33) Positively associated with wheezing (36)
Helicobacter pylori	Th1 polarization ↓Th2 response ↑ (IFN)-γ, IL-12, IL-18, IL-23 (128)	Negative association between H. pylori infection and asthma, eczema, and rhinitis (128)
Hookworm (*Ancylostoma duodenale* and *Necator americanus*)	Induction of IL-25 and ILC2s (129) ↑IgG1, IgG4, and IgE Expansion of Treg cell numbers in the gut (107, 108)	Protect against wheezing, asthma, and allergic diseases. Reduction in risk of wheeze (130)
Toxoplasma gondii	Induces IL-10 production, IL-27, and activity of lipoxins (131, 132).	Protective effect against atopy (133) Suspend the development of airway inflammation and atopy in mice (133) Decrease in specific IgE for *Dermatophagoides pteronyssinus* (134)
Toxocara spp.	↑ levels of total IgE Cross reactivity with aeroallergens	Positive skin tests to allergens, and asthma prevalence and morbidity (135, 136)
Bifidobacterium	Stimulating IL-10 or IL-12 synthesis	Protective factor for high risk of allergic asthma and atopic dermatitis in children from Turkey (137)
Bacteroides fragilis	Stimulate Th2 cytokines by biding TLR2	Risk factor in children with a positive API (138)
Penicillium	High counts in patients with atopy	Risk factor for atopic asthma (139, 140)
Aspergillus fumigatus	Decrease the expression of GCR	Aggravate airway hyper-responsiveness and increase the level of TLR2 (141)

↑*increase;* ↓*decrease.*

reporting a risk association for asthma among infected people and positively associated with wheezing (33, 35, 36).

Maternal soil-transmitted helminths (STH) infections can sensitize the individual still in the fetal phase. Cooper et al. (144) reported a strong association between Maternal STH infections during pregnancy (mainly moderate to chronic *A. lumbricoides* infection) and childhood STH infections. During pregnancy, infected mothers have an increased number of CD4 + T cells and production of IL-10 in cord blood from newborns demonstrating immunomodulation mediated by parasite antigens (145, 146). In the same study, poor hygiene conditions, with the prevalence of STH infections, were not associated with reduced eczema-asthma-rhinitis symptoms (144). Co-exposure to mites and *Ascaris lumbricoides* in the context of low worm burdens promotes allergic sensitization and asthmatic symptoms by increasing parasite-specific IgE production, mite-specific and mite–parasite cross-reacting IgE antibodies, observed mainly in urban areas, once in rural areas the exposure to helminths tends to be chronic (40). *A. lumbricoides* extract was associated with inhibition of pulmonary eosinophilia in mice sensitized with ovalbumin (OVA) and a decrease in allergic inflammation independent of IL-10 (147) (**Table 2**). In contrast, Anti-*A. lumbricoides* IgE (but not active infection), were associated with risk of wheezing in atopy in atopic children (36).

Viruses and Protozoans vs. Asthma/Allergies

Some viral and protozoan infections have been associated with decreased reactivity to skin prick tests for aeroallergens (148, 149) and asthma (150). The host's defenses against viruses are marked by a predominance of the Th1 response and interaction with different Toll-like receptors with probable biological and immunomodulatory effects on Th2 responses. *Toxoplasma gondii* infection has been reported to suspend the development of airway inflammation and atopy in mice (133) and induces IL-10 production, IL-27 and activity of lipoxins (131, 132). In addition, a negative association was reported between *T gondii* seropositivity and specific IgE to *Dermatophagoides pteronyssinus* (134). Hepatitis A virus (HAV) exposure has been inversely associated with allergies (151). In the United States, positive serology for HAV was associated with a lower chance of developing hay fever and asthma and skin reactivity to airborne allergens (152). In Turkey, the prevalence of atopy was lower among individuals with positive serology for HAV and hepatitis

B virus (anti-HAV IgG, HBsAg, anti-HBc IgG) (153). The accumulated infection burden, considering HAV, herpes simplex virus, Epstein–Barr virus, Cytomegalovirus, *Helicobacter pylori*, and *Toxoplasma gondii* (> 3 microbes), was associated with a protective effect against atopy (149, 153). According to Amedei et al. (128) *H. pylori* infection was negatively associated with asthma, eczema and rhinitis and induces Th1 polarization (**Table 2**). Moreover, BCG vaccination at an earlier age was associated with a decreased risk of atopy in children without a family history of asthma and atopy (154). However, there are controversies regarding some types of vaccines (155, 156).

Bacteria vs. Asthma/Allergies

A study (157) from the Copenhagen Prospective Study on Asthma in Childhood has shown that the lack of development of the gut microbiome in the first year of life is the determinant to the occurrence of childhood asthma, increasing asthma risk. The lower number of *Lachnospiraceae* and *Ruminococcaceae* genera was observed in asthmatic children and was associated with allergic wheezy phenotype (157). The production of SCFAs was suggested to be associated with asthma development in a study of high vegetable fiber intake by children from Manitoba Prospective Cohort Study of Allergy, Genes and the Environment, acting as a protective factor against to airway hyperresponsiveness (158, 159). *Bifidobacterium longum* has been described influencing the prevalence of allergic disease being a protective factor for allergic asthma and atopic dermatitis in children from Turkey (**Table 2**) (137). In contrast, *Bacteroides fragilis* count was significantly higher in children with a positive Asthma Predictive Index as compared with those negative (138). It seems that *Bacteroides* species maybe stimulate Th2 cytokines and some studies have found an association between this genera and higher IgG in children with allergies (138) (**Table 2**).

Fungus vs. Asthma/Allergies

Skin-test for fungal allergens is usually characterized with the presence of immediate cutaneous hyperreactivity or positive results for specific IgE antibodies to fungal antigens and has been related to be especially common in patients with life-threatening asthma (139, 160). *Aspergillus*, *Alternaria*, *Penicillium*, *Cladosporium,* and *Trichophyton*, have been described to be associated with exacerbation and severity of asthma (139). *Penicillium* species was higher in patients with atopy compared with healthy control subjects, suggesting to be a risk factor for atopic asthma since this genera is one of the most common fungi related to allergic asthma exacerbations among adults (**Table 2**) (140, 161). A study using rats with asthma shows that *Aspergillus fumigatus* may decrease the expression of glucocorticoid receptor aggravating airway hyper-responsiveness and increase the level of TLR2, involved in airway inflammation (141) (**Table 2**).

The immune response in the context of asthma and atopy as well as its development, differentiation of cell subtypes and expression of receptors and cytokines are influenced by exposures to holobionts. This immunological modulation is often accompanied by epigenetic changes. In part, such modifications that allow such plasticity of immune responses, also promote homeostasis through the balance of adaptive immune responses in certain conditions and are responsible for the maintenance and intensification of Th2 responses, increasing the risk for allergic diseases and other inflammatory diseases.

EPIGENETIC MECHANISMS: BASIC CONCEPTS

Currently, epigenetics can be defined as changes above the DNA without changing the nucleotide sequence (162). Different mechanisms of epigenetic regulation have been described, such as DNA methylation, histone modifications and non-coding RNAs. Since the first Waddington epigenetics works (163, 164), many studies have been conducted to determine the influences of epigenetics in several conditions. The epigenetic mechanisms are widespread in the different cell types of the human body, including cells that participate in an immune response pathway directly involved in the etiopathogenesis of asthma and other allergic diseases. Understanding the impact of epigenetic changes on the normal and abnormal functioning of these cells, therefore, is an important piece to compose the complex puzzle that allergic diseases represent. Bellow, are described the main mechanisms of epigenetics-induced changes in gene expression.

DNA Methylation

DNA methylation is the addition of a methyl group (CH3) to a cytosine by DNA methyltransferases, generating 5-methyl-cytosine (165). Promoter regions of genes have a large amount of CpG (cytosine-phosphate-guanine), known as CpG islands, that when methylated prevents the binding of transcription factors and represses gene expression (166).

Several factors can contribute to DNA methylation changes, such as aging, environmental exposure, cell type, and age. These modifications can be passed through cell division through either mitosis or meiosis (167). Recently, many epigenome-wide association studies (EWAS) have described the association between DNA methylation and asthma, and several genes were identified, including EPX, IL4, IL5RA, PRG2, SIGLEC8, CLU, AP2A2, and KCNH2 (168–170).

Histone Modifications

Histone is a protein involved in the organization of chromatin and regulation of gene expression. They are grouped into 8 subunits, two of each H2A, H2B, H3, and H4 forming an octameric nucleosome where the DNA coils. Histone H1 is associated with this complex and stabilizes the chromatin structure. Some modifications can occur in the N-terminal tails of histones, including acetylation, methylation, ubiquitylation, and phosphorylation (171).

Histone acetylation occurs when acetyltransferases add lysine residues to histone tails. Histone acetylation increases DNA access and facilitates the process of transcription, increasing gene expression. Previous studies reported that H3K4me3 and H3K27me3 were associated with T helper cell differentiation and IL-5 expression (172), and higher histone 3 acetylation levels at the IL13 locus were associated with higher protein levels of IL13 (173).

Methylation in histones is performed by methyltransferases and usually occurs at lysine (K) or arginine (A) residues and can increase or decrease gene expression depending on the modified residue. For instance, inactivation can occur by methylation on H3K9, H3K27, and H4K20, while activation occurs by methylation on H3K4 and H3K36 (174).

Non-coding RNAs

Non-coding RNAs are a group of RNAs that do not encode proteins but can play an important role in the regulation of gene expression acting at the post-transcriptional level (175). Regarding size, RNAs with regulatory functions are divided into short non-coding RNAs (siRNAs, miRNAs and piRNAs) and long non-coding RNAs (lncRNAs) (176). They can silence genes through the RNA interference pathway and modulate several biological processes, including immunological functions (177).

SHAPING IMMUNE RESPONSES THROUGH EPIGENETICS MECHANISMS: REGULATION OF CYTOKINE GENE EXPRESSION, TRANSCRIPTION FACTORS, AND REGULATION OF IMMUNE RESPONSES IN ASTHMA AND ALLERGY

The epigenetic mechanisms previously described are present in the different contexts and cell types of the human body, including driven immune cell pathways directly involved in the etiopathogenesis of asthma and other allergic diseases. Understanding the impact of epigenetic changes on the normal and abnormal functioning of these cells, therefore, is an important piece to compose the complex puzzle that allergic diseases represent. Some advances in this direction have recently been achieved. Thus, epigenetic modifications play a role in regulating the expression of cytokines related to T cell differentiation and transcription factors (178). The development of cell types and, consequently, the specificity of immunological responses occur through internal stimuli or driven by stimulatory molecules of microorganisms. They act on surface receptors such as TLR signaling, signal transduction proteins, and lineage-specifying transcription factors, promoting intracellular events. Even the development of T lymphocytes and maturation for helper (CD4+) and cytotoxic (CD8+) cells are influenced by epigenetic control. This promotes CD4+ silencing in CD8+ thymocytes and the development of T helper cell subsets (Th1, Th2, and Th17) accompanied by epigenetic changes (179, 180). Epigenetic changes have also been linked to the activation and polarization of macrophages (M1/M2 phenotypes) (181).

Allergic diseases, e.g., asthma, result from a strong interaction of genetic and environmental components with remarkable phenotypic heterogeneity. This heterogeneity of asthma can be partially explained by dysregulated epigenetic mechanisms correlated with environmental exposures, pharmacological treatments, and airway inflammation and function (182). There is evidence that the induction of Th2 cells, maintenance, and the resurgence of memory Th2 cells are controlled by epigenetic regulation since this induction is mediated by signal transducer and activator of transcription 6 and the consequent production of the Th2 cytokine profile (183). Hypermethylation in GATA3 CpG loci was associated with a decreased risk of asthma at birth (184), and hypomethylation of IL-13 and interleukin 5 receptor subunit alpha (IL5RA) was associated with an increased risk of asthma in teenagers (169).

Epigenetic mechanisms are essential in controlling gene expression or silencing and the consequent balance of Th1/Th2 responses. Corroborating the principle that Th1/Th2 imbalance is involved in the pathogenesis of asthma and atopy, experimental studies in mice showed hypermethylation in the IFN-ɤ gene promoter in TCD4+ cells, leading to the silencing of the *IFNG* gene (Th1 pattern) (185). During initiation of a Th2 immune response, an increase in histone acetylation was observed at the Th2 cytokine *loci*. It has been demonstrated that the *IL4* and *IL13* genes are hypomethylated in asthmatic patients, critical genes in amplifying the Th2 response (186).

A balance of histone deacetylase (HDAC) and histone acetyltransferase (HAT) activity has been considered to regulate gene expression. Reduced HDAC expression was observed among adults with severe asthma compared to mild asthma (187). In atopic asthmatic children, a relationship was found between HDAC/HAT activity and increased histone acetylation, and the degree of acetylation was associated with an increase in bronchial hyperresponsiveness (188).

miRNAs have also shown an essential role in the inflammatory response of asthma. Studies have shown that miR-155 and miR-221 are associated with modulation of the Th2 response (189) and hyperproliferation of airway smooth muscle in asthmatic patients, respectively (190). In the asthma context, non-coding RNAs have been observed as markers of disease diagnosis, phenotypes, and response to treatments. For example, a negative correlation between the levels of miR-323-3p and IL22 and IL17 was observed in PBMCs from patients with asthma, suggesting that non-coding RNA acts as negative feedback in the production of these cytokines influencing the immune response of these individuals (191). Moreover, elevated levels of miRNA-21 in the peripheral blood of children with asthma were identified, suggesting that this non-coding RNA may be a biomarker in the diagnosis of asthma (192). Furthermore, high expression of microRNA-155 and decreased expression of Let-7a were observed in the plasma of asthmatic patients and were associated with the degree of asthma severity, suggesting that these markers can be used both in diagnosis and in the prediction of the severity of disease (193).

In the context of regulatory T cells (Tregs), cells that play a substantial role in immune homeostasis through mechanisms of tolerance and immune de-activation during a regular immune response and suppression of a self-destructive immune response, the repressive phenotype of Tregs is conferred, in part, by the expression of Forkhead box protein 3 (FOXP3) (194). Hypermethylation of CpG islands in the promoter region in the *FOXP3* locus impacts transcriptional silencing and consequent reduction in Treg cell function. Air pollutants have been recognized as acting on epigenetic changes. Increased exposure to polycyclic aromatic hydrocarbons has been associated with an increase in DNA methylation at the *FOXP3* locus in peripheral

blood mononuclear cells and elevated total IgE with significant effects on asthmatics (195). Furthermore, an increase in *FOXP3* DNA methylation has been associated with an increased risk of asthma and persistent wheezing (196).

Figure 1 shows different factors shaping asthma and allergy, such as environmental factors, epigenetic changes, and exposure to holobionts components which can be modulated by disturbances in the homeostasis.

EPIGENETIC CHANGES ASSOCIATED WITH HOLOBIONT INTERACTIONS

Since immune dysregulation is linked to allergies and asthma through the lack of certain environmental exposure, one could think that potential epigenetic mechanisms may play a role in this phenomenon. A question raised by these new findings is at "what point in the development of the human being the epigenetic mechanisms could act to drive the maturation of the immune system in early life?." In this sense, in recent years, the hygiene hypothesis has been expanded to encompass the potential effect of prenatal exposure to microbial agents on modulating the individual risk of asthma and other allergic diseases (115). Although some evidence in this regard was already available through epidemiological studies that assessed the impact of maternal microbial exposure on the risk of developing allergic conditions in the offspring (116), the elucidation of the molecular mechanisms underlying these processes has been a relatively new and fascinating field of investigation.

Alterations in the gut microbiota, called dysbiosis, is related to infections and inflammatory diseases and comes with irregular immune responses, e.g., particular inflammatory cytokines (66, 75). Changed gut microbiota can also increase the production of NK-κ-B and TNF-α and the overexpression and activation of Th1 and Th17 cells (197). Studies have shown that changes in the gut bacterial composition and the production of its metabolites can influence epigenetic levels, such as reducing methylation and inhibiting histone deacetylases (197). Specifically, the metabolites influencing epigenetic enzyme activity are a substrate needed for epigenetic changes (197). For example, Butyrate, a metabolite from microbiota, can also inhibit HDAC, increasing the expression of FOXP3 through the acetylation of histone H3 in the promoter and enhancing Treg generation (164).

One of the first mechanistic studies on the allergoprotective effects of maternal exposure to microbes used a mouse model with the farm bacterium *Acinetobacter lwoffii* (198). In this study, the protective effect for allergic airway inflammation (AAI) in the offspring was dependent on maternal TLR signaling, since this protection was abolished when mothers were knocked out for multiple TLR genes. The authors also demonstrated that the immune dampening observed in the progeny of pregnant mice was not due to microbial components able to pass the fetus-maternal interface and directly activate the developing fetal immune system. This last observation suggests the possible involvement of epigenetic factors operating in the fetuses of mothers exposed to *Acinetobacter lwoffii*. Indeed, another study by the same group reported epigenetic changes in Th1/Th2 cytokine genes in offspring from pregnant mice exposed to *A. lwoffii* (199). While the IFNγ promoter on CD4+ T cells exhibited significant protection against the loss of histone 4 (H4) acetylation, with the consequent increase in IFN-ɤ expression in OVA-induced AAI, the IL4 promoter showed a significant decrease in H4 acetylation and diminished gene expression. A protective effect against induced AAI has also been shown in the progeny of pregnant mice exposed to *Helicobacter pylori* extracts (200). An epigenetic consequence observed in the offspring was the enhanced demethylation of the regulatory T cell-specific demethylated region in Foxp3+ Treg cells. Intriguingly, this protective effect extended to the second generation (F2) of mice exposed to *H. pylori* antigens during pregnancy, with both sexes exhibiting similar levels of protection. This indicates that the epigenetic changes in the offspring induced by transmaternal exposure to *H. pylori* may extend to chromosomal *loci* other than just the TSDR linked to the X chromosome. The transfer of allergoprotective effects during the prenatal phase through maternal infection with the helminth *Schistosoma mansoni* has also been previously investigated in experimental models of AAI in mice (201). Interestingly, this protective effect was dependent on the stage of the immune responses to *S. mansoni* in the females at the time of mating. While the offspring of the mothers mated during the Th1 and regulatory phases showed protection against OVA-induced AAI, those born to mothers mated during the Th2 phase showed an exacerbation of the allergic inflammatory response compared to the controls. The authors also demonstrated that the protective effect of transmaternal exposure to *S mansoni* was mediated by maternally produced IFN-ɤ and not by the transfer of helminth antigens to the fetus. Potential epigenetic changes in the offspring associated with the protective immune phenotype, however, were not further investigated and remain to be clarified (202).

In humans, data on epigenetic changes induced by pre- or post-natal exposure to microbial agents and their relationship to asthma and other allergic conditions are still scarce. A pilot study evaluated the effect of maternal exposure to the farm environment on offspring epigenetic changes for genes known to be associated with asthma and allergies (203). Significant differences between non-asthmatic children born to mothers exposed to the farm environment and asthmatic children born to unexposed mothers were observed for the methylation pattern of the *ORMDL3* and *STAT6* genes in cord blood. In a recent study, Lund et al. (204) reported that changes in the methylation pattern in chromosomal previously linked to asthma, such as the SMAD3 promoter at 15q22.33 and intronic regions of the DDO/METTL24 genes at 6q21, were associated with atopic asthma in children with early rhinovirus-induced wheezing. In turn, DNA methylation changes linked to the prostaglandin D2 synthase gene were associated with non-atopic asthma in children with rhinovirus etiology at the first severe wheezing episode (204). This suggests that the epigenetic changes triggered by the same microbial agent may differ according to the specific phenotype of asthma or other allergic diseases, which needs to be further investigated in the future.

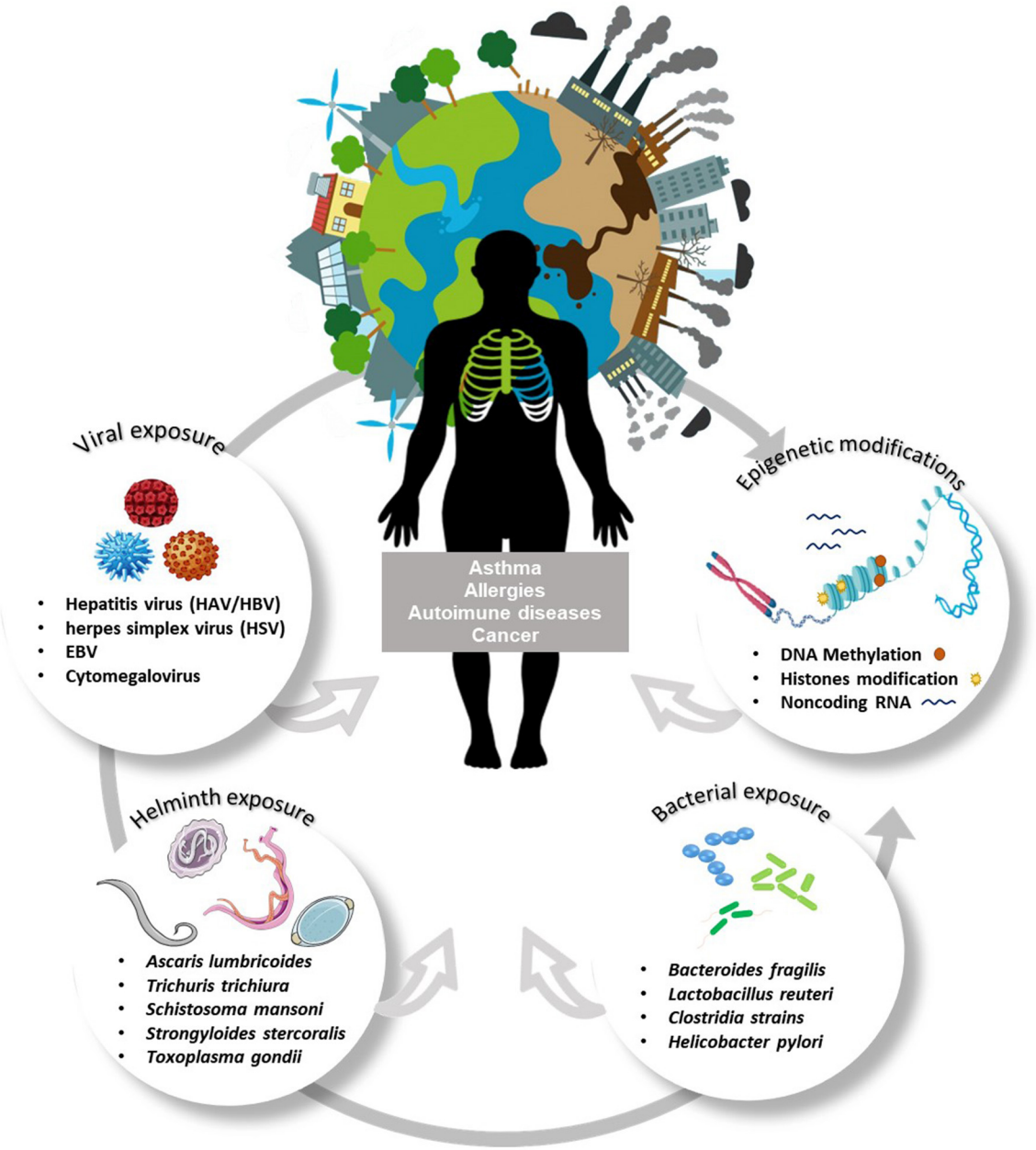

FIGURE 1 | Interaction between multiple environmental exposures and epigenetic changes: impact on immune-mediated diseases. Polluting agents, environmental exposure, diet, age, drugs, and especially exposure to organisms (species of bacteria, fungi, protozoan, and helminths) act as inducers of epigenetic changes. Among the epigenetic modifications are DNA methylation and histone modifications. Histone acetylation increases DNA access and facilitates the process of transcription, increasing gene expression. The addition of a methyl group in CpG islands prevents the binding of transcription factors and represses gene expression. The interaction between environmental exposures and epigenetic variations begins in the embryonic period and continues throughout life, being strongly dependent on the environmental experiences/challenges of everyone. Such alterations can be highly modifiable by instantaneous adaptation to the environment or generate inheritable epigenetic patterns and consequences to offspring. Epigenetic mechanisms influence cell differentiation and polarization of immune responses, these events modulate biological responses, and can interfere with the development of different immune-mediated diseases such as cancer, asthma, allergies, autoimmune diseases.

Taken together, although several studies related to epigenetics of asthma and allergies have been published so far, very few initiatives explores the role of the environmental changes, in special, exposure to organisms such as bacteria, fungi, protozoan and helminths as important modulators of those biochemical changes in human DNA. Further studies are needed to better understand such associations.

FUTURE THERAPIES AS POTENTIAL MODULATORS OF EPIGENETICS CHANGES IN ASTHMA AND ALLERGIES: OBSERVATIONS AND FUTURE PERSPECTIVES

The usual immunotherapy and pharmacological therapy in the treatment of asthma and allergies act in the modulation of immune responses, with a focus on reducing inflammation and increasing immunological tolerance. This immunological modulation is almost always accompanied by epigenetic changes. It is even possible to distinguish different epigenetic signatures between untreated individuals and individuals under treatment (205).

The use of inhaled corticosteroids (ICS) in the management of moderate to severe asthma is recommended by asthma management guidelines (206) and several studies have shown that corticosteroids are potent epigenetic modifiers (207–209). Children with better response to corticosteroids have been shown to have hypermethylation in Vanin-1 (VNN1) promoter compared to the group with poor response, in addition VNN1 mRNA expression was higher among good responders. VNN1 appears to have an important role in corticosteroid responsiveness among asthmatics, and can be used as a biomarker for treatment response (209, 210). Acetylation of histones by HATs activity was reported to be reduced in asthmatics treated with inhaled steroids (211). Variations in serum IgE concentrations can be influenced by DNA methylation patterns. An association between total serum IgE concentration and low methylation at 36 *loci* has been demonstrated, this observation may be useful in optimizing therapies with anti-IgE antibodies such as omalizumab (212).

Studies evaluating the effectiveness of peanut oral immunotherapy found a great suppressive function of Treg cells and higher levels of FOXP3 hypomethylation among treated individuals (213). In addition, a study involving cow's milk allergy children and dietary intervention using probiotic *Lactobacillus rhamnosus* (abundant in butyrate-producer bacteria strains) demonstrated that oral tolerance in children with IgE-mediated CMA involves epigenetic regulation of the FOXP3 gene. Difference in the methylation status of FOXP3 was found among children who developed oral tolerance after probiotic therapy (205). Prenatal administration of *Acinetobacter lwoffii* F78 in murine demonstrated a modulation in Th1/Th2 balance genes, with protection for asthma in the progeny, accompanied by changes in DNA acetylation (199).

Although some studies using probiotic supplementation in animal models have indicated a protective effect of probiotics on asthma and allergic Rhinitis (214, 215), studies in humans are still limited due to couple limitations such as the duration of supplementation.

Many efforts have been focused on understanding and developing microbial therapies using technological approaches involving parasitology, genomics, transcriptomics, and proteomics methods. Currently with the help of bioinformatics and helminth genome sequencing initiatives it is possible through *in silico* analyzes to identify molecules with potential immunomodulatory properties. These databases are available on WormBase Parasite, HelmDB, and Heminth.net (216–218).

The identification of genomic sequences of helminth parasites known to down-modulate the immune system of mammalian hosts such as *Ascaris suum*, *Necator americanus*, *Schistosoma mansoni*, *Strongyloides* spp. as mentioned in previous topics in this review, have motivated the development of recombinant helminth proteins with therapeutic potential for immune-mediated diseases such as protease inhibitors, cytokine homologs and lectins (219). High immunogenicity has been observed for these therapeutic recombinant proteins (220) which may be able to mimic the immunomodulation observed in helminth infections. However, standardized studies in humans as well as adequacy of doses and treatment duration are still necessary.

Epigenetic mechanisms play an important role in the regulation of immune response and are strongly influenced by microbial exposures and drug use, advancing the knowledge about such interactions may be used to both development of future target therapeutic strategies for asthma and allergies but also to discover new biological properties in current drugs in use.

The genetic susceptibility to allergic disorders is known be polygenic and recent studies have established that the presence of the gut microbiota is essential for normal gene expression (221, 222). The presence of certain bacterial species in the gut, such as *Helicobacter pylori* increases the CpG methylation in the promoter region of O6-methylguanine DNA methyltransferase, which ends up decreasing the expression of this DNA methyltransferase in gastric mucosa cells (222).

Lactobacilli and *Bifidobacteria* are the major source of butyrate and the absence of these species is important. By inhibiting HDACs, butyrate suppresses nuclear NF-κB activation, upregulates PPARγ expression, and decreases IFNγ production in the residing gut immune cells, promoting an anti-inflammatory gut environment (222). In a study with patients with allergic rhinitis, blocking the HDAC activity restored the integrity of the nasal epithelium and restored mucosal function and prevented the development of airway inflammation and hyperresponsiveness in experimental models (223).

Studies in dietary manipulation have demonstrated that diets high in methyl-donating nutrients are associated with hypermethylation of the epigenome, impacting the gene expression, especially during early development when the epigenome is first established, and can have long-term effects in adult life (224, 225). According to Bae et al. (225), in humans, methyl donors for DNA methylation are mostly derived from dietary methyl groups nutrients such as folate, vitamin B12, and choline. Methyl donors affect DNA methylation and immune responses such as Th17, Th1/Th2 balance, and Treg generation (225).

Additional studies are needed to better characterize the mechanisms underlying the different asthma phenotypes and their correlation with clinical characteristics, and those that contemplate the complex interaction of different epigenetic

mechanisms and those that focus on a single-cell type or investigations at the single cell level (221, 226). In this sense, EWAS can be useful to identify patterns of epigenetic signatures among asthma and allergy phenotypes and clinical characteristics, which reinforces the potential of epigenetic changes as future biomarkers for diagnosis and target personalized therapies.

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BF, HF, PM, CM, TS, and CF have contributed for the first draft. HF designed the figure. CF designed the work. All authors listed co-authored and proofread the manuscript and approved the final manuscript.

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Bovine Holo-Beta-Lactoglobulin Cross-Protects Against Pollen Allergies in an Innate Manner in BALB/c Mice: Potential Model for the Farm Effect

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The lipocalin beta-lactoglobulin (BLG) is a major protein compound in cow's milk, and we detected it in cattle stable dust. BLG may be a novel player in the farm protective effect against atopic sensitization and hayfever. In previous studies, we demonstrated that only the ligand-filled holo-form of BLG prevented sensitization to itself. Here, we investigated whether holo-BLG could, in an innate manner, also protect against allergic sensitization to unrelated birch pollen allergens using a murine model. BALB/c mice were nasally pretreated four times in biweekly intervals with holo-BLG containing quercetin–iron complexes as ligands, with empty apo-BLG, or were sham-treated. Subsequently, mice were intraperitoneally sensitized two times with apo-BLG or with the unrelated birch pollen allergen apo-Bet v 1, adjuvanted with aluminum hydroxide. After subsequent systemic challenge with BLG or Bet v 1, body temperature drop was monitored by anaphylaxis imaging. Specific antibodies in serum and cytokines of BLG- and Bet v 1-stimulated splenocytes were analyzed by ELISA. Additionally, human peripheral blood mononuclear cells of pollen allergic subjects were stimulated with apo- versus holo-BLG before assessment by FACS. Prophylactic treatment with the holo-BLG resulted in protection against allergic sensitization and clinical reactivity also to Bet v 1 in an unspecific manner. Pretreatment with holo-BLG resulted in significantly lower BLG-as well as Bet v 1-specific antibodies and impaired antigen-presentation with significantly lower numbers of CD11c+MHCII+ cells expressing CD86. Pretreatment with holo-BLG also reduced the release of Th2-associated cytokines from Splenocytes in BLG-sensitized mice. Similarly, *in vitro* stimulation of PBMCs from birch pollen allergic subjects with holo-BLG resulted in a relative decrease of CD3+CD4+ and CD4+CRTh2 cells, but not of CD4+CD25+CD127– Treg cells, compared to apo-BLG stimulation. In conclusion, prophylactic treatment with holo-BLG protected against allergy in an antigen-specific and -unspecific manner by decreasing antigen presentation, specific antibody production

and abrogating a Th2-response. Holo-BLG therefore promotes immune resilience against pollen allergens in an innate manner and may thereby contribute to the farm protective effect against atopic sensitization.

Keywords: allergy, beta-lactoglobulin, holo-BLG, ligands, tolerance, cross-protection, immune resilience, protective farm effect

INTRODUCTION

Cow's milk allergy (CMA) is associated with a low quality of life in children and their families, as milk and milk products are considered essential food in early lifetime. As milk avoidance can be difficult (1), different preventive strategies to reduce the allergenicity of major allergens in cow's milk have been conducted all over the world (2–9).

The phenomenon of CMA is in striking contrast to studies showing that consumption of unprocessed cow's milk is considered to represent an important factor associated with the protective effect of cattle farms against atopic sensitization, asthma, and hayfever (10–13).

Milk processing, especially pasteurization, can affect the physiologic structure of several milk proteins, thereby increasing their allergenic potential (12, 14, 15). Heating milk above 65°C structurally alters the thermolabile milk proteins, in particular the whey fraction, and causes aggregates (16). This leads to an increase in the antigenicity of the whey protein beta-lactoglobulin (BLG) (16) and the appearance of several new epitopes on BLG (17) during protein unfolding (18). Several other milk constituents may be affected by processing, too. For example, during the defatting process involving centrifugation and homogenization, the milk lipid fraction, *e.g.* ω-3 polyunsaturated fatty acids, which is considered to be a precursor of anti-inflammatory mediators (19), is diminished.

BLG belongs to the lipocalin protein family (20, 21), which is capable of carrying molecules such as retinoids (22), fatty acids, hormones, vitamins, and iron-chelating agents (20, 23, 24) in their large, calyx-like pocket (25). In our previous studies, we showed that the holo-BLG loaded with the flavonoid quercetin–iron complex is not allergenic (22). Holo-BLG rather created a tolerogenic environment through promotion of regulatory cells (23) by delivering ligands, thereby activating the anti-inflammatory aryl hydrocarbon receptor (AHR) pathway and down-tuning the antigen presentation skills of antigen presenting cells. BLG is not only present in milk, but is also secreted in the cattles' urine. Its presence can be detected in air samples and in dust samples in and around cattle stables (26) and (Pali-Scholl et al., manuscript in review).

Here, we went a step further, showing *in vivo* that the spiked holo-BLG is not an allergen, but protects against the onset of allergies in an antigen-specific as well as antigen-non-specific manner, similar to the observed allergy-protective farm effect.

MATERIALS AND METHODS

Preparation of Apo-BLG

Commercially available bovine beta-lactoglobulin (≥90% pure, Sigma Aldrich, Steinheim, Germany) was dialyzed four times against 10 μM deferoxamine mesylate (DFO) by using snakeskin dialysis tube (ThermoScientific, MWCO 3.5 K), followed by four times dialyzation against deionized water.

Generation of Holo-BLG

The holo-form of BLG was generated by incubating apo-BLG with flavonoid quercetin–iron complexes (FeQ2) in a molar ratio BLG:quercetin:iron of 1:2:1 as previously described (23).

Animals

5–7 weeks old female BALB/c mice were purchased from Charles River (Sulzfeld, Germany), maintained on milk-free chow and treated under conventional housing conditions according to the European Community rules of animal care. All experiments were approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Education, Science and Culture (BMWF-66.009/0133-WF/V/3b/2016).

Experimental Design: Intranasal Prophylaxis and Protection Against the Same Allergen (BLG)

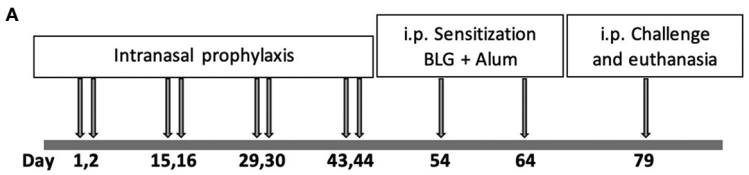
Sample sizes for the mouse experiments were based on the literature. No randomization was performed and protocols were designed as follows:

Prophylaxis: Mice (n = 11 per group) were intranasally (i.n.) pretreated with 10 μl per mouse (5 μl per nostril) containing apo-BLG (10 μg of apo-BLG (0.5 nM) plus 0.3 mg of deferoxamine (0.5 nM) to prevent loading of BLG during nasal application) or holo-BLG, corresponding to BLG loaded with the flavonoid quercetin–iron complex (10 μg BLG plus 338 ng quercetin and 28 ng iron) four times on two consecutive days at 14 days interval or sham-pretreated with distilled water (n = 10).

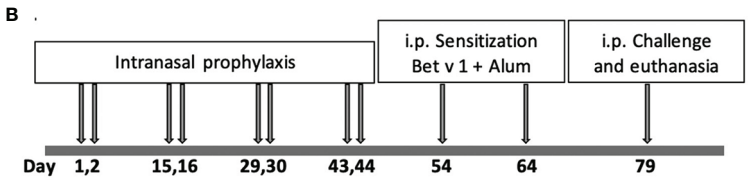
Sensitization: For systemic sensitization, BLG (5 μg/mouse) adjuvanted with 50 μl aluminum hydroxide (alum, Serva, Heidelberg, Germany), was intraperitoneally (i.p.) injected two times in a 10-day interval. Two weeks after the last sensitization, all mice were intraperitoneally (i.p.) challenged with apo-BLG (50 μg/50 μl 0.9% NaCl/mouse) to induce an acute allergic response before they were sacrificed by gradual introduction of CO_2. Pooled results from two independent experiments were compared. A schematic overview of the experimental design is depicted in **Figure 1A**.

Experimental Design: Intranasal Prophylaxis and Protection Against Allergen Bet v 1

The experimental design of the second experiment is comparable to the one described above (**Figure 1B**). Shortly, a new/another set of mice were i.n. pretreated four times on two consecutive



Prophylaxis (i.n.)	Sensitization (i.p.)	Challenge (i.p.)
Apo-BLG	BLG + Alum	BLG
Holo-BLG	BLG + Alum	BLG
Water	BLG + Alum	BLG



Prophylaxis (i.n.)	Sensitization (i.p.)	Challenge (i.p.)
Apo-BLG	Bet v 1 + Alum	Bet v 1
Holo-BLG	Bet v 1 + Alum	Bet v 1
Water	Bet v 1 + Alum	Bet v 1

FIGURE 1 | Schematic overview of the *in vivo* protocols. **(A)** Intranasal prophylactic treatment of mice to protect against sensitization to the same allergen (BLG). Mice were intranasally treated four times in biweekly intervals with either apo-BLG (n = 11), holo-BLG (n = 11), or water (as a control, n = 10). This was followed by two i.p.-sensitization steps with with BLG (5 μg/mouse) adjuvanted with 50 μl aluminum hydroxide within ten days. Thereafter, mice were i.p. challenged with apo-BLG (50 μg/mouse) and allergic response was monitored before euthanasia. **(B)** Intranasal prophylactic treatment of mice to protect against sensitization to an unrelated, non-milk allergen (Bet v 1). Mice were pre-treated as in protocol A, but were subsequently immunized twice i.p. with Bet v 1 (5 μg Bet v 1/mouse adjuvanted with 50 μl aluminum hydroxide). Thereafter, they were challenged with Bet v 1 (50 μg Bet v 1/mouse i.p.), and body temperature as well as physical activity was monitored before euthanasia.

days at 14 days interval with distilled water as sham-treatment, apo-BLG (20 μg) or holo-BLG (20 μg BLG plus 676 ng quercetin and 56 ng iron). Each group consisted of 11 mice except for the sham-treated group, consisting of 10 mice. Two weeks after the last treatment, mice were i.p. sensitized with Bet v 1 (5 μg/mouse) adjuvanted with 50 μl aluminum hydroxide two times at 14-day intervals. Two weeks later, all mice were i.p. challenged with Bet v 1 (50 μg/50 μl 0.9% NaCl/mouse) to assess the occurrence of allergen-induced acute anaphylactic reaction. Results of two independently performed experiments were combined.

In Vivo Evaluation of the Allergic Reaction: Anaphylaxis Read-Outs

Two weeks after the last sensitization, all mice were i.p. challenged with the allergen BLG or Bet v 1 (50 μg allergen/50 μl 0.9% NaCl). Over a period of 20 min, the anaphylactic shock-induced drop in body temperature and horizontal movement impairment were measured using a non-invasive heat imaging system (Biomedical Int. R+D, Vienna) (27). The severity of symptoms after the challenge was evaluated according to the scoring system previously described (16): 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, pilar erection, reduced activity and/or decreased activity with increased respiratory rate; 3 = wheezing, labored respiration and cyanosis around the mouth and the tail; 4 = No activity upon stimuli, tremor or convulsion; 5 = death. All assessments were performed in a blinded fashion.

Antigen-Specific Antibodies

BLG and Bet v 1 specific IgG1, IgA, IgG2a, and IgE levels were measured by ELISA. Microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated (100 μl/well) with either BLG (10 μg/ml) or Bet v 1 (5 μg/ml) in carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Serum was added diluted 1:100 for IgG1, IgA, IgG2a, and 1:15 for IgE (100 μl/well) after washing and blocking with 1% BSA in PBS (RT/1 h) and incubated overnight at 4°C. Also, serial dilutions of mouse IgG1 (Southern Biotech, clone 15H6), IgG2a (Southern Biotech, clone HOPC-1), IgE (BD Biosciences, Clone IgE-3), and IgA (Southern Biotech, S107) standards were used and were directly coated. Monoclonal rat anti-mouse antibodies (eBiosciences), IgG1 (clone A85-1), IgG2a (clone R19-15), IgG2b (clone R12-3), IgA (clone c10-1), or IgE

(clone R35-72) were applied, followed by incubation with polyclonal peroxidase-labeled goat anti-rat IgG antibodies (GE Healthcare). Tetramethylbenzidine (eBiosciences) was used as substrate and 1.8 M sulfuric acid was used as stop solution followed by optical density measurement at 450 nm.

In Vitro Stimulation

After sacrifice, the spleens were harvested. Cell suspensions of individual spleens were prepared immediately by grinding and filtering through 40 µm nylon meshes (BD Biosciences, Schwechat, Austria) under sterile conditions. After erythrocytes lysis and washing, cells were counted and plated (4×10^6 cells/well) in sterile round-bottom 48-well tissue culture plates (ThermoScientific) in RPMI medium. Splenocytes were stimulated with apo-BLG (5 and 25 µg/ml), Bet v 1 (25 µg/ml), and positive control concanavalin A (Con A) (2.5 µg/ml) or left unstimulated for 96 h at 37°C and 5% CO_2. The supernatants were harvested and stored at −20°C until further use for cytokine measurement.

Cytokines Detection

Cytokine concentrations in the undiluted supernatants of stimulated splenocytes were analyzed using an ELISA specific for murine IL-5, IL-10, IL-13, and IFN-γ (eBiosciences), according to the manufacturer's instructions.

Flow Cytometric Assessment of Co-Stimulatory Molecules on DCs

Single-cell suspensions of splenocytes (0.5 million cells) were incubated for 30 min under dim condition with anti-CD11c PE (eBioscience, clone N418), anti-MHC Class II I-Ad APC (clone AMS-32.1) and anti-CD86 FITC (clone GL1) in staining buffer (eBioscience). Afterwards, cells were washed two times with Hepes-buffer (20 mM Hepes, 150 mM NaCl, pH 7.2). Doublets were excluded, before gating on the living cells and using calcein-AM (Thermo-Fisher), as a living marker. Afterwards, cells were gated on CD11c+ in the living population, before gating on MHC Class II I-Ad+ CD86+ cells. Fluorescence Minus One (FMO) controls were used to identify gating boundaries. Acquisition and analysis were performed on a FACS Canto II flow cytometer (BD Bioscience, San Jose, CA, USA) using the FACSDiva Software 6.0.

Isolation and Stimulation of Human PBMCs From Pollen Allergic Donors

The study was approved by the institutional ethics committee of the Medical University of Vienna and conducted in accordance with the Helsinki Declaration of 1975. Fourteen birch and/or grass pollen allergic volunteers donated 15 ml blood. All subjects gave their full written informed consent.

Heparin-treated blood was mixed with equal volumes of 0.9% sodium chloride solution before applying to 10 ml Ficoll-Paque (GE Healthcare) and centrifuged at 400 g for 30 min without brake as already described. After density gradient separation, the lymphocyte fraction was isolated and washed twice with 0.9% sodium chloride solution before being diluted to a concentration of 1×10^6 cells/ml in DMEM medium containing neither phenol red nor fetal calf serum. Isolated PBMCs (0.5 Mio/ml) were incubated with apo-BLG (5 µM) and holo-BLG (5 µM BLG plus 10 µM quercetin and 5 µM iron) for 18 h.

Subsequently, cells were stained with combinations of Calcein Violet 450 AM (Thermo-Fisher) as a living marker, CD3-APC-Cy7 (Biolegend, clone SK7), CD4-PE-Cy7 (Biolegend, clone SK3), CD25-APC (biolegend, clone BC96), CD127-PE (Biolegend, clone A019D5) and CRTH2-FITC (Biolegend, clone BM16) and combinations of Calcein Violet 450 AM (Thermo-Fisher), CD14-APC (Biolegend, clone M5EZ), HLADR-PE (Biolegend, San Diego, Calif, clone L243PC), and CD86-PE-CY7 (Biolegend,clone IT2.2) for flow cytometric analysis. Doublets were excluded before gating the living lymphocytic population for CD3+ cells, and on the living monocytic population for CD14+ gating on the FSC/SSC plot. Samples were acquired by FACS Canto II machine (BD Bioscience, San Jose, CA, USA). Recorded events were analyzed with the FlowJo software version 10.3.

Supernatants of stimulated PBMCs were investigated for cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α; and IFN-γ by multiplex system in FACS (LEGENDplex™ Human Th1/Th2 Panel 8-plex, Biolegend).

Statistical Analyses

Mouse groups and cellular studies were compared by performing ANOVA following the Tukey multiple comparisons test. Anaphylactic shock symptom score was analyzed with Kruskal–Wallis non-parametric test with Dunn's multiple correction. To compare the effects of different treatments on primary cells, we applied repeated measures one-way ANOVA following the Tukey multiple comparisons test. All tests were two sided, and the results were considered significant when P was less than 0.05.

RESULTS

Intranasal Application of Holo-BLG Decreases Sensitization Levels in Mice

We first investigated whether the loading condition of BLG is decisive for protection against BLG sensitization. Therefore, mice were intranasally treated four times at biweekly intervals with the ligand-filled holo-BLG, with empty apo-BLG, or sham-treated with water, then i.p. sensitized and challenged with BLG (scheme of treatment in **Figure 1**). As depicted in **Figures 2A, B**, treating mice with holo-BLG prior to BLG-sensitization decreased the sensitization level and therefore protected against clinical reactivity upon BLG challenge and significantly prevented the anaphylactic temperature drop when compared to the group pretreated with apo-BLG. This was also reflected by the significantly lower mean symptom score of one in the holo-BLG group compared to apo-BLG exposed group with a mean score of three (**Figure 2C**). Also, horizontal movements of individual mice monitored upon the specific allergen challenge showed that the physical capacity was better—though not reaching statistical significance—in mice exposed to holo-BLG

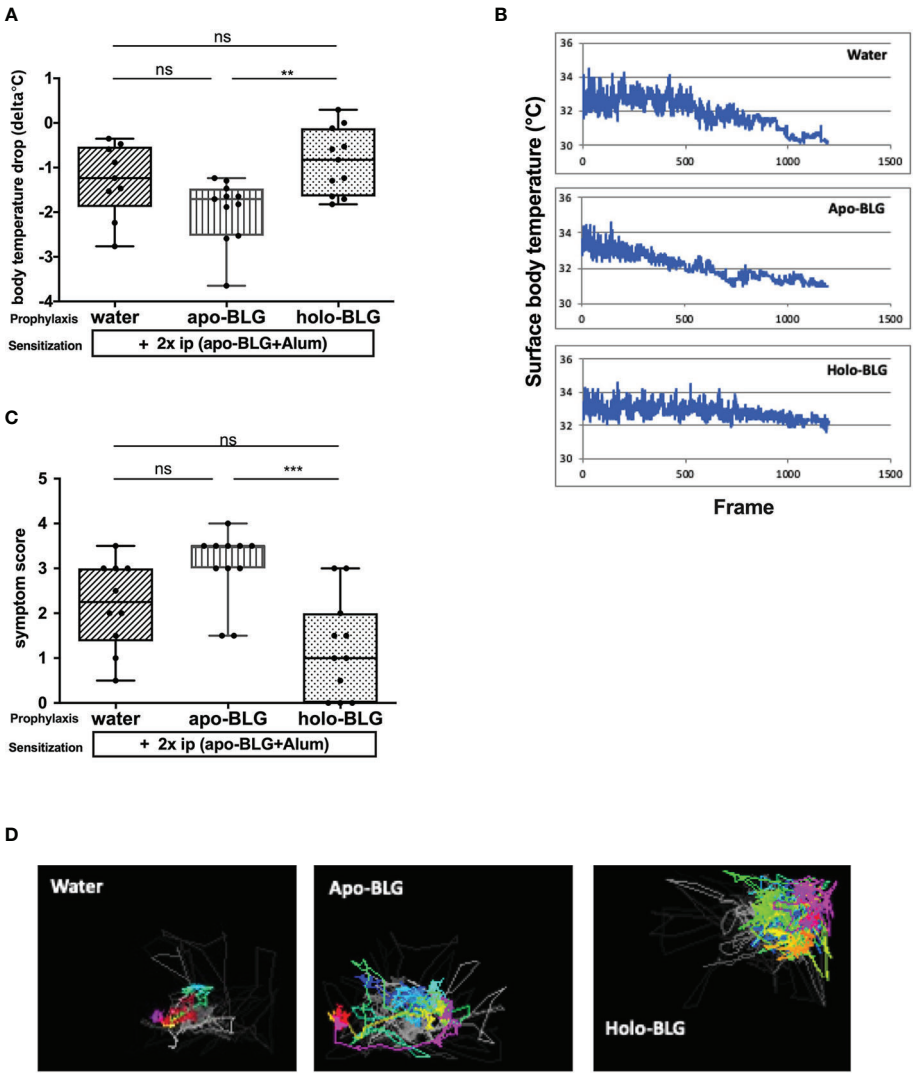


FIGURE 2 | Holo-BLG pre-treatment protected against acute allergic symptoms to BLG. Pretreated mice were sensitized to BLG and thereafter challenged with BLG i.p., and allergic response was monitored. **(A)** Body temperature drop 20 min after i.p. challenge (ANOVA followed by Tukey's multiple comparison test). **(B)** Representative examples of temperature drop during 20 min. observation period, x-axis represents number of frames (1 frame/s). **(C)** Anaphylactic shock symptom score (Kruskal–Wallis test). **(D)** Representative images of horizontal movements (lines) recorded by the imaging cage after systemic challenge with BLG in the differently treated groups. Pooled results from two independents experiments are shown. Panels in **(A, C)** show medians represented by a box whisker plot; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, non-significant.

compared to the group pretreated with apo-BLG in which the reduced horizontal physical activity reflected their anaphylactic reactions (**Figure 2D**).

Holo-BLG Protects Against Sensitization to Itself With Strong Suppression of Th2 Cytokines

We assessed which BLG-specific immunoglobulins were induced in the mice to understand the differences in the differently treated groups. Mice exposed to holo-BLG prior BLG-sensitization showed significantly lower levels of BLG-specific IgE, IgG1, IgA and IgG2a antibodies than the other groups despite the sensitization regimen applied (two i.p.-shots with BLG in combination with Alum as adjuvant) (**Figure 3A**).

In analogy to the humoral responses observed in the holo-BLG pretreated group, the BLG-stimulated splenocytes secreted significantly less IL-5, IL-13, but also IL-10 cytokines compared to apo-BLG (**Figure 3B**) due to targeted delivery of ligands to immune cells, synergizing in immune resilience (23). The Th2-associated cytokines, IL-5 and IL-13, were significantly lower in the holo-BLG groups, whereas IFN-γ levels were comparable between the apo- and holo-group. Hence, the Th2/Th1-ratio (IL-13/IFN-γ) was significantly higher in the apo-BLG group, emphasizing that the empty form of BLG evoked a strong Th2-response (**Figure 3B**).

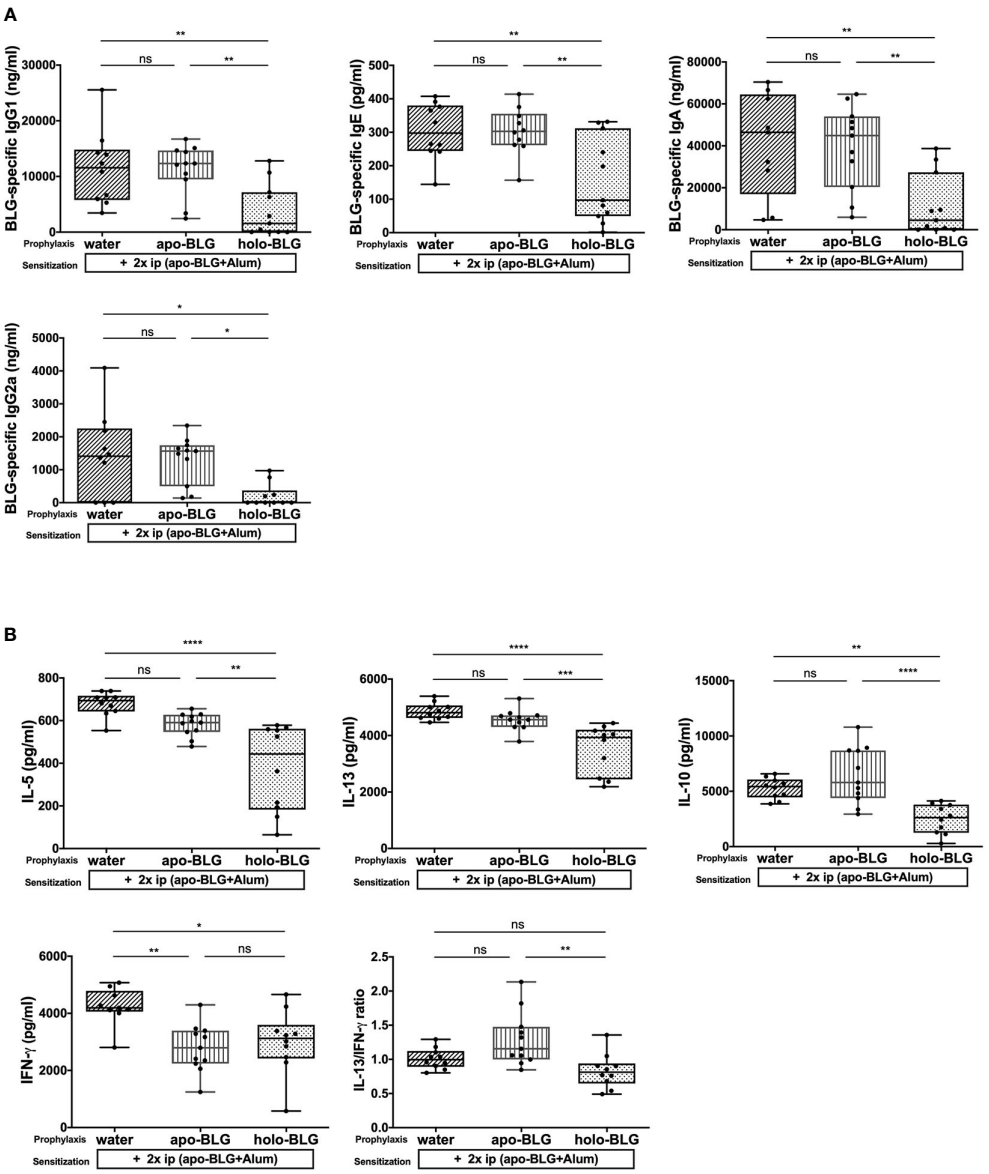


FIGURE 3 | Holo-BLG pretreatment reduced antibody- and cytokine response in BLG-sensitized mice. **(A)** BLG-specific antibody-serum levels of mice treated with apo-BLG, holo-BLG, or water prior to BLG-sensitization and challenge with apo-BLG. **(B)** Concentrations of IL-5, IL-13, IL-10, IFN-γ and the ratio of IL-13/IFN-γ in supernatants of splenocytes stimulated with BLG for 4 days (37°C, 5% CO_2). Pooled results from two independents experiments are shown. Groups were compared by ANOVA following Tukey's multiple comparisons test. The panels show medians represented by a box whisker plot; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, non-significant.

Impaired Antigen Presentation by Holo-BLG Pretreatment

Antigen-presenting cells are one of the first cells that encounter and process antigens and hence are critical for activating or suppressing the immune system. Consequently, we analyzed the co-stimulatory molecules on splenic dendritic cells (DCs) of each individual mouse. As depicted in **Figure 4**, the relative number of CD11+ dendritic cells expressing MHC Class II I-Ad+ and CD86+ was significantly reduced in mice pretreated with holo-BLG despite the strong subsequent sensitization scheme. Hence, holo-BLG pretreatment may impair the antigen presentation capacity of the dendritic cell population causing immune resilience (23) and may participate in tolerance induction, as antigen presentation in the absence of co-stimulatory molecules leads to anergy.

Holo-BLG Cross-Protects Against Anaphylactic Reaction to Bet v 1

As holo-BLG seemed to promote tolerogenic dendritic cells, we investigated whether this protective effect extends to the

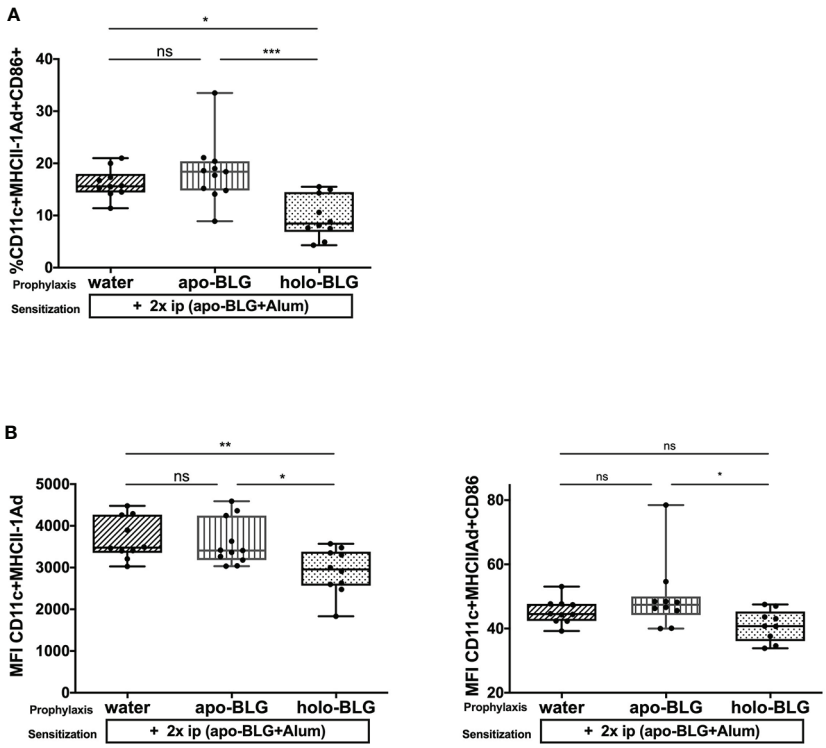


FIGURE 4 | Impaired antigen presentation by holo-BLG pre-treatment in mice. **(A)** Splenocytes of the differently treated groups were analyzed for relative number of CD11c+ DCs expressing MHCII+CD86. **(B)** The mean fluorescence intensity (MFI) was measured for the co-stimulatory markers MHCII+CD86+ on CD11c+ DCs. Pooled results from two independent experiments are shown. Groups were compared by ANOVA after testing for normal distribution, followed by Tukey's multiple comparisons test. The panels show medians and interquartile ranges represented by box whisker plots; *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant.

protection against other allergens. In a similar protocol as mentioned before, mice were either sham-treated with water, apo- or holo-BLG before sensitizing them twice, this time with the major birch pollen allergen Bet v 1 adjuvanted with Alum (**Figure 1B**). Indeed, as demonstrated in **Figures 5A, B**, initial mucosal exposure of mice to holo-BLG protected against clinical reactivity, preventing a body temperature drop (as a sign of anaphylaxis) compared to mice pretreated with apo-BLG or water alone before Bet v 1-sensitzation and challenge. This was also reflected in a significantly lower anaphylactic symptom score (**Supplementary Figure 1**) and protection from impaired physical activity (**Figure 5C**), compared to mice that had received apo-BLG or water prior to Bet v 1-sensitization.

Holo-BLG Prevents Cross-Sensitization to Unrelated Allergen Bet v 1

The prevention of allergic reactions upon pretreatment with holo-BLG before Bet v 1 sensitization was accompanied by reduced levels of Bet v 1-specific IgG1 and IgE, besides a trend towards lower Bet v 1-specific IgA, and IgG2a levels (**Figure 6A**). Hence, the reduced immune response to holo-BLG resulted in protection against allergic sensitization also to non-related antigens such as Bet v 1. However, we were not able to detect differences in the 96 h cytokine-secretion pattern in Bet v 1-stimulated splenocytes in the differently treated groups (**Figure 6B**). As in these late time points cytokines derive mostly from T-cells, the data suggest that prevention of allergy-development takes place rather during antigen presentation by impaired cross-presentation than on a T cellular level.

Holo-BLG Hinders Antigen Presentation and Down-Regulates the Number of CD3+CD4+ Th2 Cells

We investigated the impact of apo-BLG and holo-BLG on surface marker expression of PBMCs from pollen allergic individuals *in vitro* after 18 h incubation. As depicted in **Figure 7A**, holo-BLG reduced the relative numbers of CD14+ monocytes/macrophages. Consequently, the relative numbers of CD14+ cells, expressing the co-stimulatory molecules, HLADR+ and CD86+, were significantly decreased. Holo-BLG reduced CD14+ expression, which is in line with the already described impact on DCs in an *in vivo* murine model in previous studies (23) and link holo-BLG exposure to an overall reduced antigen presentation capacity.

We further analyzed in greater detail the impact of holo-BLG on the T-cell compartment. As previously published, holo-BLG reduced the relative numbers of T helper cells (CD3+CD4+, **Figure 7B**). Within this compartment, holo-BLG suppressed the

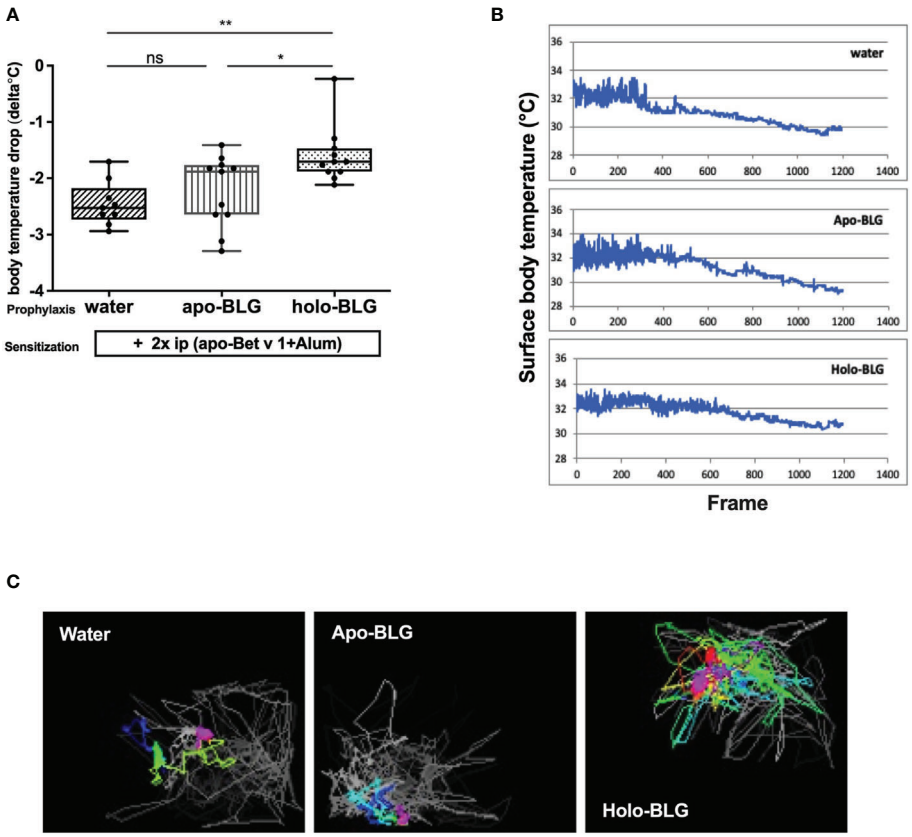


FIGURE 5 | Holo-BLG treatment protected against acute allergic symptoms in an antigen-unspecific manner. Pretreated mice were sensitized to Betv1, thereafter challenged with Bet v 1, and the allergic response was monitored. **(A)** Body temperature drop determined 20 min after i.p. challenge (ANOVA followed by Tukey's multiple comparison test). **(B)** Representative examples of temperature drop during the 20 min. observation period. **(C)** Representative images of horizontal movements (lines) recorded by the imaging cage after systemic challenge with Bet v 1 in the different treated groups, x-axis represents number of frames (1 frame/s). Combined results from two independents experimental rounds are shown. Medians with interquartile ranges are represented in box whisker plots; *P < 0.05; **P < 0.01; ns, non-significant.

apo-BLG induced upregulation of CRTH2 on Th2-cells (**Figure 7C**). In contrast, the relative numbers of regulatory T-cells (CD3+CD4+CD25+CD127−) remained stable and were comparable to PBMCs stimulated with apo-BLG (**Figure 7D**).

Upon assessment of cytokine levels in supernatants of PBMCs, stimulation with holo-BLG showed only a trend towards lower levels of IL-4 and IL-13 (**Supplementary Figure 2**), which might reflect that all donors were allergic, and the cytokine pattern of their PBMCs could not be influenced any further in the *ex vivo* treatment.

DISCUSSION

Exposure to cattle stables and barns, as a consequence of growing up on a farm, have been reported to protect against allergy in humans (28–30). In addition, several studies have shown that consumption of raw, unprocessed, cow's milk is one of the distinctive farm factors being inversely associated with allergy and asthma (10, 13, 19). The allergy-protective effect of raw cow's milk is related to the heat-sensitive native whey proteins (10, 15, 31), which are known to have immunomodulatory function (25, 31, 32). However, also other factors in milk, such as microbial components, fatty acids, TGF-beta, IL-10, IgG, microRNA and oligosaccharides have been discussed to contribute to the protective farm effect (33).

In our previous studies, we showed that proper loading of the lipocalin BLG, the major whey compound of milk, with iron–flavonoid complexes can modulate immune-reactivity and induce immune resilience. Its iron-chelating ligands were found to strongly activate the aryl hydrocarbon receptor (AHR) (23), which is expressed by different immune cells (34–36) and considered anti-inflammatory (37). Ligands such as retinoic acid (22, 24) or iron–flavonoid complexes (23) in the calyx of BLG can mask major T-cell linear epitopes, suggesting an increased stability towards endolysosomal enzymes such as cathepsin S and hampering antigen processing (22). The natural innate function of holo-BLG delivering complexed iron particularly to antigen-presenting cells provides an anti-inflammatory signal and further dampens antigen presentation (38, 39).

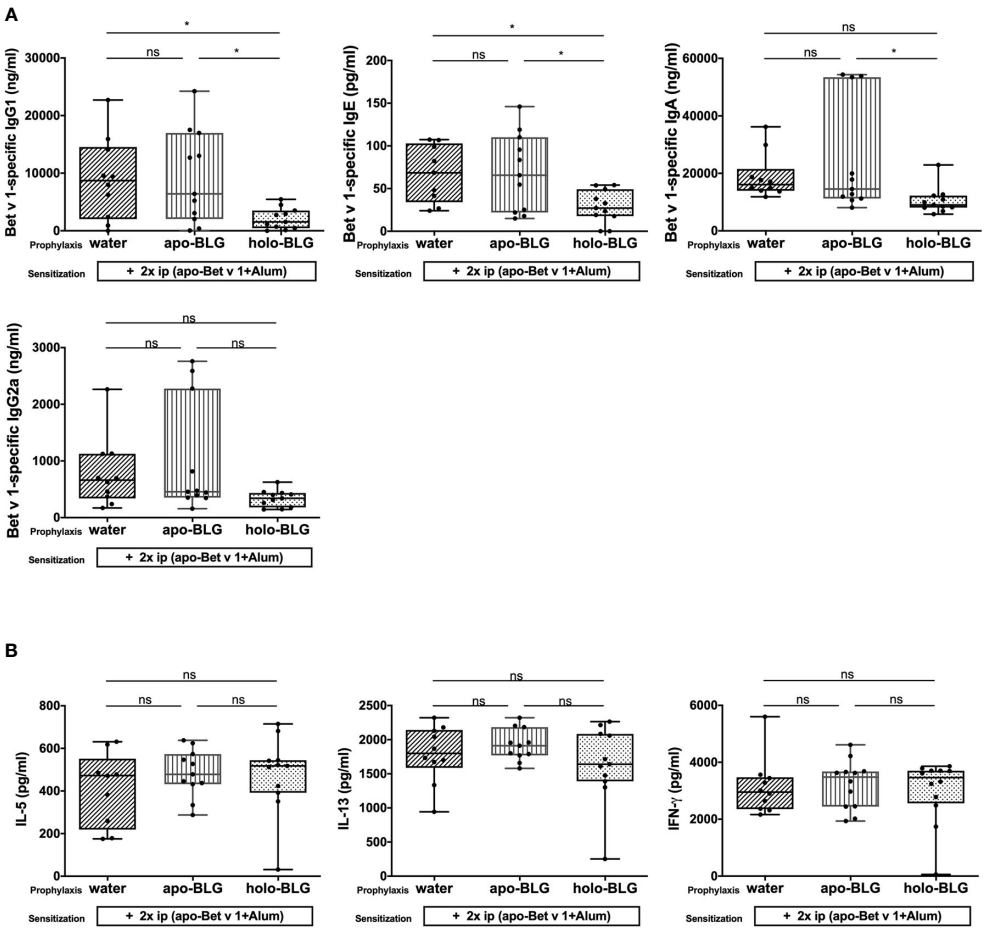


FIGURE 6 | Holo-BLG pretreatment reduced antibody production, but not cytokine response in mice sensitized to Bet v1. **(A)** Bet v 1-specific antibody-levels were measured in serum of mice sensitized and challenged with Bet v 1 after being prophylactically treated intranasally with apo-BLG, holo-BLG, or water. **(B)** IL-5, IL-13, and IFN-γ concentrations were measured in supernatant after *ex vivo* stimulation of splenocytes with Bet v 1 for 4 days (37°C, 5% CO_2). Pooled results from two independents experiments are shown. Groups were compared by ANOVA following Tukey's multiple comparisons test. The panels show medians represented by a box whisker plot; *P < 0.05; ns, non-significant.

Indeed, all tested components are present in milk. BLG content in milk ranges from 2 to 5 g/l (corresponding to 100 to 500 µM BLG) (40); the polyphenol content in milk depends on the forage composition and ranges from 3.7 to 35.8 g per liter milk. Quercetin concentration has been measured in milk to be up to 0.68 g/l (this would correspond to up to 2 mM quercetin) (41). Iron concentrations range from 57 µg to 1,500 µg/l (42) (corresponding to roughly 1 to 26 µM Fe). Polyphenolic compounds are highly available in feed plants and constitute part of regular cow diet (41) and polyphenols, *e.g.* quercetin in milk increases after feeding polyphenol-rich diets to lactating animals (43). There are numerous reports showing the iron-binding abilities of BLG (44–47) as the major component in whey (48), leading to improved iron absorption (49–52). On the other hand, milk processing such as pasteurization has been shown to cause aggregation of whey proteins (16), as well as a decrease in copper and iron content in milk. Therefore, there are numerous indirect evidences that at least BLG from milk is indeed loaded with various ligands and that processing can affect its ligand- and iron-binding properties.

These data explain why raw, unprocessed cow's milk is protective, despite the presence of BLG, which otherwise is best known as the major milk allergen Bos d 5. Our data propose that loading of BLG with ligands possessing anti-inflammatory properties, such as in unprocessed raw milk, maintains it tolerogenic. The likelihood of losing these ligands is particularly increased during industrial milk processing. Additionally, animal welfare may play a role as stressful situations, infections, and supply of forage may influence the ligand loading process.

In the present *in vivo* study, we demonstrate that prophylactic treatment with holo-BLG protects not only against the onset of allergy to this milk protein itself, but the protective impact of

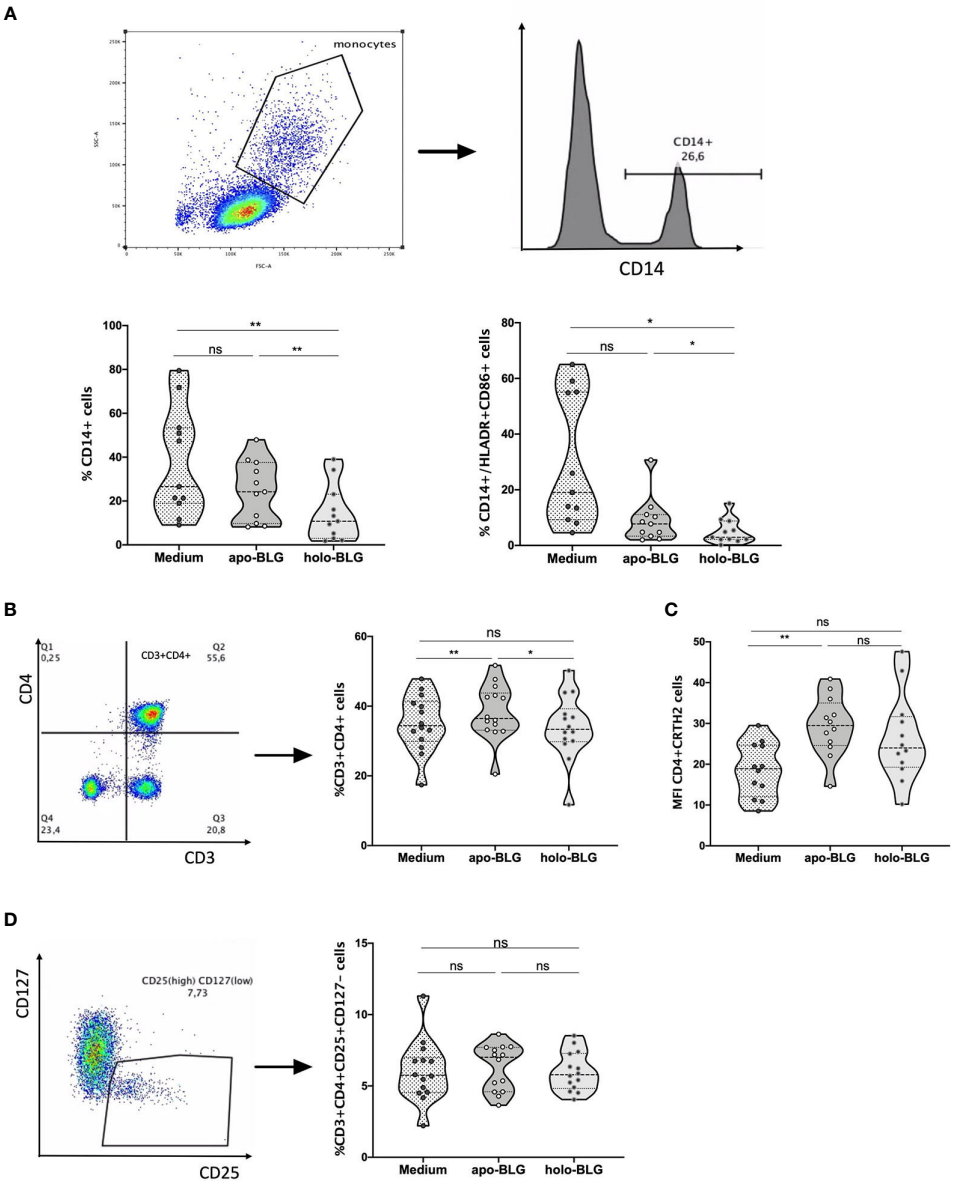


FIGURE 7 | Holo-BLG hinders antigen presentation and decreases the relative number of Th2 cells. PBMCs from 14 pollen allergic donors were stimulated with apo-BLG or holo-BLG and incubated overnight in iron-free media before flow cytometric analysis. **(A)** CD14 positive cells were gated and further analyzed for their HLADR+CD86+ expression. The percentage of different cell subsets was determined in PBMCs after antigen stimulation: **(B)** CD3+CD4+ cells, **(C)** CD4+CRTH2+ cells, **(D)** CD4+CD25+CD127-cells. Data from four independently performed experiments with a total of 14 subjects are shown. Groups were compared by repeated measures one-way ANOVA following the Tukey multiple comparisons test. *P <.05; **P <.01; ns, non-significant.

holo-BLG is extended to another independent allergen, Bet v 1, in an antigen-unspecific manner. In previous experiments, we could show that iron–quercetin complexes *per se* did not induce a temperature drop or had any impact on serum antibodies and splenocyte cytokine release. Therefore, FeQ2 was no longer included as control group here. Therefore, we cannot fully exclude that FeQ2 could have an allergy preventive effect in the present experimental setup. Holo-BLG prophylaxis targeted antigen presenting cells such as dendritic cells. Levels of the co-stimulatory molecules CD86 were suppressed on dendritic cells (DCs), blocking the co-stimulatory T-cell activation signals and inducing immune tolerance. Several studies showed that CD86 level is upregulated in patients with asthma and allergic diseases (53–55) and is closely associated with Th2 reactions and airway inflammation (56). Importantly, decreasing the expression of the co-stimulatory molecules CD80 and CD86 in DCs has been reported as a potential target for the treatment of allergic diseases (57, 58).

Also, specific antibody production was prevented by prophylactic exposure to holo-BLG *in vivo*. Binding of FeQ2 complex within the calyx of BLG may render BLG more stable towards cathepsin S degradation during antigen processing, resulting in a hampered T-cell-stimulation in an antigen-specific manner (23). Additionally, holo-BLG might have a direct impact on B-cells as antigen presenting cells itself may affect antibody production. Similar to our previous findings, in our mouse model, holo-BLG prevented allergy development, inhibited antigen-specific antibody generation and abrogated Th2 differentiation and Th2 cytokine release to the same allergen. Holo-BLG also prevented allergy development, inhibited antigen-specific antibody generation to the unrelated pollen allergen Bet v 1. This points towards non-antigen specific protection, presumably *via* tolerogenic aryl hydrocarbon receptor pathways and blocking of mast cell degranulation *via* iron transport into mast cells (23).

In line with the *in vivo* data, *in vitro* stimulation of human PBMCs of allergic patients with holo-, but not apo-BLG, lowered the relative numbers of CD14+ monocytes/macrophages, important contributors to the pathogenesis of allergic asthma (59, 60). Consequently, this population revealed less CD86 surface expression, demonstrating an immunosuppressive effect with impaired antigen presentation. Additionally, holo-BLG was able to reduce the relative numbers of CD3+CD4+ T-cells compared to cells stimulated with apo-BLG, and a significant increase of CRTH2 expression was inhibited on CD3+CD4+ T-cells. CRTH2 induces Th2 cells to release type 2 cytokines and is involved in recruiting and activating eosinophils and basophils, which further contribute to amplification of type 2 inflammation (61–64), whereas blocking of CRTH2 by antagonists suppresses allergic inflammation (65, 66). Furthermore, we show that spiked holo-BLG repressed CRTH2 expression and thus seems able to attenuate a Th2-associated response. Stimulation of these PBMCs with holo-BLG showed only a trend towards lower levels of IL-4 and IL-13, which might reflect that all donors were allergic, and the cytokine pattern of their PBMCs could not be influenced any further by *ex vivo* treatment.

Intriguingly, when the major milk protein BLG in its holo-form transports ligands to immune cells, this will result in innate immune resilience (23, 24). In line with this novel molecular concept, the results of our current *in vivo* study suggest that exposure to the loaded holo-BLG can in an antigen-nonspecific manner protect against allergic sensitization. This phenomenon might be due to targeted delivery of ligands from BLG to immune cells, supplementation of intracellular iron, and quercetin activating the AhR, altogether synergizing in non-antigen-specific immune tolerance.

The results of the present study propose that ligand-bound BLG contributes to the protective farm effect, as it is a major constituent of raw milk, and its abundant presence has been identified in the dust of cattle farms (26) and (Pali-Scholl et al., manuscript in review).

Further studies need to address i) how farming conditions, the cows' health status, as well as the forage composition, affect the richness of ligands available for BLG in milk, and ii) how industrial processing can be adapted to prevent changes in the ligand composition and protein integrity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional ethics committee of the Medical University of Vienna and conducted in accordance with the Helsinki Declaration of 1975. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Education, Science and Culture (BMWF-66.009/0133-WF/V/3b/2016).

AUTHOR CONTRIBUTIONS

SMA conducted all mouse and cell stimulation experiments, performed statistical analysis, provided support and wrote the manuscript. IP-S obtained the legal requirements for and helped in the mouse experiments, provided support and contributed to writing and editing of the manuscript. KH contributed in the mouse experiments and to writing. GH provided support and contributed to the writing. ME-B provided support and contributed to writing. FR-W contributed in the mouse experiments, conceived, and directed the research, interpreted the data and contributed in writing. EJ-J financed and directed research, designed the experiments, and contributed in manuscript writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.611474/full#supplementary-material

Supplementary Figure 1 | Holo-BLG treatment protected against acute allergic symptoms in an antigen-unspecific manner. Pretreated mice were sensitized to Betv1, thereafter challenged with Bet v 1 and anaphylactic shock symptom score (Kruskal–Wallis test) was monitored in a blinded fashion. Representative data from two independent experiments are shown. Medians with interquartile ranges are represented in box whisker plots; *$P < 0.05$; ns = non-significant.

Supplementary Figure 2 | PBMCs from 14 pollen allergic donors were stimulated with apo-BLG or holo-BLG and incubated overnight in iron-free media. Supernatants of PBMCs were assessed for **(A)** IL-2, **(B)** IL-6, **(C)** IL-4, **(D)** IL-13, **(E)** IL-10 and **(F)** IFN-γ by multiplexing in FACS. Data from four independently performed experiments with a total of 14 subjects are shown. Groups were compared by repeated measures one-way ANOVA following the Tukey multiple comparisons test. *P <.05; **P <.01; ****P < 0.0001; ns = non-significant.

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EJ-J and FR-W are inventors of EP2894478, LCN2 as a tool for allergy diagnostic and therapy. EP 14150965.3, Year: 01/2014; US 14/204,570, owned by Biomedical International R+D GmbH, Vienna, Austria.

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Reduction of Allergic Lung Disease by Mucosal Application of *Toxoplasma gondii*-Derived Molecules: Possible Role of Carbohydrates

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Background: The hygiene hypothesis suggests a link between parasitic infections and immune disorders, such as allergic diseases. We previously showed that infection with *Toxoplasma gondii* or systemic application of *T. gondii* tachyzoites lysate antigen (TLA) in a prophylactic, but not therapeutic protocol, prevented allergic airway inflammation in mice. Here we tested the effect of prophylactic and therapeutic application of TLA *via* the mucosal route.

Methods: Mice were intranasally treated with TLA either i) prior to sensitization, ii) during sensitization and challenge, or iii) after sensitization with ovalbumin (OVA). Recruitment of inflammatory cells to the lung, cytokine levels in restimulated lung and spleen cell cultures as well as levels of OVA-specific antibodies in serum were measured. In parallel, the effect of native TLA, heat-inactivated (hiTLA) or deglycosylated TLA (dgTLA) on sensitized splenocytes was evaluated *ex vivo*.

Results: When applied together with OVA i) during systemic sensitization and local challenge or ii) exclusively during local challenge, TLA reduced infiltration of eosinophils into the lung, OVA-specific type 2 cytokines in restimulated lung cell cultures, and partially, type 2 cytokines in restimulated spleen cell cultures in comparison to allergic controls. No beneficial effect was observed when TLA was applied prior to the start of sensitization. Analysis of epitope sugars on TLA indicated a high abundance of mannose, fucose, N-acetylglucosamine, and N-acetylgalactosamine. Deglycosylation of TLA, but not heat-inactivation, abolished the potential of TLA to reduce type 2 responses *ex vivo*, suggesting a significant role of carbohydrates in immunomodulation.

Conclusion: We showed that mucosal application of TLA reduced the development of experimental allergy in mice. The beneficial effects depended on the timing of the application in relation to the time point of sensitization. Not only co-application, but also therapy in sensitized/allergic animals with native TLA reduced local allergic responses. Furthermore, we show that TLA is highly glycosylated and glycoconjugates seem to play a

role in anti-allergic effects. In summary, given the powerful modulatory effect that TLA exhibits, understanding its exact mechanisms of action may lead to the development of novel immunomodulators in clinical application.

Keywords: *Toxoplasma gondii*, tachyzoites lysate antigen, allergic airway inflammation, immunomodulation, deglycosylation, hygiene hypothesis, parasites, carbohydrates

INTRODUCTION

Allergic diseases of the airways affect millions of people and the prevalence has been increasing continuously. Allergic asthma is a heterogeneous and complex inflammatory disease of the airways that develops upon allergic sensitization with allergens, such as house dust mites, cockroaches, animal dander, grass and tree pollens, and fungal spores (1). These inhaled allergens stimulate the production of type 2 cytokines, such as IL-4, IL-5, and IL-13, leading to airway eosinophilia, airway hyperresponsiveness, mucus hypersecretion, and elevated IgE in serum (1, 2).

The lack of preventative, curative, and disease modifying strategies for asthma establishes substantial unmet medical need. Although symptoms can be treated e.g. with inhaled glucocorticosteroids, bronchodilators, or monoclonal anti-IgE antibodies (3–5), specific immunotherapy (SIT) is the only available curative treatment of allergy, inducing desensitization and long-term allergen-specific immunological tolerance (6). In patients with mild to moderate asthma, SIT can reduce allergic symptoms, such as dyspnea, cough, wheeze, chest tightness, and medication requirements, but studies showing the effects on overall lung function have been inconclusive (6–9). A noteworthy drawback of current SIT is the lack of high-quality allergen extracts with well-defined composition (10). Another clear disadvantage is that SIT may lead to novel sensitization to antigens in the formulation, or even to anaphylactic reactions (11).

Toxoplasma gondii is a ubiquitous, obligate intracellular protozoan parasite which replicates sexually in felids (12, 13). Shed oocysts can be transmitted *via* the fecal-oral route, while tissue cysts containing bradyzoites can be ingested upon the consumption of undercooked meat, infecting warm-blooded animals including humans (13, 14). Upon ingestion, oocysts release sporozoites to the lumen of the gut, which in turn differentiate into tachyzoites (15). Infection with *T. gondii* remains mostly asymptomatic in healthy individuals. However, severe disease has been reported in immunocompromised patients and in congenitally infected newborns (13, 14).

Since the postulation of the hygiene hypothesis in the late 1980s (16), multiple studies suggested a link between the prevalence of allergic diseases and increased hygiene standards, alongside an early use of antibiotics, reduced bacterial exposure, and low incidences of parasitic infections in industrialized countries (17–19). Epidemiological studies reported that humans that were infected with *T. gondii* exhibited reduced prevalence of allergic diseases (20). Indeed, we and others confirmed these epidemiological findings by showing that infections with *T. gondii* reduced allergic airway inflammation in mice (21, 22). Of note, the prevention of allergy was achieved also by non-infectious molecules derived from *T. gondii*, such as oocyst lysate antigen (OLA), when applied intraperitoneally and in the presence of Freund's complete adjuvant GERBU (23).

Harvesting *T. gondii* oocysts for the preparation of OLA requires passaging through feline hosts, such as cats. The yields are relatively low in proportion to the efforts and collected material may be contaminated with bacterial or host-derived components (24). On the other hand, culturing *T. gondii* tachyzoites *in vitro* in Vero cells is ethically, and also economically, advantageous compared to harvesting of oocysts. Furthermore, *in vitro* culturing enables high yields of tachyzoites while posing a significantly reduced risk of contamination (25, 26). Recently, we have shown that not only OLA, but also adjuvanted tachyzoite lysate antigen (TLA), applied *via* the parenteral route, reduced experimental allergic inflammation in mice (24).

The mucosal application of immunomodulatory substances or drugs, e.g. *via* the intranasal route, offers several benefits over parenteral application, such as a facilitated, needle-free administration, thereby reducing the need for trained medical personnel, as well as a lung-targeted delivery (27, 28). Along these lines, we previously demonstrated the high potential of the intranasal route to reduce allergic responses in the lung by treating mice with probiotic bacterial strains (29–31).

To date, there is only limited understanding which components of TLA are essential players in allergy-prevention. TLA consists mostly of proteins, carbohydrates (32, 33), and membrane-associated lipids (34, 35). We previously showed that compared to native and heat-inactivated TLA, sodium metaperiodate-treated deglycosylated TLA exhibited reduced potential to induce cytokines *in vitro* in naïve splenocytes (24).

Here we tested the effect of intranasally applied TLA on experimental allergic airway inflammation. Furthermore, we investigated whether mucosal immunological tolerance could be established in sensitized mice in a therapeutic treatment protocol. Finally, we characterized the glycosylation pattern of TLA and evaluated if carbohydrates play an important role in TLA-induced immunomodulation.

MATERIAL AND METHODS

Preparation of TLA

T. gondii tachyzoites (strain S-48) were cultivated in Vero cells, harvested and TLA extracts were prepared as described previously (24). Briefly, TLA was prepared by three freeze-thaw cycles in liquid nitrogen, followed by sonication and

subsequent centrifugation at 10,000 x *g* (60 min at 4°C). The supernatant was sterile filtered (0.22 µm Millex®GV Filter Unit; Merck Milipore) and its protein concentration was assessed with a BCA Protein Assay Reagent kit (Pierce Peribo). Endotoxin levels were determined by Limulus Amoebocyte Lysate (Endpoint Chromogenic LAL Assay; Lonza LTD) and were below 0.1 EU in 1 µg of extract. TLA was stored at −80°C until further use.

Heat-Inactivation and Sodium Metaperiodate Treatment

TLA was heat-inactivated by incubation for 15 min at 95°C (hiTLA) and deglycosylation (dgTLA) was performed by sodium metaperiodate treatment according to a modified protocol (36). Briefly, 3 mg TLA (1 mg/ml in PBS) were mixed with 3 ml 100 mM sodium metaperiodate in 100 mM acetate buffer, at pH = 4.5. TLA was incubated at 37°C for 30 min in the dark. The oxidation reaction was stopped with 300 ml 0.5 x PBS. The volume was then reduced to 5 ml by centrifugation at 2,900 x *g* at 4°C using Ultracel®-3K centrifugal filters (Amicon® Ultra-15; Merck Millipore). Volume was further decreased with a Vacuum Concentrator Centrifuge (Univapo 150H; Uniequip). The protein concentration was assessed as above and modification of glycan moieties was verified with a Western blot using biotinylated Concanavalin A (ConA) as described below.

SDS-Page and Western Blot

10 µg TLA and dgTLA were separated with SDS-PAGE according to the manufacturer's protocol using NuPAGE™ 4–12% Bis-Tris Protein Gels (10-well and 12-well; Invitrogen) and PagerRuler™ Prestained Protein Ladder (10–180 kDa; Thermo Scientific). Protein bands were transferred to nitrocellulose membranes (Whatman Protram Nitrocellulose Transfer Membrane; Whatman) and a Western blot was performed using biotinylated *Anguilla anguilla* agglutinin (AAA) (1 mg/ml, 1:5,000 dilution), biotinylated *Lycopersicon esculentum* lectin (LEL) (1 mg/ml, 1:5,000 dilution), biotinylated *Wisteria floribunda* agglutinin (WFA) (0.5 mg/ml, 1:2,500 dilution), biotinylated ConA (1 mg/ml, 1:5,000 dilution), or biotinylated *Ulex europaeus* agglutinin I (UEA-I) (1 mg/ml, 1:5,000 dilution). The membranes were subsequently incubated with monoclonal anti-biotin–alkaline phosphatase antibody produced in mouse (1:10,000 dilution; Sigma) and stained with SIGMAFAST™ BCIP®/NBT staining tablets (Sigma).

Enzyme Linked Lectin Assay

Abundance of epitope sugars was determined by a modified enzyme-linked lectin assay (ELLA) protocol as described elsewhere (37). Briefly, plates were coated with 5 µg TLA in 200 µl PBS overnight at 4°C, blocked for 2 h at room temperature using 3% BSA in TBS-buffer (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH = 7.6), and subsequently incubated for 1 h with 1 µg biotinylated lectins in 100 µl TBS and 0.05% Tween-20 (TBS-T). The following lectins were used: *Griffonia simplicifolia* (GS II), Wheat germ agglutinin (WGA), WFA, *Maackia amurensis* agglutinin (MAA), *Galanthus nivalis* agglutinin (GNA), *Dolichos biflorus* agglutinin (DBA), *Bauhinia purpurea* agglutinin (BPA), LEL, *Ricinus communis* agglutinin II (RCA II), UEA-I, *G. simplifolica* 1B4 (GS1B4), phytohemagglutinin (PHA-L), AAA, *Helix pomatia* agglutinin (HPA), Peanut agglutinin (PNA), ConA and *Sambucus nigra* agglutinin (SNA). Next, plates were incubated with horseradish peroxidase avidin (HRP-avidin) (1:250, eBioscience™ Avidin HRP; Invitrogen™; ThermoFisher Scientific) in 50 µl TBS-T and epitope sugars were detected with brief incubation with 3,3′,5,5′-tetramethylbenzidine (TMB). The colorimetric reaction was stopped with 0.18 M H_2SO_4 and absorption was measured at 450 nm with a SparkControl Magellan plate reader (Tecan).

Mice

Female, 6 to 8 weeks old wild-type BALB/c mice were purchased from Charles River (Sulzfeld, Germany). Mice were kept under conventional housing conditions. Experiments were approved by the Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Research (BMWFW-66.009/0358-WF/V/3b/2015 and BMBWF-66.009/0277-V/3b/2019).

Experimental Design

On days 0 and 14 mice were intraperitoneally immunized with 10 µg ovalbumin (OVA; grade V; Sigma-Aldrich) in PBS and 67% (v/v) alum (Alu-Gel-S Suspension; Serva Electrophoresis) in a total volume of 150 µl or with PBS and alum alone. Mice were challenged intranasally with 100 µg OVA in PBS in a total volume of 30 µl or 30 µl PBS alone on days 21 to 23. For the prophylactic approach, mice were treated intranasally with 30 µg TLA in 30 µl PBS or PBS alone on days -7 to -5. For the co-application strategy, mice were treated intranasally with 30 µg TLA in 30 µl PBS or 30 µl PBS alone 30 min prior sensitization and challenge on days 0, 14, 21, 22, and 23. For the therapeutic setup, mice were administered intranasally 30 µg TLA or hiTLA in 30 µl PBS or PBS alone on days 18 to 20 and 30 min prior challenge on days 21 to 23. Before each challenge and TLA treatment, mice were anesthetized with 5% (v/v) isoflurane (Isocare; Inhalation vapor, Animalcare Ltd.) at an airflow rate of 3 L/min in a UniVet Porta anesthesia machine (Groppler Medizintechnik). In the co-application model, 24 h after the last challenge, airway hyperresponsiveness (AHR) was assessed with whole-body plethysmography (Buxco Electronics Inc., DSI). Mice were conscious and unrestrained while they were exposed to 0, 12.5, and 50 mg/mL of aerosolized methacholine (acetyl-β-methyl-choline chloride; Sigma-Aldrich) in PBS. AHR was expressed by the dimensionless parameter enhanced pause (PenH) as previously described (38).

Differential Cell Counts in Bronchoalveolar Lavage Fluid (BALF)

Lungs were lavaged with 1 ml ice-cold PBS. BALF was centrifuged (300 × *g* for 5 min at 4°C). Pelleted cells were resuspended in PBS and 4 x 10^4 cells were spun onto microscope slides (800 x *g* for 3 min; Shandon Cytospin, Shandon Southern Instruments), air-dried and stained with hematoxylin and eosin (H&E; Hemacolor®, Merck). At least 130 cells (macrophages, eosinophils, lymphocytes, and

neutrophils) per slide were counted under a light microscope (1000 x magnification; Nikon Eclipse, Nikon).

Lung Cells Isolation and Stimulation *Ex Vivo*

Lungs of terminally anesthetized mice were excised and processed as described elsewhere (39). Briefly, lungs were minced and digested in 6 ml RPMI-1640 media (Gibco®, Thermo Fisher Scientific) containing 0.05 mg/ml Liberase TL (Roche) and 0.5 mg/ml DNAse (Sigma-Aldrich) for 45 min at 37°C in 5% CO_2 atmosphere. Next, the digested tissue was forced through a 70 µm cell strainer and erythrocytes were lysed in 3 ml ACK Lysing Buffer (BioWhittaker®, Lonza) for 90 s. Lung cells were resuspended (5 x 10^6 cells/ml) in complete RPMI (RPMI-1640 containing 10% FCS, 2 mM mercaptoethanol, 2 mM L-glutamine and 100 µg/ml gentamycin; Sigma-Aldrich). One hundred microliter cell suspensions were plated into 96-well plates and incubated either with complete RPMI or with 100 µg/ml endotoxin-free OVA (Endo-Grade; Hyglos) in complete RPMI for 72 h at 37°C in 5% CO_2 atmosphere. After incubation, supernatants were collected and analyzed for the production of cytokines (IL-4, IL-5, IL-13, and IFN-γ) with ELISA kits following the manufacturer's instructions (Ready-SET-Go!™ Kit, eBioScience™, Thermo Fisher Scientific).

Spleen Cells Isolation and Stimulation *Ex Vivo*

Spleens of terminally anesthetized mice were processed to single-cell suspensions. Briefly, spleens in 10 ml RPMI-1640 media were pressed through a metal net and the disrupted tissue was subsequently forced through a 70 µm cell strainer. Erythrocytes were lysed in 3 ml ACK Lysing Buffer for 60 s. Similar to lung cells, spleen cells were resuspended (5 x 10^6 cells/ml) in complete RPMI and restimulated and assessed for cytokine production following the same protocol that was used for lung cells, except for *ex vivo* restimulations analyzing the immunomodulatory properties of differently treated TLA. Here, 100 µl spleen cell suspensions of allergic mice were incubated with 50 µl complete RPMI or 0.75 µg native, heat-inactivated or deglycosylated TLA in 50 µl complete RPMI for 1 h at 37°C in 5 % CO_2 atmosphere. Next, cells were incubated on top with either 50 µl complete RPMI or with 20 µg endotoxin-free OVA (Endo-Grade; Hyglos) in 50 µl complete RPMI for 72 h at 37°C in 5 % CO_2 atmosphere. After incubation, supernatants were collected and analyzed for the production of cytokines (IL-4, IL-5, IL-10, and IFN-γ as above).

Lung Histology

Lungs were infiltrated with 7.5% (v/v) formaldehyde for histology. Formalin-fixed lungs were dehydrated with a series of ethanol solutions, followed by xylene, and subsequently embedded in paraffin. Sections (3 µm) were stained either with H&E or with Periodic acid-Schiff (PAS; Sigma-Aldrich). The histological pathology score was evaluated according to Zaiss et al. (40) with modifications. Stained sections were scored according to following criteria regarding i) perivascular and peribronchiolar inflammation (H&E) (0 = no inflammation; 1 = single scattered leukocytes; 2 = aggregates less than 10 cells thick; 3 = aggregates more than 10 cells thick; 4 = numerous coalescing aggregates more than 10 cells thick) and ii) number of leukocytes in alveolar spaces (H&E) {0 = not present; 1 = rare [2 to 4 cells in 400 × HPF (high power field)]; 2 = moderate (5 to 10 cells); 3 = high (more than 10 cells)}. The numbers of PAS-positive mucus producing goblet cells in the bronchial epithelium were counted and expressed per one millimeter of basement membrane according to Skevaki et al. (41).

Collection of Blood Serum

At the beginning of the experiment (day -7 or day 0) as well as on days 13 and 25, approximately 100 µl blood were collected by puncturing the facial vein. Serum was obtained by centrifuging the blood in microtainer® SST™ tubes (BD) at 15,000 x *g* for 5 min. Serum was stored at −20°C until analysis.

Detection of OVA-Specific Antibodies

Microtiter plates were coated with OVA (5 µg/ml; grade V) and blocked with 1 % (w/v) BSA and 0.05% (v/v) Tween in PBS for 6 h and subsequently incubated with BALF or with serum samples at 4°C overnight. BALF supernatant was tested neat and sera were diluted 1:2,000 for IgG1, 1:500 for IgG2a and 1:20 for IgE. The next day, plates were washed and incubated with rat-anti mouse IgG1, IgG2a or IgE (1:500, BD Pharmingen™) at 4°C overnight. On the next day, plates were washed and incubated with horseradish peroxidase-conjugated mouse anti-rat IgG (1:2,000; Jackson ImmunoResearch Laboratories Inc.) for 1 h at 37°C, followed by incubation for 1 h at 4°C. Plates were washed again and 1 mM ABTS (Sigma-Aldrich) in 70 mM citrate-phosphate buffer (pH = 4.2; Sigma-Aldrich) was added for colorimetric measurement. Absorption was measured at 405 nm with a SparkControl Magellan plate reader.

Rat Basophil Leukemia (RBL) Cell-Based Assay

RBL cell mediator release assay was performed as described elsewhere (41). Briefly, RBL 2H-3 cells were plated into 96-well plates (4 x 10^4 cells/well) and incubated with serum samples (1:300) from the beginning and the end of the experiment for 2 h at 37°C in a 5 % CO_2 atmosphere. Cells were washed with Tyrode's buffer [137 mM NaCl, 5.6 mM D glucose, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 10 mM HEPES and 0.1 % (w/v) BSA; pH = 7.4; Sigma-Aldrich] and degranulation of cells was induced by incubation with 0.3 µg/ml OVA in Tyrode's buffer. Supernatants were analyzed for β-hexosaminidase content by incubation with 80 µM 4-methylumbelliferyl-N-acetyl-β-d-glucosaminide (Sigma-Aldrich) and measuring fluorescence at λex: 360 nm/λem: 465 nm with a SparkControl Magellan plate reader. Results show percentage of total β-hexosaminidase release after adding 1 % (v/v) Triton X-100 (Sigma-Aldrich) in ddH_2O.

Statistics

The comparison of cytokine levels and histopathology scoring of all treatment groups was performed with two-way analysis of variance, followed by Bonferroni's multiple comparison test for the prophylactic model or followed by Tukey's multiple

comparison test for the co-application and therapy models. Significance between all treatment groups in BALF as well as multiple time points in RBL assays and serum antibody ELISA was assessed with two-way analysis of variance, followed by Bonferroni's multiple comparison test. The comparison of all treatment groups of *ex vivo* TLA stimulation and the comparison of OVA-specific IgG2a in BALF was performed with one-way analysis of variance, followed by Tukey's multiple comparison test. Statistical comparisons were performed using GraphPad Prism Software 7 (GraphPad Software Inc.). All data are shown as mean ± SEM. Significant differences were considered at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

RESULTS

Prophylactic Intranasal TLA Treatment of Mice Before OVA Sensitization and Challenge Does Not Reduce Allergic Airway Inflammation

We tested the ability of TLA to reduce the development of experimental allergy when applied prophylactically *via* the mucosal route. Mice were sensitized by two i.p. injections of OVA in alum followed by three intranasal challenges of OVA one week later on days 21 to 23 (**Figure 1A**). In the OVA-sensitized and challenged control mice (PBS/OVA), high levels of macrophages and eosinophils and low levels of neutrophils and lymphocytes were detected in BALF (**Figure 1B, Supplementary Figure S1A**). Additionally, high levels of OVA-specific IgG2a in BALF (**Figure 1C**) and high numbers of inflammatory cells and PAS-positive goblet cells were detected in the lung of these mice (**Figures 1D, E**). In parallel, restimulation of lung and spleen cell cultures with OVA led to production of high levels of type 2 cytokines IL-4, IL-5, and IL-13, and of IL-10 in comparison to cultures which were stimulated with media only (**Figures 1F, G**). In lung cells, the levels of IFN-γ were similar upon restimulation with media or OVA (**Figure 1F**), while in spleens the levels of IFN-γ were increased upon restimulation with OVA compared to restimulation with media in the PBS/OVA group (**Figure 1G**). The level of sensitization in PBS/OVA mice was documented by increased OVA-specific IgG2a (**Figure 1H**) and OVA-specific β-hexosaminidase release after incubation of RBL cells with serum collected on day 25 compared with serum from day -7 (**Figure 1I**). Intranasal application of TLA on days -7 to -5 (**Figure 1A**) prior to allergic sensitization with OVA had no significant effect on the total counts of macrophages, eosinophils, neutrophils, and lymphocytes in the lung (**Figure 1B, Supplementary Figure S1A**). Similarly, the levels of OVA-specific IgG2a in BALF (**Figure 1C**), the histopathology score in the lung (**Figures 1D, E**), the production of OVA-induced cytokines in restimulated lung (**Figure 1F**) or spleen cell cultures (**Figure 1G**) were unaffected by TLA treatment. In serum, no significant changes of OVA-specific IgG2a levels (**Figure 1H**) were detected. Similarly, no effect of TLA-treatment on OVA-specific IgE-mediated β-hexosaminidase release by RBL cells was observed (**Figure 1I**). Of note, reduced numbers of PAS-positive goblet cells were detected in the lung in TLA-treated mice compared to allergic controls (**Figure 1D**).

Intranasal Application of TLA Concurrent With OVA Sensitization and Challenge Reduces Allergic Airway Inflammation

Mice were treated intranasally with TLA 30 min before each systemic application of OVA in alum on days 0 and 14 and before each intranasal challenge with OVA on days 21 to 23 (**Figure 2A**). Allergic airway hyperresponsiveness (**Figure 2B**) and eosinophilia (**Figure 2C**, **Supplementary Figure S1B**) were reduced in TLA-treated mice (TLA/OVA) compared to allergic controls (PBS/OVA). No effect on the levels of OVA-specific IgG2a in BALF was observed (**Figure 2D**). Although the TLA treatment did not reduce the histopathology score or the number of PAS-positive goblet cells in the lung (**Figures 2E, F**), reduced levels of type 2 cytokines and IL-10 were measured in OVA-restimulated lung cell cultures of TLA/OVA mice in comparison to PBS/OVA controls (**Figure 2G**). Production of IFN-γ in the cultures was not affected by TLA-treatment (**Figure 2G**).

Similar to the local responses, OVA-restimulated spleen cells of TLA/OVA mice expressed lower levels of IL-4, IL-5, and IL-10 compared to PBS/OVA mice, while no significant difference was detected for IFN-γ (**Figure 3A**). In serum, OVA-specific levels of IgG1 were comparable between PBS/OVA and TLA/OVA mice, while OVA-specific IgG2a was increased upon TLA treatment compared to PBS/OVA mice (**Figure 3B**). Moreover, TLA led to a reduction of OVA-specific IgE-mediated release of β-hexosaminidase in RBL cells compared to PBS/OVA mice (**Figure 3C**).

Therapeutic Intranasal TLA Application Reduces Th2 Responses in the Lung and Spleen

Next, we investigated whether also a therapeutic application of TLA reduces allergic responses. Additionally, we tested if heat-inactivation of TLA impairs its immunomodulatory effects. Sensitized mice were treated intranasally with native TLA on days 18 to 23 (**Figure 4A**). Another group of mice received heat-inactivated TLA (hiTLA/OVA). Both groups that were treated with TLA and hiTLA exhibited reduced numbers of eosinophils in the BALF (**Figure 4B, Supplementary Figure S1C**), but no changes were seen in the levels of OVA-specific IgG2a in BALF, in degree of histopathology scores or in the number of PAS-positive goblet cells in the lung (**Figures 4C–E**) in comparison to allergic controls. The production of type 2 cytokines was reduced in OVA-restimulated lung cells of TLA/OVA and hiTLA/OVA mice compared to PBS/OVA mice, while no effect was seen on the production of IL-10 and IFN-γ (**Figure 4F**). Similarly, levels of type 2 cytokines and also IL-10 were reduced in OVA-restimulated spleen cells of both TLA/OVA and hiTLA/OVA mice compared to PBS/OVA mice (**Figure 5A**). Levels of IFN-γ in OVA-restimulated spleen cells of TLA/OVA and hiTLA/OVA treated groups was comparable with sham treated mice and

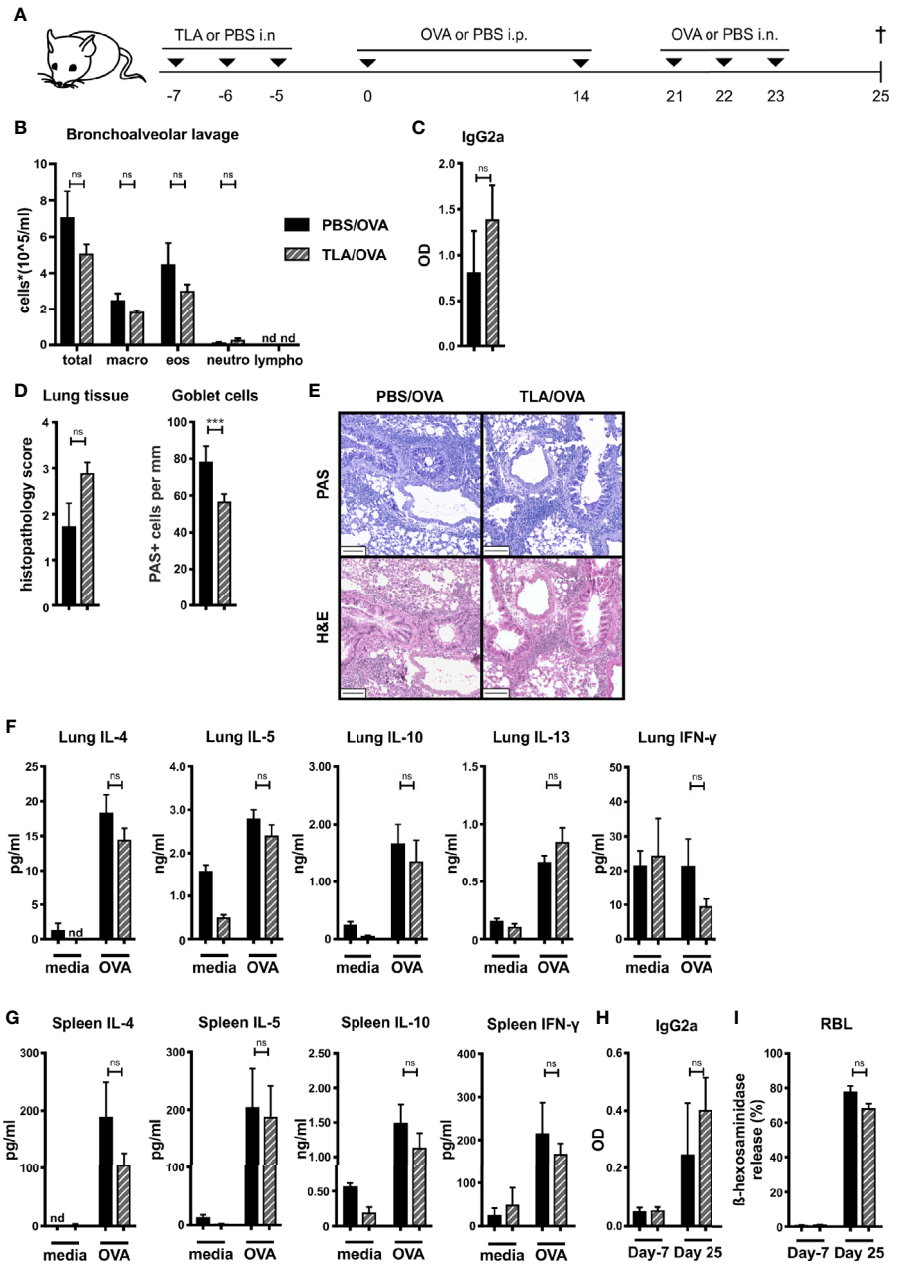

FIGURE 1 | Prophylactic treatment with TLA fails to prevent allergic airway inflammation. **(A)** Experimental design. **(B)** Differential cell counts in bronchoalveolar lavage (BALF). **(C)** Levels of OVA-specific antibody IgG2a in BALF collected at the end of the experiment. **(D)** Average histopathology score and number of Periodic acid-Schiff (PAS)-positive goblet cells of lung sections. **(E)** PAS or hematoxylin and eosin (H&E)-stained lung sections from 1 representative example from each group (n = 5); scale bars, 100 µm. **(F)** Levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ after medium and ovalbumin (OVA) restimulation of lung cells. **(G)** Levels of IL-4, IL-5, IL-10, and IFN-γ after medium and OVA restimulation of spleen cells. **(H)** Levels of OVA-specific antibody IgG2a in serum collected at the beginning and at the end of the experiment. **(I)** Release of β-hexosaminidase by rat basophil leukemia (RBL) cells. Graphs show results from 1 representative experiment from 2 independent experiments with 5 mice per group (**Figures 1B–I**). Error bars show mean ± SEM. *TLA,* tachyzoites lysate antigen; *OVA,* ovalbumin; *i.n.,* intranasal; *i.p.,* intraperitoneal; *macro,* macrophages; *eos,* eosinophils; *neutro,* neutrophils; *lympho*, lymphocytes; OD, optical density; *nd*, not detectable; *ns*, not significant; ***P < 0.001.

reduced compared to allergic controls (**Figure 5A**). Levels of OVA-specific IgG1 in serum were comparable between TLA/OVA, hiTLA/OVA and PBS/OVA mice (**Figure 5B**). TLA treatment increased OVA-specific IgG2a levels in serum compared to PBS/OVA and hiTLA/OVA mice (**Figure 5B**). There was no difference in OVA-specific IgE-mediated β-hexosaminidase release from RBL in serum samples between all allergen-exposed groups (**Figure 5C**).

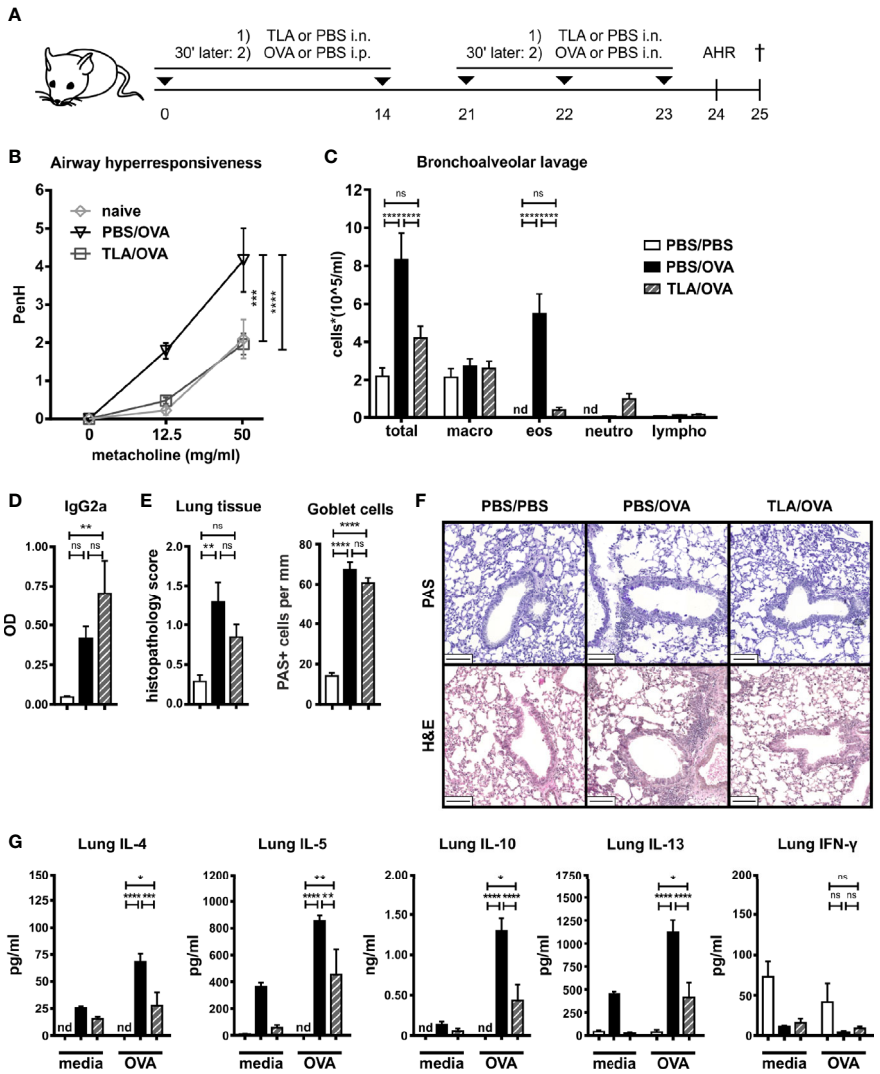

FIGURE 2 | TLA reduces allergic airway inflammation in a co-application model. **(A)**, Experimental design. **(B)** Airway hyperresponsiveness in response to methacholine. **(C)** Differential cell count in bronchoalveolar lavage (BALF). **(D)** Levels of OVA-specific antibody IgG2a in BALF collected at the end of the experiment. **(E)** Average histopathology score and number of Periodic acid-Schiff (PAS)-positive goblet cells of lung sections. **(F)** PAS or hematoxylin and eosin (H&E)-stained lung sections from 1 representative example from each group (n = 5); scale bars, 100 µm. **(G)** Levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ after medium and OVA restimulation of lung cells. Graphs show results from 1 experiment with 5 mice per group (**Figure 2B**, PBS/PBS group in **Figures 2C–G**) or from 1 representative experiment from 2 independent experiments with 5 mice per group (groups PBS/OVA and TLA/OVA, **Figures 2C–G**). Error bars show mean ± SEM. *TLA*, tachyzoites lysate antigen; *OVA*, ovalbumin; *i.n.*, intranasal; *i.p.*, intraperitoneal; *macro*, macrophages; *eos*, eosinophils; *neutro*, neutrophils; *lympho*, lymphocytes; *nd*, not detectable; *ns*, not significant; *OD*, optical density; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Characterization of TLA With Lectins Reveals a Complex Pattern of Epitope Sugars

The immunomodulatory properties of native and hiTLA were comparable as shown in **Figures 4** and **5**, suggesting that not proteins, but rather heat-stable components, such as carbohydrates, might play a role in immunomodulation. Hence, we next characterized the diversity and abundance of epitope sugars with ELLA. Several lectins bound to TLA, indicating a complex glycosylation pattern. WFA, (specifically binding to β-GalNAc), followed by AAA, LEL, UEA-I, and ConA, (specifically binding to α-Fuc, β-GlcNAc, α-Fuc/Arabinose, and α-Man/α-Glc, respectively) showed the highest absorbance, suggesting that these epitope sugars are the most prevalent in TLA (**Figure 6A**). Based on the ELLA results, we performed Western blotting of TLA probed with lectins which exhibited the highest signal when measured by ELLA (**Figure 6B**). The lectin UEA-I was binding to proteins with a size between 40 and 115 kDA, while AAA was binding to proteins with a size of approximately more than 60 kDa. LEL-binding was distributed evenly between smaller and larger proteins and WFA-binding was detected on distinct bands of smaller and mid-sized proteins ranging from approximately 20 to 80 kDa. By probing with

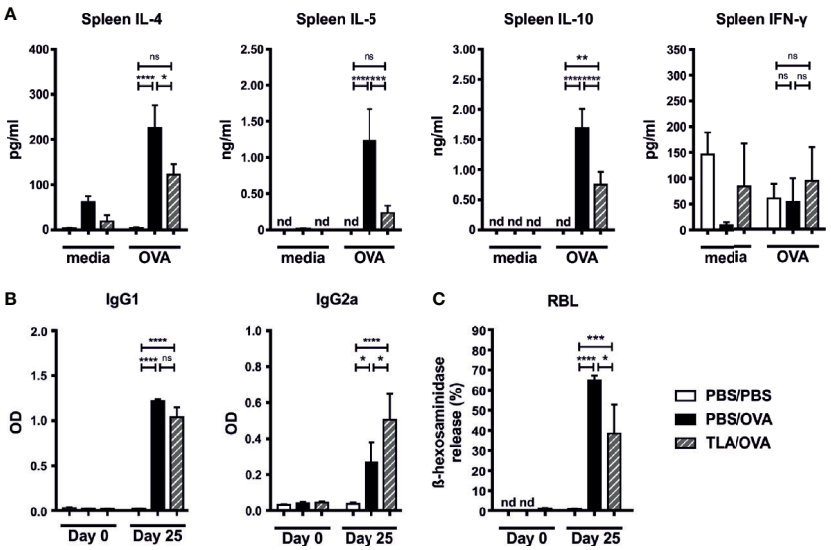

FIGURE 3 | TLA reduces systemic IL-4 and serum IgE-levels in a co-application model. **(A)** Levels of IL-4, IL-5, IL-10, and IFN-γ after medium and ovalbumin (OVA) restimulation of spleen cells from mice treated as in **Figure 2A**. **(B)** Levels of OVA-specific antibodies IgG1 and IgG2a in serum collected at the beginning and at the end of the experiment. **(C)** Release of β-hexosaminidase by rat basophil leukemia (RBL) cells. Graphs show results from 1 experiment with 5 mice per group (PBS/PBS) or 1 representative experiment from 2 independent experiments with 5 mice per group (PBS/OVA and TLA/OVA). Error bars show mean ± SEM. *TLA*, tachyzoites lysate antigen; *OD*, optical density; *nd*, not detectable; *ns*, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

ConA, Western blot analysis showed a strong band at approximately 50 kDa. Additional proteins covered with α-Man/α-Glc epitope sugars were detected from 30 kDa size upwards.

Native and Heat-Inactivated, But Not Deglycosylated TLA Reduced Type 2 Cytokines in Splenocytes of Allergic Mice *Ex Vivo*

Next, we tested whether deglycosylation of TLA impairs its immunomodulatory properties. The effects of TLA, hiTLA, and dgTLA on spleens of allergic mice were examined *ex vivo*. The removal of glycan moieties in dgTLA was verified with a ConA-specific Western blot (data not shown). Single-cell suspensions of allergic spleens were pre-incubated 60 min either with media, native TLA, hiTLA or dgTLA followed by 72 h restimulation with either media (only for non-stimulated cells) or OVA. Pre-incubation with TLA and hiTLA, but not with dgTLA, followed by restimulation with OVA reduced levels of OVA-specific IL-4 and IL-5 compared to sham-treated controls (**Figure 7**). While the levels of IL-10 were comparable between all groups, the production of IFN-γ was increased after pre-incubation with TLA and hiTLA, followed by restimulation of OVA compared to media/OVA, while pre-incubation with dgTLA had no effect on the levels of IFN-γ (**Figure 7**).

DISCUSSION

Epidemiological studies have shown inverse associations between allergy and infection with *T. gondii* (20, 42, 43). These findings were confirmed in experimental settings, where we and others have shown that infection with *T. gondii* (21, 22) or intraperitoneal injection of extracts of different *T. gondii* developmental stages, such as oocysts and tachyzoites, admixed to a potent adjuvant, such as alum or Freund's complete adjuvant, prevented allergy in mice (23, 24). Here we show that the less invasive mucosal application of TLA *via* the nose reduced the development of allergic airway inflammation in mice. Importantly, the beneficial effect was achieved by co-application of TLA during the allergic sensitization and challenge as well as by therapeutic treatment in sensitized mice. Of note, TLA was applied in the absence of any adjuvant.

To induce tolerance by immunomodulatory substances, the mucosal route of application is advantageous over the parenteral route for several reasons: i) the active compound is absorbed slower when applied *via* the mucosa compared to parenteral application which might be beneficial for achievement of a sustained protective effect, ii) the needle-free application allows for easy self-administration, and iii) the mucosal route tolerates lower purity of the drug compared to the parenteral route (44, 45). The preferred mucosal site of the application, either the nose, mouth, or vagina, may vary depending on various factors, such as the frequency of administration, the desired drug dosage and formulation, or the target organ (46). In our present study, the intranasal TLA application reduced the development of allergy in the lung, but also modulated systemic immune responses, such as levels of allergen-specific antibodies in serum or allergen-specific recall responses in the spleen. These results are in agreement with previous findings, where intranasal application of recombinant probiotic *Escherichia coli* Nissle 1917 prevented allergic airway inflammation in mice accompanied by reduced local and systemic allergic responses (30). Similarly,

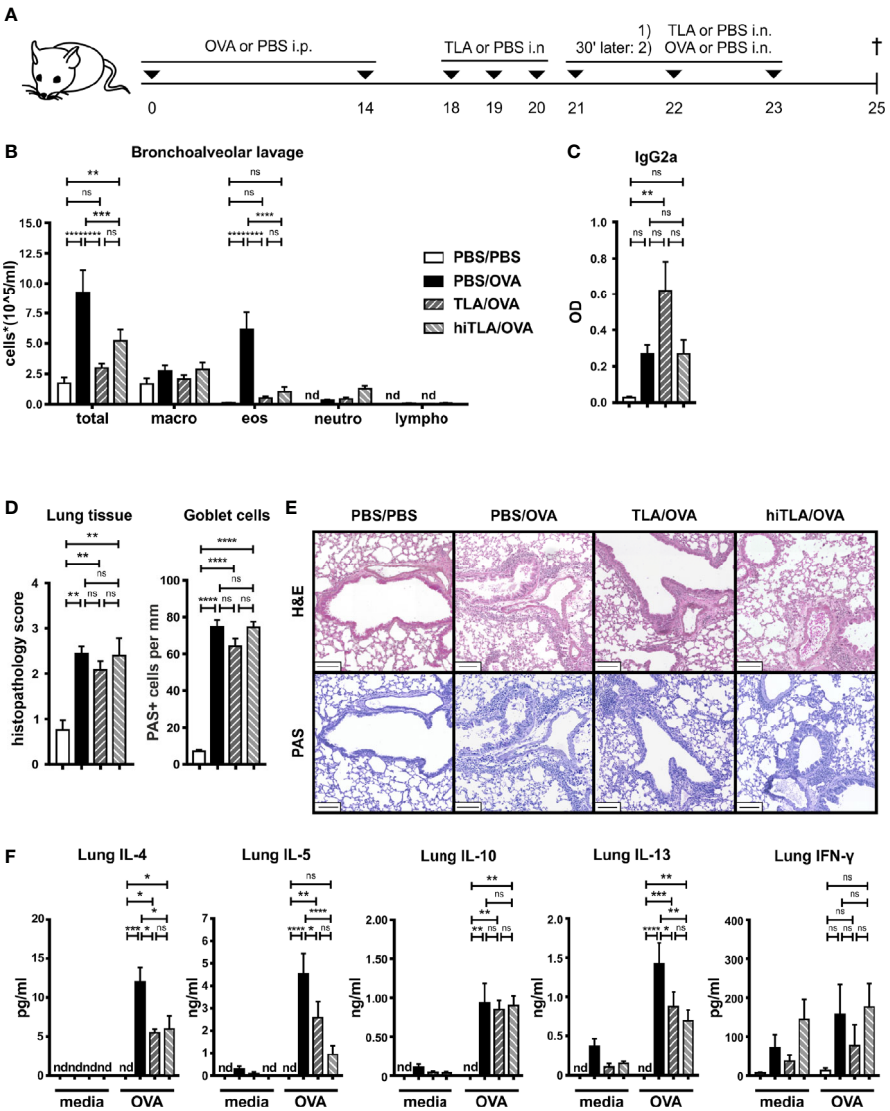

FIGURE 4 | Therapeutic treatment with native or heat-inactivated TLA reduces allergic airway inflammation. **(A)** Experimental design. **(B)** Differential cell count in bronchoalveolar lavage (BALF). **(C)** Levels of OVA-specific antibody IgG2a in BALF collected at the end of the experiment. **(D)** Average histopathology score and number of Periodic acid-Schiff (PAS)-positive goblet cells of lung sections. **(E)** PAS or H&E-stained lung sections from 1 representative example from each group (n = 5); scale bars, 100 µm. **(F)** Levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ after medium and OVA restimulation of lung cells. Graphs show results from 1 representative experiment from 2 independent experiments with 5 mice per group. Error bars show mean ± SEM. *TLA*, tachyzoites lysate antigen; *hiTLA*, heat-inactivated; OVA, ovalbumin; i.n., intranasal; i.p., intraperitoneal; macro, macrophages; eos, eosinophils; neutro, neutrophils; lympho, lymphocytes; n.s., not significant; OD, optical density; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

nasal vaccines induced protective local and systemic responses in humans (47).

We and others have shown previously that mice which underwent allergic sensitization and challenge in the course of *T. gondii* infection were protected from the development of allergy (22, 48). Here we show that allergic sensitization and challenge in the close proximity to TLA-exposure had a similar protective effect. However, when mice were treated with TLA seven to five days before the beginning of the sensitization, the development of allergy was not prevented. Altogether, these observations suggest that in order to reach mucosally-induced immunological tolerance, the host needs to be exposed to the *T. gondii* immunomodulatory molecules during the allergen exposure, suggesting a possible application of TLA in seasonal treatment protocols.

Generally, it is more challenging to cure patients with established allergic diseases compared to prophylactic approaches (49). We show that intranasal application of TLA to sensitized mice exclusively during intranasal challenge with OVA reduced local as well as systemic allergic responses. The sensitization status was confirmed by increased levels of OVA-specific IgG1 in serum and elevated IgE-mediated

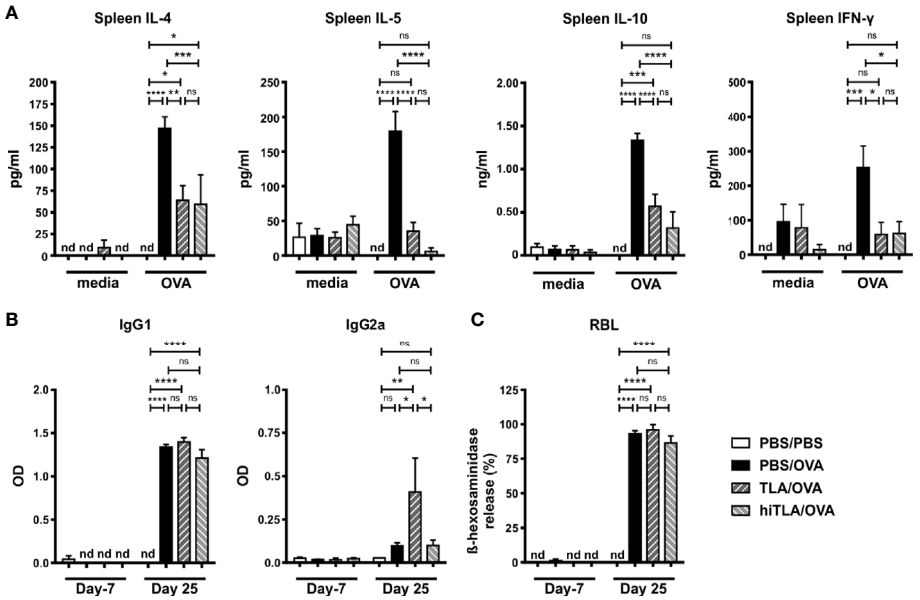

FIGURE 5 | Therapeutic treatment with native or heat-inactivated TLA reduces systemic type 2 responses. **(A)** Levels of IL-4, IL-5, IL-10, and IFN-γ after medium and ovalbumin (OVA) restimulation of spleen cells from mice treated as in **Figure 4A**. **(B)** Levels of OVA-specific antibodies IgG1 and IgG2a in serum collected at the beginning and at the end of the experiment. **(C)** Release of β-hexosaminidase by rat basophil leukemia (RBL) cells. Graphs show results from 1 representative experiment from 2 independent experiments with 5 mice per group. Error bars show mean ± SEM. *nd*, not detectable; *TLA*, tachyzoites lysate antigen; *hiTLA*, heat-inactivated TLA; *ns*, not significant; *OD*, optical density; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

β-hexosaminidase release by RBL cells in comparison to sham-treated controls (data not shown). In contrast to our previous study, where therapeutic intranasal treatment with *E. coli* O83 failed to reduce eosinophilia (29), therapeutic TLA application reduced the recruitment of eosinophils to the lungs. We hypothesize that the protective effect of TLA in adult mice lasts only for a limited time and repetitive applications might be required to reach a prolonged protective effect. However, the temporary character of suppressed type 2 responses might be favorable to treat patients suffering from seasonal allergies. For example, birch or grass pollen allergic patients are exposed to the relevant allergens only during the pollen season (50), thus, the

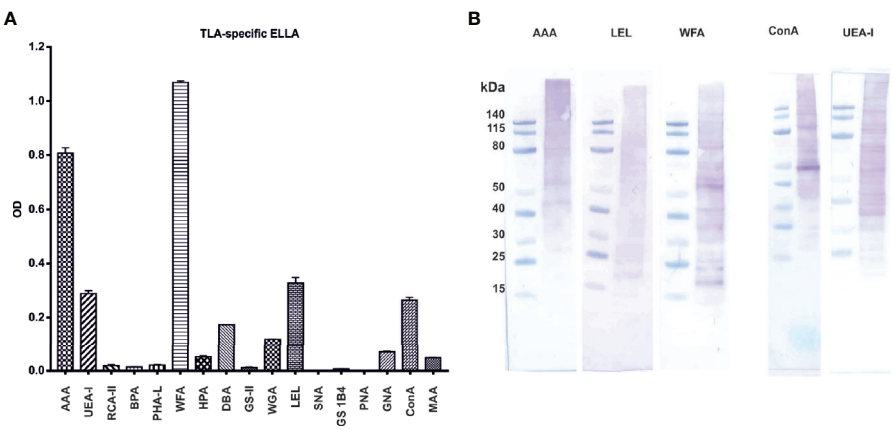

FIGURE 6 | Characterization of TLA with enzyme-linked lectin assay (ELLA) and lectin-Western blots reveal a complex pattern of epitope sugars: **(A)** abundance of tachyzoite lysate antigen's (TLA)s epitope sugars evaluated with ELLA. **(B)** Distribution of epitope sugars on proteins and peptides of TLA. *AAA Anguilla anguilla* agglutinin; *UEA-I Ulex europaeus* agglutinin-I; *RCA-II Ricinus communis* agglutinin II; *BPA Bauhinia purpurea* agglutinin; *PHA-L* phytohemagglutinin; *WFA Wisteria floribunda* agglutinin; *HPA Helix pomatia* agglutinin; *DBA Dolichos biflorus* agglutinin; *GS-II Griffonia simplicifolia* agglutinin; *WGA* Wheat germ agglutinin; *LEL Lycopersicon esculentum* lectin; *SNA Sambucus nigra* agglutinin; *GS1B4 Griffonia simplifolica*-1B4; *PNA* peanut agglutinin; *GNA Galanthus nivalis* agglutinin; *ConA* Concanavalin A; *MAA Maackia amurensis* agglutinin. *TLA*, tachyzoites lysate antigen; *OD*, optical density.

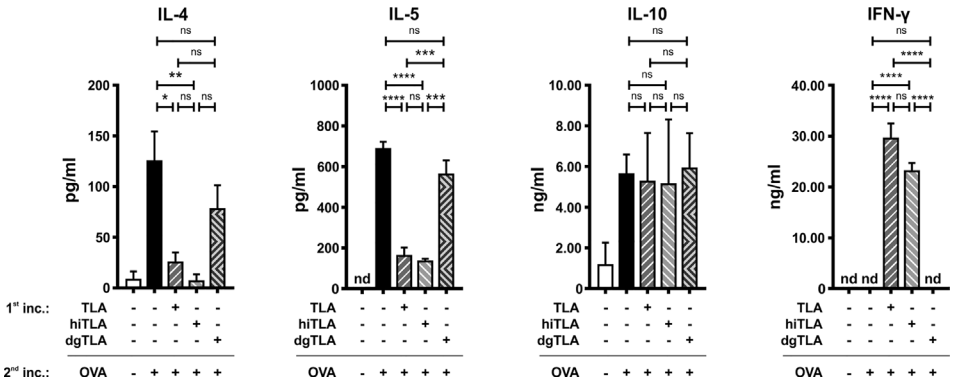

FIGURE 7 | *Ex vivo* stimulation with TLA and hiTLA, but not dgTLA reduces type 2 cytokines and elevates IFN-γ. Levels of IL-4, IL-5, IL-10, and IFN-γ after stimulation with medium, TLA, hiTLA or dgTLA, followed by stimulation with medium or ovalbumin (OVA) of splenocytes excised from allergic control mice after sensitization and challenge. Graphs show results from 1 representative experiment from 2 independent experiments with 3 mice per group. Error bars show mean ± SEM. *nd* not detectable; *TLA*, tachyzoites lysate antigen; *hiTLA*, heat-inactivated TLA; *dgTLA*, deglycosylated TLA; *inc*, incubation. *ns*, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

therapeutic application of immunomodulators exclusively during this period could be sufficient to reduce the disease progression.

The significance of IFN-γ in resolving allergy was highlighted by Coyle et al. (51), showing that IFN-γ receptor knockout mice exhibited prolonged eosinophilia compared to wild type controls (51). IFN-γ is also a crucial player in host defense to control an infection with *T. gondii* (52, 53) and extracts derived from this parasite, such as TLA or OLA, are potent inducers of this cytokine both *in vivo* and *in vitro* (23, 24). In a mouse model of allergy, we have previously demonstrated that infection with *T. gondii* or intraperitoneal application of TLA admixed to OVA in alum reduced type 2 responses accompanied by increased levels of allergen-specific IFN-γ in restimulated splenocytes compared to allergic controls (22, 24). Similarly, Fenoy et al. (48) detected increased levels of OVA-specific IFN-γ in sensitized and challenged mice during acute *T. gondii* infection, but the production of IFN-γ was decreased in chronically infected mice.

Of note, mucosal application of *Lactobacillus paracasei* NCC 2461, a probiotic bacterial strain which is a potent inducer of IFN-γ *in vitro*, reduced not only allergic type 2 responses, but also allergen-specific IFN-γ in restimulated splenocytes (31, 54). Here we show that TLA increased levels of OVA-specific type 1 isotype IgG2a in serum in the co-application and the treatment protocol. As the BALF closely reflects the immunological processes in the lung, we also measured levels of OVA-specific IgG2a and levels of IFN-γ and IL-17AF in BALF. The data show that TLA did not influence the local type 1 responses (data for IFN-γ and IL-17AF not shown).

The success of SIT in human patients is *inter alia* reflected by a reduction of allergen-specific IgE (55, 56). Here, we show that TLA, applied concurrently with sensitization and challenge, reduced the activity of OVA-specific IgE in serum in comparison to controls. However, the reduction of IgE-dependent β-hexosaminidase release by RBL cells was not observed in mice, where i) the interval between the last TLA-treatment and the start of sensitization was five days or ii) the treatment was applied to mice with completed sensitization. Although the therapeutic TLA application did not reduce the β-hexosaminidase release, the treatment reduced the recruitment of eosinophils to the lung and production of allergen-specific type 2 cytokines in the lung and spleen. Similarly, it has been shown that, although SIT led to clinical improvement in allergic patients, the levels of IgE remained initially high and declined gradually over months or even years (56). Here, in the therapeutic experiment, serum was collected already seven days after the TLA was first introduced to mice, and thus the question remains whether analysis of sera collected at a later time point or after prolonged treatment with TLA would lead to a reduction of humoral type 2 responses in sensitized animals.

It has been shown that carbohydrates and carbohydrate-binding proteins play an essential role in adhesion or invasion of certain parasites into host cells (57). Furthermore, parasite-derived carbohydrates can trigger the host´s innate and adaptive immune responses. *Toxoplasma* assembles polysaccharides and many cellular proteins and lipids are glycosylated. A recent study revealed that the *Toxoplasma* genome encodes a set of predicted glycogenes with a possible role in assembling of N-glycans, O-glycans, a C-glycan, GPI-anchors, and polysaccharides (58). Applying a double-CRISPR/Cas9 strategy indicated an important role of certain glycan-biosynthesizing enzymes (e.g. glycosyltransferase responsible for assembling novel Glc-Fuc–type O-glycans) for *in vitro* growth of *Toxoplasma* (58).

Here, we applied ELLA, a lectin ELISA, to characterize the glycosylation pattern of TLA. ELLA is commonly used for analysis of glycoconjugates and was previously employed to determine immunogenic epitope sugars of parasites, such as *Tritrichomonas foetus* or *Trypanosoma cruzi* (59, 60). Our data showed a strong binding of the lectins WFA, AAA, ConA, and

LEL to TLA, which indicate high abundances of terminal GalNAc, Fuc, Man/Glc, and GlcNAc, respectively. Previously, terminal Fuc(1→4)GlcNAc as well as the terminal motives Fuc (1→3)GalNAc and Fuc1→4(Fuc1→3)GlcNAc, which are commonly found in *S. mansoni eggs*, were shown to be immunogenic (61, 62). Furthermore, terminal fucosylation was shown to play a critical role in colonization and initial evasion of the host's immune system in infections with *Helicobacter pylori* (63). On the other hand, it was suggested that mannose plays a pivotal role in the virulence of *Leishmania mexicana* (64). Host cells, such as dendritic cells, Langerhans cells, and lymphocytes, express a plethora of carbohydrate-binding proteins including C-type lectins, selectins, and galectins which may recognize and interact with parasite-derived glycosylation patterns (65, 66).

We have shown previously *in vitro* that heat–inactivation of TLA does not affect its immunomodulatory properties (24). Here, we confirmed this observation *in vivo* and additionally, by using an *ex vivo* model, we could show that deglycosylation of TLA abolished its anti-allergic effects. However, more studies are needed to understand the precise role of *T. gondii* carbohydrates in allergy prevention.

In addition to carbohydrates, also *T. gondii*-specific lipids play an essential role for interacting with host cells and the host's immune system resulting in the establishment and maintenance of long-term persistence in the host (67). For example, fatty acids present in *T. gondii* tachyzoites exhibit immunomodulatory effects on murine macrophages (68). However, the link between the *T. gondii* lipidome and allergy prevention remains to be elucidated.

Although clinical trials using infections with nematode parasites opened new possibilities to treat immune-mediated inflammatory diseases in humans (69), the ultimate goal is the use of parasite-derived extracts or single molecules to replicate the immunomodulatory effect of the infection without causing any of the obvious disadvantages. Indeed, promising results have been seen in studies where parasite products were administered in mouse models (24, 70, 71), but to date no parasite-derived molecules have been applied as a treatment for humans yet. Molecules present in TLA have evolved to act in the environment of the host´s immune system and thus represent natural biologicals with high potential in future translational research.

CONCLUSION

In the present study, we show that mucosal application of TLA during sensitization and challenge, as well as therapeutic treatment of sensitized mice, reduces allergic exacerbations in a mouse model of allergic airway inflammation. Additionally, we show that TLA is highly glycosylated and that removal of carbohydrates impaired its immunomodulatory properties. We therefore believe that further investigations focusing on glycosylation patterns of TLA will strengthen our understanding of host-parasite interactions and will pave the way for novel, therapeutic treatment strategies against allergic disorders.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the raw data supporting the conclusions of this article will be made available by the authors upon reasonable request. Requests to access the datasets should be directed to IS, irma.schabussova@meduniwien.ac.at.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Research (BMWFW-66.009/0358-WF/V/3b/2015 and BMBWF-66.009/0277-V/3b/2019).

AUTHOR CONTRIBUTIONS

IS and UW designed and supervised the study and acquired funding. EK and IS prepared documents for ethics approval. RP cultivated *T. gondii* tachyzoites. MD and EK prepared TLA. EK performed experiments, analyzed and interpreted the data. NG performed histological analysis of lung tissue samples. EK and IS wrote the initial draft of the manuscript. EK provided visualization of the data. EK, IS, AI-K, AJ, MD, and AW critically reviewed and edited the manuscript. IS, UW, and AJ provided resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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frontiers in Immunology

MINI REVIEW
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The Hygiene Hypothesis – Learning From but Not Living in the Past

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Postulated by Strachan more than 30 years ago, the Hygiene Hypothesis has undergone many revisions and adaptations. This review journeys back to the beginnings of the Hygiene Hypothesis and describes the most important landmarks in its development considering the many aspects that have refined and generalized the Hygiene Hypothesis over time. From an epidemiological perspective, the Hygiene Hypothesis advanced to a comprehensive concept expanding beyond the initial focus on allergies. The Hygiene Hypothesis comprise immunological, microbiological and evolutionary aspects. Thus, the original postulate developed into a holistic model that explains the impact of post-modern life-style on humans, who initially evolved in close proximity to a more natural environment. Focusing on diet and the microbiome as the most prominent exogenous influences we describe these discrepancies and the resulting health outcomes and point to potential solutions to reestablish the immunological homeostasis that frequently have been lost in people living in developed societies.

Keywords: hygiene hypothesis, allergy, asthma, immune tolerance, T cell-response, microbiome

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Last year we celebrated the 30th anniversary of the Hygiene Hypothesis. Since Strachan framed the Hygiene Hypothesis in 1989 (1) his fundamental idea to explain the origins of allergic diseases development has survived the test of time. The basic idea of how humans, their microbiota, and a continuously modernizing environment have interacted to drive immune dysregulation has persisted and become part of the popular imagination. Here, we aim to provide an editorial overview on the history of the Hygiene Hypothesis and related topics to offer a framework for the articles collected in the special edition research topic "The Hygiene Hypothesis and its Immunological Implications."

A CHRONOLOGICAL OVERVIEW

The epidemiological basis for the Hygiene Hypothesis became apparent long before the Hygiene Hypothesis was postulated. Two simple observations were made in the 1960s and in the 1970s. First, a Swedish study described differences in the prevalence of asthma and socio-medical conditions between populations living in urban or rural sites (2). A few years later, in a population-based study conducted in Saskatchewan, Canada, showed that allergies were less frequent in native tribes living traditionally in rural sites compared to Caucasian Canadians living in urban habitats (3). Moreover, the authors postulated that frequent bacterial infections in childhood might be responsible for the inverse association with allergic diseases. Strachan's observations made in the late 80s in a British population corroborated these findings and he later named this concept "Hygiene Hypothesis"

in 2000. Briefly, Strachan suggested that transfer of early childhood infections between siblings is associated with protection against allergies later in life (4).

The hypothesis was further substantiated and extended by studies that compared asthma and allergy prevalence directly after the "Fall of the Iron Curtain" between Western and Eastern Germany, a decade later (5, 6). Interestingly, these studies triggered a paradigm shift in allergy research. Until then, environmental pollution was broadly regarded as the leading force for allergy development. Environmental data clearly indicated a higher level of pollution by industrial emissions in Eastern Germany compared to the Western part and the study team therefore hypothesized that the prevalence of allergic diseases was higher in children from Eastern Germany. Surprisingly, the researchers found their hypothesis disproved, as children in Western Germany showed a higher prevalence of allergies. Hence, it was postulated that other exposures than pollutants influence the development of atopic diseases. Socio-demographic and –economic factors, as well as household hygiene turned out to be further discriminatory factors between both parts of German population. Improved sanitation and hygiene were positively associated with atopic diseases. Another decade later a follow-up further validated this hypothesis and found life-style differences and the prevalence of atopic diseases began to equilibrate within 10 years after the reunification. In consequence, the Hygiene Hypothesis became the leading postulate to explain underlying relationships and mechanisms for the development of allergic diseases in a societal context (6).

Based on this paradigm shift, Rook published the "Old Friends-Hypothesis" which argues that infectious diseases have a long co-evolutionary history with human development, and appropriate levels of exposure to these microorganisms early in life might protect against immune deviation and allergic diseases. These early-life exposures to potential pathogens might educate the developing immune system from a type-2-dominated *in utero*-milieu toward a more defensive T helper (h)1 response (7).

The next milestone involved findings obtained from the so-called "Alpine farm studies" conducted at the turn of the millennium. Von Mutius and Braun-Fahrländer recognized the unique situation that the Alpine traditional farming environment represents a socio-cultural and ecological niche which significantly differs from the post-modern and urbanized life-style. In a number of epidemiological studies they identified traditional farming characteristics such as consumption of unprocessed farm milk and close contact with farm animals to act allergoprotective and found these parameters to be associated with a higher microbial load. These Alpine farm studies added substantial evidence to Strachan's basic idea and led to a broader view and understanding of the relationship between human health and (early life) exposure to microbes (8, 9).

Further evidence was added by studies conducted in Northern Europe. In the late 1990s studies conducted in Scandinavian und Baltic children described microbial factors to be associated with a lower prevalence of allergic diseases in the Eastern countries (10–12). Next, the Karelia Study, conducted on both sides of the Finnish-Russian border, addressed the impact of the environmental microbial burden on the development of allergic diseases in Finnish and Russian Karelian children that share the same ethnic background but have different life-styles (13). These studies corroborated the Alpine farm studies and point to the microbial environment as a major factor in allergy development.

Furthermore, these studies demonstrate that the diversity and the richness of an immune-stimulating microbial world in human habitats is crucial to establish a competent, tolerogenic and defensive immune system configuration while absence or depletion of those stimuli as found in post-modern environments foster immune deviation and development of allergic diseases (14).

Moreover, two relevant studies [the cross-sectional study "Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle (PARSIFAL)" and the multi-center, pregnancy/birth cohort study "Protection against Allergy: Study in Rural Environments (PASTURE)"] support the idea that the "window of opportunity" in which the appropriate education of the immune system starts already in the mother's womb (15–17). The PARSIFAL Study demonstrated that maternal exposure to a farm environment rich in microbial compounds is inversely associated with the development of atopic sensitization and correlated with an upregulation of receptors of the innate immune system in the offspring at school age (15). Further, maternal farm activities during pregnancy were shown to modulate cord blood cytokines and allergen-specific immunoglobulin responses toward a Th1 pattern (16, 17). These findings are in line with the Barker theory (18), postulating that pathological pathways occurring in adolescence and adulthood are paved already in prenatal life.

THE HYGIENE HYPOTHESIS AND THE BACTERIAL WORLD

Even before high-throughput sequencing techniques were established that allow a deeper view into the microbial world on our body surfaces, Noverr and Hufnagle proclaimed the "Microbiota Hypothesis" by which they claimed the microbiota to be indispensable for developing and maintaining a tolerogenic immune status (19). A similar idea concept was proposed earlier by Holt, Sly and Björkstén (20). The rediscovery of the microbiota and its powerful metabolic and immunologic interplay with the mucosal surfaces of the host underlined and complemented the principals of this basic idea (21, 22). Microbiome research has made significant achievements over the past 15 years; here we can emphasize only a few aspects that might be relevant in the context of the Hygiene Hypothesis and the development of allergies.

Phylogenetic Impacts

An intriguing concept to better understand the complex symbiotic interplay at organ surfaces was suggested by McFall-Ngai in 2007. In her evolutionary perspective she shed light on findings made in invertebrates which not only lack an endoskeleton but also an adaptive immune system. Thus, invertebrates have to exclusively rely on their innate immune system, which to our current understanding, lacks an immunological memory. Analyses of the intestinal microbiota

in such animals have shown—in contrast to vertebrates—a rather low diversity in the community of their microbial residents. Only a handful of strains could be identified as stable colonizers on the gastrointestinal surfaces while most bacteria travel through as transient visitors. Some invertebrates, like insects, separate bacterial colonies from epithelial host cells by a peritrophic matrix composed of chitin and other compounds (24). During the course of evolution, the microbial colonization of epithelia started to get more complex and in turn the host was challenged to develop new strategies to manage these diversifying communities. To permanently recognize a specific bacterium as beneficial or harmful, an adaptive immune response that provides an immunological memory over generations of immune cells was needed. Mutual adaption of both partners, the bacterial community, as well as the complex network of adaptive immune cells, led to a sophisticated metabolic and immunologic interplay with a highly digestive and defensive performance. This symbiosis is based on early education of the host's immune cells by a diverse microbial community to successfully discriminate dangerous pathogens from beneficial symbionts and own healthy cells. Finally McFall-Ngai stated, that complex systems might be prone to failure and allergies and autoimmune disorders might be a consequence of this (23).

Ontogenetic Impacts

A number of recently published reports substantiated the impact of the early life microbiota on immune maturation [recently reviewed in (25)] and the development of allergic disorders in early infancy [recently reviewed in (26)]. The developmental starting point of the infant gut microbiota is still unknown, but undoubtedly, the process of delivery seems to be a key point in the development of the neonatal microbiota (27). Meconium, the neonate's first intestinal discharge, was shown to contain various bacterial strains indicating that the perinatal gut is colonized by bacteria (28, 29). In a landmark study, Dominguez-Bello et al. reported that the neonatal microbiota differs between vaginally born infants and neonates delivered by Caesarian (C)-section. The authors found a high abundance of *Bacteroides, Bifidobacterium,* and *Lactobacillus spec.* in meconium samples obtained from vaginally delivered newborns, while *Staphylococcus*, *Streptococcus, Corynebacterium,* and *Propionibacterium spp*. were found predominantly in meconium samples of C-section born neonates (30).

Colonization of the neonate's colon by *Lactobacilli* and *Bifidobacteria* transferred from the maternal vaginal compartment during vaginal passage might provide advantages for the newborn due to the metabolic properties of these bacteria that foster the adaptation to milk-based feeding. These bacteria are capable of metabolizing breast milk-derived lactose and human milk oligosaccharides (HMOS) (31) and were shown to provide immune-modulating short chain fatty acids (SCFAs) (32) and conjugated trans-linoleic acids (tCLAs) (33), which are shown to reduce pro-inflammatory eicosanoid production by regulating the transcription of cyclooxygenase 2 (COX-2) (34) and to induce anti-inflammatory M2-macrophage differentiation (35).

However, how sustainable and decisive are these mode of delivery-associated differences beyond the neonatal age? Chu et al. recently showed that function and composition of the microbiota significantly diversifies in all body sites within the first 6 weeks of life, resembling the corresponding maternal body site microbiota at this time point. Infant's mode of delivery or other prenatal factors seems to have no impact on this development (36). Data from the Copenhagen Prospective Studies on Asthma in $Childhood_{2010}$ ($COPSAC_{2010}$) cohort underlined the importance of the maturation of the microbiota on the further development of the gut microbiome and the risk of asthma later in life. In that study, Stockholm et al. compared the gut microbiome of vaginally and C-section delivered infants from birth to 1 year of life in the context of asthma development at school age. Marked differences between C-section and vaginally delivered infants were observed by 1 week and by 1 month of life, but only minor differences between these groups were found by 1 year of age. An increased risk for school-age asthma was only observed in a subgroup of C-section-born infants that maintained the C-section-associated composition for at least 1 year. The authors conclude that vaginal delivery and/or subsequent maturation of the infant microbiota might support a more robust and stable microbiota in the offspring that is more adaptive to the challenges later in life (37). Further exposure to the maternal microbiota (38), as well as nutritional impacts (e.g., cessation of breastfeeding) (39) within the first month of life, might foster the maturation of the gut microbiome in early infancy.

Nutritional Impacts

How is the microbiota linked to the rising atopic epidemic observed in the recent decades? A recently published study conducted in indigenous tribes living in the Brazilian Amazonas-Orinoco Basin may help to answer this question (40). In this study the gut microbiome of the semi-nomadic gatherer/hunter people of the Yanomami who maintained a primitive close-to-nature life-style was compared to subjects representing populations that are characterized by a westernized or non-ancestral life-style in rural and urban settings. The Yanomami microbiota was significantly more diverse than those of the westernized counterparts. Moreover, an additional study comparing Venezuelan with Brazilian Yanomami indicated a high level of adaptability to specific environmental conditions of the microbiota in these peoples. While a high taxonomic diversity was found in both sub-tribes, the composition of microbiota was significantly different (41). These findings point to environmental and life-style factors that influence the composition of the microbiota the absence of which may thus foster the loss of taxonomic and metabolic diversity in westernized societies (42).

Diet is one of the most prominent environmental factors that differ between modern and ancient life-styles. While dietary habits in indigenous people such as the Yanomami strongly depend on the sometimes limited food supply due to seasonal cycling, people living in developed societies have access to high in calories food ready at any time and in abundance. Moreover, diet in indigenous cultures is often based on high-fiber products derived from plants that are easy to culture

such as plantain, manioc or sweet potatoes, all rich in inulin (43). High-fiber diet and, in particular inulin, is known as an effective enhancer of beneficial bacteria such as *Bifidobacteria* in the colon that stabilize gut homeostasis (44). Translating these findings into a clinical approach, McLoughlin et al. applied soluble inulin to asthmatics in a short-term placebo-controlled-trial and could report an array of beneficial effects in patients orally treated with inulin. In comparison to the placebo group, inulin-treated patients displayed a significantly reduced number of eosinophils in the sputum and, overall, reported a significantly improved asthma control. Inhibition of histone deacetylase 9 (HDAC9) in sputum cells upon a combined application of inulin and a multi-strain probiotic mixture of *Lactobacillus acidophilus, Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subspecies *lactis* indicated that epigenetic pathways are involved in the mechanisms by which lactic acid bacteria modulate host responses in combination with the prebiotic gavage (45).

A number of recently recognized metabolites released by beneficial symbiotic bacteria convey immunomodulatory effects, mainly in the gut but also on other mucosal surfaces (46).

In particular, SCFAs derived from dietary fibers and released in the lumen of the colon contribute to immune modulation and inhibition of pro-inflammatory cytokines when absorbed by gut epithelial cells (47). By binding to chemoattractant G protein 43 receptor, SCFAs are capable of regulating inflammatory responses (48) as shown for intestinal inflammation (49). Tryptophan, an amino acid produced by an array of beneficial microorganisms, is degraded to indole derivatives which may bind to the aryl hydrocarbon receptor (AHR) and by this regulate the activity of immune cells at the epithelial barrier. That involves AHR-dependent differentiation of regulatory T cells associated with anti-inflammatory IL-10 expression. Further, Th2-cells are inhibited on the transcription factor level in favor of a Th1 response (50).

A number of beneficial bacteria contribute to the orchestration of T cell subsets at the gut epithelial barrier. *Bacteroides sp*. and *Clostridium* clusters IV and XIVa colonizing the gut epithelium are known to stimulate intestinal epithelial cells to release thymic stromal lymphopoietin (TSLP), transforming growth factor (TGF)-ß and interleukin (IL)-25 which in combination may induce tolerogenic effects in dendritic cells (DCs) (51), e.g., by secretion of TGF-ß and retinoic acid. Both factors initiate differentiation of naïve T cells to regulatory T cells upon activation of the nuclear transcription factor forkhead box P (FoxP3) (52). These regulatory mechanisms are challenged by "pathobionts" or other damage factors. In presence of these stressors, overexpansion of Th1, Th2 and Th17 effector cell subsets might result in an inflammatory response in the infected organ or, by migration of these cells, at distant sites. Namely, *Clostridium difficile,* which is associated with wheezing and atopic sensitization, was shown to initially disturb the intestinal balance when acquired early in childhood (53).

Traveling from the gastrointestinal to the respiratory tract the microbiota established in the lung might also play a role in the development of allergic disorders, namely of allergic asthma. Though the gut is known to play a major role in establishing and regulating immune defense mechanisms, the "gut-lung axis" alone might not completely explain the rise of allergic asthma (54). As many studies focused on the lung microbiome, it has become clear that there is a strong relationship between frequently inhaled environmental microbes, microbial colonization of the respiratory tract, and the prevalence of allergic asthma (55). For example, results from the "Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community (GABRIEL) Advanced Studies (GABRIELA)" study suggested a transfer of built-environment-associated bacteria into the respiratory tract. Indoor dust samples from farm houses and nasal swabs from farm children displayed a higher bacterial diversity than those samples collected in rural non-farm children (56). New evidence was added recently by studies conducted in the Finnish part of the PASTURE-study. Kirjavainen et al. reported that the ecological diversity of the so-called "indoor microbiota" is inversely linked to the prevalence of allergic asthma. Substantiating former farm studies, this report further validated the hypothesis that microbial diversity and composition in the natural environment is linked to a reduced risk of early-onset allergic asthma and that traditional farming is a proxy for this effect (57).

But what are the cellular and molecular mechanisms associated with high microbial diversity? Interestingly, the farm studies consistently showed an inverse association between a highly diverse environmental microbiota and allergic asthma, but this did not account to other allergic manifestations such as hay fever or atopic sensitization. On the other hand, endotoxin exposure protects against atopy but fosters the risk of non-allergic asthma and early onset of wheeze when inhaled in higher concentrations. These findings derived from the farm studies still challenge the Hygiene Hypothesis and might point out that microbial colonization and exposure to microbial compounds have to be considered separately (58). Integration of beneficial environmental bacteria into the microbial community of the respiratory tract leads to a tolerogenic mucosal symbiosis that establishes a local T-cell balanced anti-inflammatory milieu at the epithelium, probably enhanced by a well-balanced gut microbiota. Endotoxins are potent activators of innate TLR-signaling and can attenuate B cell driven sensitization and formation of IgE-antibodies (59). Already in 2003, Vercelli postulated a switch from Th2-driven allergic responses at low endotoxin exposure to a pronounced Th1 response in the lung under high levels of environmental endotoxin. This might explain the elevated prevalence of non-allergic asthma in environments overloaded with endotoxin (60).

CONCLUSIONS

The many aspects and facets of the Hygiene Hypothesis have been supported by concepts and findings coming from a variety of scientific disciplines such as epidemiology, immunology, microbiology and anthropology. Within the last three decades we obtained a multiplicity of new insights into the complexity and plasticity of T cell networks which led us to recognize

the complexity and significance of a powerful and well-regulated adaptive immune response in relation to exogenous factors (61). Early developmental findings characterizing pre and postnatal life events highlighted the initial role of the innate immune system as an early warning system that orchestrates, educates and shapes subsequent immune responses (62, 63). Evidence from evolutionary biology and anthropology enabled us to understand how host-environment interactions are refined throughout evolutionary adaption (58, 64). Microbiology added fundamental knowledge about the micro-ecosystem that is established throughout the human body as a unique symbiosis between humans and microbes. And finally, coming back to the introductory statement, epidemiological observations such as those initially made by Strachan and von Mutius about 30 years ago still challenge and refine the hypothesis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Hygiene Hypothesis and New Perspectives—Current Challenges Meeting an Old Postulate

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During its 30 years history, the Hygiene Hypothesis has shown itself to be adaptable whenever it has been challenged by new scientific developments and this is a still a continuously ongoing process. In this regard, the mini review aims to discuss some selected new developments in relation to their impact on further fine-tuning and expansion of the Hygiene Hypothesis. This will include the role of recently discovered classes of innate and adaptive immune cells that challenges the old Th1/Th2 paradigm, the applicability of the Hygiene Hypothesis to newly identified allergy/asthma phenotypes with diverse underlying pathomechanistic endotypes, and the increasing knowledge derived from epigenetic studies that leads to better understanding of mechanisms involved in the translation of environmental impacts on biological systems. Further, we discuss in brief the expansion of the Hygiene Hypothesis to other disease areas like psychiatric disorders and cancer and conclude that the continuously developing Hygiene Hypothesis may provide a more generalized explanation for health burden in highly industrialized countries also relation to global changes.

Keywords: hygiene hypothesis, allergy, asthma, non-communicable inflammatory diseases, chronic inflammation

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INTRODUCTION

Throughout its history, the Hygiene Hypothesis has shown itself to be adaptable and flexible whenever it has been challenged by innovation in science (1). A number of new findings need to be considered in this ongoing revisiting process: The originally proposed Th1/Th2 paradigm is challenged by currently elucidated new classes of effector and regulating immune cells pointing out to a more complex immune network involved in allergy development (2). Studies on biomarkers and deep phenotyping techniques changed our understanding of asthma as a uniform disease in favor of distinct phenotypes that are driven by different causations (3). The emerging field of epigenetics enables us to fill the black box of "gene-by-environment interactions" with conveying mechanisms (4). Currently recognized epigenetic pathways overlapping between chronic inflammatory diseases and other disorders such as psychiatric conditions or cancer might extend the Hygiene Hypothesis toward a model explaining in a broader sense the rise of health burdens in westernized societies (5). Finally, the world-wide challenge caused by the climate changes will not

leave the consequences of Hygiene Hypothesis unaffected. Changing life-styles are closely related to measures implemented to slow down CO_2 emissions and to stabilize the world climate (6).

CHALLENGES FROM IMMUNOLOGY – THE IMMUNE SYSTEM BECOMES MORE COMPLEX

Parallel to the revisions that Hygiene Hypothesis has undergone over time (7), our perception of the mechanisms underlying cellular and humoral immune responses has changed fundamentally over the last decades. High-resolution flow cytometry and cell sorting and, most recently, single cell multiomics-based analyses provided a deeper insight into the phenotypic characterization, function, and development of diverse classes of hematopoietic cell types. The dichotomous model of divergent Th1 and Th2 responses was significantly expanded by the discovery that T lymphocytes represent a branched network of subsets, characterized by a high level of plasticity and adaptability (8). Namely, Sakaguchi's discovery of regulatory T-cell (Treg) subsets provided a significant new impetus to researchers investigating the immunological origin of allergic and autoimmune diseases and their prevention under healthy conditions and pointed out new strategies to combat those maladies (9, 10). Moreover, the discovery of new classes of effector cells and their cellular interactions added relevant evidence to the field. As one example, innate lymphoid cells (ILC) became of main interest as they have been shown to be both directly and indirectly associated with and involved in the development of allergic responses (11). This unique class of effector cells lacks a clonally distributed antigen receptor which thus resemble innate immune cells characterized by (antigen) unspecific activation, however, they exert T helper (Th)-like effector cell activities (12). According to their expression of effector cytokines and transcription factors ILC have been classified into three groups: ILC1, ILC2, and ILC3 (13). While ILC1 produce interferon-gamma (IFNγ) and tumor necrosis factor α (TNFα) and, similarly to Th1 cells, express T-bet, ILC2 are able to produce Th2 cytokines such as IL-5 and IL-13, like Th2 cells under the control of the transcription factor GATA-3. ILC3 are similar to Th17 cells and release IL-17A and IL-22 as well as granulocyte macrophage colony stimulating factor (GM-CSF). In animal models of allergic airway inflammation as well as in human allergic asthmatics ILC2 are present at elevated frequencies within the lung and airways epithelial compartments where they were found to produce high amounts of the type-2 cytokines IL-5 and IL-13 (14). Within the last years, ILC2 have been recognized as early promoters to establish and maintain allergic airway inflammatory responses but also as protectors promoting repair processes of the lung epithelium (15, 16).

A potential link between the Hygiene Hypothesis and the function of ILC lineages comes from the gut. The symbiotic interaction between immune cells and the microbiota in the gut is principally decisive for the development of tolerance or pathogenicity. The ILC3 lineage is essential in the development of lymphoid follicles and Peyer's patches in the gut and was shown to be crucial for the maintenance of a well-balanced symbiosis with the microbiota (17). The host microbiota itself might play an important role in determining ILC subsets specificity as indicated by results coming from experimental approaches. Sepahi et al. very recently reported that short chain fatty acids (SCFA) arising from dietary fibers by microbial fermentation in the intestine induced expansion of prevailing ILC subsets. By triggering ILC subset expansion *via* G-protein-coupled receptors (GPCR) those dietary metabolites contribute to the homeostasis in the local compartment (18). Another mechanism to induce repair and homeostatic conditions at epithelial surfaces is mediated *via* IL-22-producing ILC3 in response to the microbiota. In interaction with IL-18 produced by the epithelial cells, IL-22 is involved in the promotion of repair and remodeling processes as well as in the maintenance of the gut homeostasis (19). By acting as mediators between the microbiota and the host ILC are recognized as crucial in the early host response to microbial stimuli.

CHALLENGES FROM CHANGING ENVIRONMENTS – CAN EPIGENETICS PROVIDE THE MISSING LINK TO EXPLAIN "GENE-BY-ENVIRONMENT INTERACTIONS"?

Very recently, damaging factors that jeopardize the normal development or disturb the balance of an established immune system have come into the focus of research on allergic diseases. Environmental changes caused by in- and outdoor pollution (20, 21) and the global warming impact the atopic epidemic and some attempts were undertaken recently to integrate these scenarios into the concept of the Hygiene Hypothesis on the basis of epigenetic changes driven by gene-by-environment interactions (22).

In contrast to our ancestors who spent most of their life time outdoors and thus close to a natural environment, post-modern and mainly urban life-styles are characterized by a significantly higher proportion of indoor activities. These changing habits underline the potential importance of indoor air composition on the development of allergic diseases and further emphasize the role of the environmental microbiota (23). Indoor air in urban homes is often burdened with elevated levels of molds which are found to be harmful to the airways and favor the development of airway inflammation and asthma (24). Against the background of growing climate awareness and the resulting increased efforts to reduce energy consumption and CO_2 emissions, current research on these indoor exposures in homes with improved house insulation points out to an up-coming health problem. Enrichment of volatile organic compounds released from furniture or brought in by tobacco smoke as well perennial

allergens and molds will jeopardize mainly infants as the developing immune system and the growing lung are highly susceptible to these damage factors (25). Already the fetus might become affected by these components (26). This was exemplarily shown for tobacco smoke in a transgenerational case control study conducted to assess the risk for asthma by prenatal smoking. Grandmothers and mothers of asthmatic and non-asthmatic children were asked about smoking habits during their own pregnancy. The study reported an odds ratio twice as high for children to develop asthma in families where grandmothers frequently smoked during the mother's fetal period (27).

At that point the Hygiene Hypothesis was in line with an upcoming general idea that non-inherited/non communicable diseases like allergies and asthma develop on the background of an inappropriate interaction between environmental exposures and a given genotype to shape a specific (disease) phenotype. Though based on the concept of a so-called epigenetic landscape postulated by Waddington already in the 50ties of the last century, the underlying molecular mechanisms of epigenetic programming had still been the "missing link" in the scenario of gene-by-environment-interactions (28). By discovering mechanisms such as DNA methylation, diverse histone modifications and microRNA regulation as molecular mechanisms underlying epigenetic regulation of gene expression, an exciting new field of research was opened that currently has a strong impact on research aiming to unravel the still existing mysteries of allergy development and prevention (29–31).

Indeed, epigenetic mechanisms have meanwhile clearly been demonstrated to be involved in mediating the effects of environmental factors increasing or decreasing the risk of allergy development (4). Pro-allergic environmental influences can be exemplified by pollution. For instance, higher *in utero* exposure to polycyclic aromatic hydrocarbons (PAH) has been shown to be associated with increased cord blood leukocyte DNA methylation at the promoter of the IFNγ-encoding gene (32, 33). Moreover, in Treg isolated from peripheral blood mononuclear cells, higher PAH exposure has been correlated with elevated DNA methylation at the promoter of the gene encoding FOXP3, a master regulator of Treg development and activities, with the effect being stronger in asthmatic than in non-asthmatic children (34).

After epidemiological studies had demonstrated an association between spending early life time in specific agricultural environments and protection against the development of allergies in childhood (35, 36), functional investigations of various types started to clarify which elements of farming, such as contact with farm animals, consumption of raw cow's milk, exposure to so-called farm-dust, and others, mechanistically underlie this observation. DNA demethylation at the FOXP3-encoding locus related to higher expression of the gene and activation of Treg (37) has been associated in cord blood with maternal consumption of raw cow's milk (38) and in children's whole blood with early-life ingestion of raw cow's milk (39). Compared to processed shop milk, pretreatment with raw cow's milk reduced features of the disease in mice subjected to a model of food allergy and this effect was mediated by changes in histone acetylation patterns at crucial T cell-related genes (40, 41). Interestingly enough, unprocessed cow's milk has been shown to contain miRNAs potentially affecting the expression of important allergy-related immune genes, which might contribute to its protective effects against asthma (42). Several bacteria have been isolated from the farming environment, for instance *Acinetobacter lwoffii* (*A. lwoffii*), which were demonstrated to diminish the development of allergic symptoms in murine models (43). *A. lwoffii*-mediated protection against allergic airway inflammation has been observed in mouse models also transmaternally and shown to be IFNγ–dependent, with this effect being at least partly mediated by preservation of histone H4 acetylation at the promoter of the IFNγ-encoding gene as observed in $CD4^+$ T cells isolated from spleens of the offspring (44, 45).

CHALLENGES FROM THE CLINICS AND LESSONS FROM ANIMAL MODELS – ASTHMA PHENOTYPES AND THE HYGIENE HYPOTHESIS

A recurrent debate flared up in the field of asthma research excellently summarized at the time being in a review by Wenzel in 2012 (46). Coming from clinical heterogeneity of asthma patients she highlighted that basic inflammation patterns differ in asthma patients which in turn determines the success of the applied therapeutic strategy. As an early diagnosis and adequate treatment may prevent the development of a severe asthma phenotype later on, novel strategies to discriminate children at risk from those who will not develop asthma are required (47). Following the clinical definition of a phenotype as a result of an interaction between a given genotype and the environment Wenzel and colleagues expressed the strong medical need for novel molecular and genetic biomarkers indicative for the characterization of such phenotypes and defining the specific requirements for stratified therapies. Based on differences between Th2-driven atopic asthma and non-atopic asthma a number of subtypes were defined that evolve and differ with age and respond differentially to standard drug treatment regimes. It quickly became clear that the search for a specific biomarker that clearly identifies a respective phenotype would not be successful. Rather, the synopsis of all data collected from a subject known as "deep phenotyping" may lead to better understanding of complex asthmatic conditions (48). Deep phenotyping in the era of OMICS goes along with a tremendous increase in data that needs to be analyzed. To handle these big data-sets new approaches become increasingly employed involving models of statistical data dimension reduction and machine-learning strategies (49, 50). The idea behind these data-driven approaches is to mine data collections and classify them based on so far hidden patterns behind the data. The hypothesis-free latent class analysis (LCA) approach represent one of the most promising tools to identify new or verify proposed asthma (and

other allergic disease) phenotypes. A first LCA approach was carried out in two cohorts of adult asthmatics. Based on clinical and personal characteristics Siroux et al. described two distinct phenotypes in two independent cohorts, a severe phenotype in which asthma is already established in childhood and a second type that starts in adulthood with milder outcomes (51). In line with the Hygiene Hypothesis, these results pointed out specific preconditions in infant age which pave the pathway to severe asthma later in life. LCA analyses in children substantiated the link between early onset and later disease since early clinical signs such as current unremitting wheezing episodes are ascribed to indicate a higher risk for asthma development later in life while transient wheezing seems to have no pathological consequences (52).

LCA approaches using data from patient studies elucidated that there might be phenotypic asthmatic manifestations that could be explained by the Hygiene Hypothesis while other phenotypes that might have different pathomechanistic origins failed to be covered by this supposition (53). Among others, this discrepancy led to new approaches in pre-clinical animal-based experimental set-ups as well as investigations based on human data. New animal models were employed to prove the postulate of such phenotypes that can be discriminated on the immunological and histological levels. By switching from the well-established Ovalbumin (OVA) model, where the sensitization was mainly achieved by a rather artificial intraperitoneal allergen sensitization in the presence of the type-2 driving adjuvant alum, to a more flexible administration of standardized house dust mite extracts (HDM) *via* the nasal route, it was feasible to induce a more natural and broader spectrum of inflammatory phenotypes ranging from typical allergic eosinophil-dominated respiratory inflammation to airway inflammatory conditions almost exclusively dominated by the influx of neutrophils (54–56). Such more flexible model systems allow deeper and more precise investigations of the mechanisms underlying the development of different phenotypes and a much better characterization of the orchestration of different regulatory and effector T cell subsets in dependence of allergen administration on a continuum between Th2 and Th1/Th17-driven inflammation. In addition, these mouse models mimic the natural situation more closely by using common allergens and a potential natural route of sensitization and thus became helpful for understanding the diverse clinical phenotypes of allergic and non-allergic as well as mild and severe asthma (57, 58). By switching between different effector T cell responses in these experimental set-ups substantial knowledge is currently added to our understanding of clinical manifestations in asthma. In combination with LCA helping to elucidate clinical phenotypes these recent research developments strongly boosted a better discrimination between transient and persistent pediatric allergic conditions as well as allergic and non-allergic asthma later in life. This new evidence might lead us to the current limits of the Hygiene Hypothesis. While IgE-driven allergic asthma undoubtedly fits to the Hygiene Hypothesis, it is still unclear whether this holds true also for non-atopic asthma phenotypes the development of which is much more strongly determined by factors different from a missing (microbial) education of the immune system. Thus and to further fine-tune the Hygiene Hypothesis, continuous efforts are required to distinguish between environmental conditions (such as early life infection with pathogenic viruses) that are either associated with the induction of a disease phenotype and/or just contribute to a shift between distinct inflammatory manifestations of allergic disease phenotypes (59, 60) and those that really result in a general or a phenotype/endotype-specific prevention of disease in line with the Hygiene Hypothesis (43, 61).

CHALLENGES FROM A VIEW OVER THE FENCE – THE HYGIENE HYPOTHESIS IN PSYCHIATRIC DISORDERS AND CANCER

The French scientist Bach was the first who made the principal observation of a general inverse correlation in the prevalences of infectious versus non-communicable chronic inflammatory diseases within the last seven decades (62). Meanwhile we know that abundant exposure to a high diversity of infectious or even harmless microbes resulting in repeated, low-grade acute inflammatory episodes in early life, associates with lower prevalence of chronic inflammatory disorders accompanied by low levels of inflammatory markers in adulthood. Conversely, high levels of hygiene during perinatal and early childhood developmental periods characteristic for Western countries corresponds to higher levels of inflammatory markers correlating with a higher prevalence of chronic inflammatory disorders later in life. Based on these facts, it has been hypothesized that frequent episodes of low-grade, in most cases clinically symptom-free inflammation in infancy may balance responses to inflammatory stimuli and thus reduce the rate of continuation of chronic inflammation into adulthood, most probably by adequately shaping the adaptive immunity-dependent regulation (23).

Interestingly, this observation considers a broader spectrum of chronic inflammatory conditions beyond allergies that might fit under the umbrella of the Hygiene Hypothesis such as multiple sclerosis, irritable bowel disease or diabetes type 1 (63). Moreover, within the last years a similar approach emerged to explain the tremendous increase in psychiatric disorders in westernized countries. Mainly affective disorders such as major depression and bipolar disorder are increasingly diagnosed in the westernized world. Patients suffering from affective and anxiety disorders depict an array of features that mirror inflammatory conditions such as pro-inflammatory cytokines in the blood and the central nervous system accompanied by elevated levels of circulating C-reactive protein (CRP), activation of lymphocytes and inflammatory cellular signaling pathways (MAPK and NF-κB), with the question of causality remaining a chicken or egg problem (64). Nevertheless, based on genetic predispositions and epigenetic modifications in the brain (nervous system) and the periphery (immune system), both kinds of pathologies, mood and inflammatory disorders, might become established on the basis

of a disturbed homeostasis of otherwise tightly balanced adaptive systems of the body. Interestingly but fitting to the hypothesis, the microbiota of the gut seems to play a critical role also in the development of psychiatric disorders as shown by recently conducted studies (65). Based on an interplay between the gut and the central nervous system, persistent stress and maltreatment modifies the nervous system and thereby the endocrine hypothalamic pituitary axis (HPA) which in turn alters gut microbiota by cortisol release (66). Dysbiosis in the gut might lead to a compromised cytokine balance in the blood followed by an activation of the microglia in the brain after transfer of inflammatory mediators/cytokines through the blood-brain barrier (67). Further, degradation of beneficial bacteria in the gut microbiota might result in a loss of microbiota-derived products such as butyrate which directly results in the downregulation of γ-aminobutyrate, serotonin and dopamine, all factors directly involved in the neurological regulation circuits and thus in the genesis of neuropsychiatric disorders when dysregulated (68).

Finally, to add another example to this collection, there is increasing evidence that similar mechanisms as involved in the protection from allergies might also play a role in the prevention of oncologic diseases (69). There is no doubt that preceding infections with certain pathogens may favor initiation and further development of several tumor disease entities. However, a variety of recent studies also demonstrated positive effects of pathogen-induced "benign" inflammatory processes on cancer development, even though the underlying mechanisms of this dichotomous influence of microbial exposure-mediated immune modulation on carcinogenesis are not well understood so far (70). As one example, the origins of childhood leukemia have long been discussed in the context of microbial stimuli in early childhood. Already at the end of 20th century the question emerged whether early infections in childhood may act protectively against childhood acute leukemia by eliminating expanding aberrant leukocyte clones through well-trained and established immune mechanisms. In concordance with the Hygiene Hypothesis, Greaves propagated the "Delayed Infection Hypothesis" as an explanation for the development of childhood acute (lymphoblastic/myeloid) leukemia (ALL/AML) that peaks at the age of 2-5 years of life in affluent countries (71, 72). In his two hit model, Greaves proposed that based on a prenatally occurred chromosomal translocation or hyperdiploidy a pre-leukemic clone is already established around birth (first hit). A second hit event beyond the toddler age then leads to gene deletion or mutation and subsequent transformation to ALL/AML. While children suffering from infections and/or exposed to a rich microbial environment early in life might be ready to prevent that second aberration, predisposed children with an insufficiently educated immune system due to missing "old friends" contacts in the early postnatal life might not be able to eliminate expanding malignant cell clones (72). A number of studies aimed to prove this hypothesis by exploiting "day care attendance" before the third year of life as a proxy for infection. This concept is still a matter of debate. While the vast majority of these studies could add evidence to the Greaves hypothesis, some well-conducted studies could not support his assumptions (73, 74). Recently, a meta-analysis investigated the farm effect with regard to childhood leukemia and confirmed that contact to livestock provides protection not only against allergies but also against childhood leukemia (75). This study might point out to microbiota as a crucial player in both prevention of allergies and childhood cancer.

The challenges outlined in this mini review are intended to stimulate further exciting debates that might result in continuing revisions and adaptations of the Hygiene Hypothesis. We are aware that the examples reported in this review may only describe a limited subjective selection of the scientific topics currently discussed in context of the Hygiene Hypothesis. However, it is common to all topics that the explanations to unravel the underlying mechanisms refer to the close and beneficial relationship between man and microbes as established on the mucosal surfaces of our body. These interactions result in adequate shaping of adaptive systems of the body (mainly the immune system) that enables the whole organisms to appropriately handle diverse adverse influences. Without exaggeration, this finding might be considered one of the most fundamental insights of the life sciences within the last thirty years.

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The "Hygiene Hypothesis" and the Lessons Learnt From Farm Studies

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We celebrate the 30th anniversary of the "hygiene hypothesis", which has been a cornerstone for research into asthma and allergic diseases for many of us. It appeared while we witnessed the rapid increase of these conditions in the westernized world by the end of last century (1). It has stimulated thought of many researchers resulting in numerous more or less modified hypotheses meandering in diverse gestalt through the scientific landscape. It all started with an epidemiological observation about significantly decreased risk of allergic sensitization and hay fever in subjects having many siblings. This observation was counterintuitive at that time when the prevalent paradigm stated that viral infections cause asthma. However, the observation was confirmed many times in independent populations and is one of the most robust epidemiological findings in the context of allergy (2). Over the years the epidemiological gestalt changed from siblings to day care, oro-fecal and other infections and then to farm exposures. Interestingly, the farm effect is independent of the "sibling effect" (3). The gestalt also took on various immunological garments from a Th1-Th2 dichotomy to regulatory networks. Lately, the technological progress allowing exploration of the world of microbiomes has revitalized the debates around the "hygiene hypothesis" with tantalizing findings from mouse experiments and population-based studies.

The "hygiene hypothesis" has also been a cornerstone of my scientific life resulting in my continued interest in the farm populations that I have been following with many colleagues since the beginning of this century. In this modified gestalt, the farm exposures may be considered strong support for a hypothesis that may be rephrased as pointing to the importance of microbial exposures for the development of childhood asthma and allergies. The concept is intuitively easily understandable which has resulted in a widespread perception by the lay press. Yet, we still wrestle with the identification and mechanistic understanding of the relevant building blocks that may allow translation into prevention of childhood asthma and allergies. In the following, I attempt to distil some lessons from the farm studies.

The protective effect of a traditional farm exposure on the development of childhood asthma and allergies as documented in numerous studies is very robust. Similar to the allergy protective "sibling effect" and the asthma risk by exposure to moulds and active/passive smoking it is a remarkably reproducible finding across populations and continents. Moreover, the effects are strong. The most consistent finding, which relates to allergic sensitization and hay fever, shows odds ratios around 0.5 suggesting halving of risk (4). Findings for asthma seem somewhat weaker and less reproducible which may be attributable to the many facets of the asthma syndrome. These observations may suggest a strong extrinsic factor that once identified could serve as novel prevention strategy for these illnesses.

We have identified two main pillars of the protective farm effect, one being the exposure to animal sheds, in particular cowsheds and the second the consumption of unprocessed cow's milk (5). It is tempting to speculate that one unifying exposure may underlie these seemingly distinct exposures. The working group of Erika Jensen-Jarolim proposes that ß-lactoglobulin, which is found in cow's milk and urine and thus also in ambient air of cowsheds, carries farm-specific ligands which render this lipocalin tolerogenic rather than allergenic (6). Thereby ß-lactoglobulin could act as important transport protein presenting its allergy- and potentially asthma-protective cargo to competent immune cells. This concept awaits however, confirmation in mouse studies of experimental allergic asthma and allergy. If substantiated the nature of the cargo needs further investigation and the relative contribution of the transporter *versus* the cargo (and the diversity of the cargo) must be resolved. It seems conceivable that the farm environment confers not only the protective exposures, but also the transporters that enhance the protective effects by optimizing presentation to competent immune cells.

From the epidemiological observations, diversity of exposures in the farm environment has however been a central theme. We have so far not found one single component conferring protection. One must bear in mind that cowsheds and unprocessed cow's milk are "soups" containing myriads of potentially relevant elements. We have shown that an increased diversity of food introduction protects from food allergy, atopic dermatitis and asthma (7). Moreover, the diversity of farm animal exposure during pregnancy has been associated with lower risk of atopic dermatitis and higher IFN-y and TNF-α levels in supernatants of cord blood mononuclear cells stimulated with LPS (8, 9). Finally, the diversity of the environmental and human nasal microbiome, respectively, have been associated with lower risk of asthma in the farm populations (10).

A strong signal with diversity results in a low likelihood to find the one "magic bullet" explaining the protective associations. It can in turn be interpreted as a multitude of additive (weak) effects interacting with a multitude of host factors in the general population which is made up of subjects with very diverse genetic and immune response backgrounds. Alternatively, diversity may harbour a limited number of relevant, necessary and sufficient elements or hubs in exposure networks which drive the protective effects. We have some evidence that these necessary elements exist because in experimental studies of farm exposure, i.e. extracts from cowshed dust extracts do no longer protect mice from allergic asthma when they are devoid of MYD88/TRIF and epithelial A20 signalling (11, 12). Thereby, innate immune responses may be essential elements for the protective effect, but the precise nature of these elements stills awaits elucidation.

The complexity of the interplay of protective elements may be further increased by the multitude of exposure routes that may matter. Environmental exposures such as the indoor microbiome or the stay in cowsheds may be inhaled or ingested. In fact, we have seen that both the nasal and gut microbiome are influenced by these external exposures because young children breathe in airborne matter and put their contaminated fingers in their mouth thereby ingesting external compounds. In addition, ingestion of relevant exposures such as unprocessed cow's milk or a diverse introduction of solid foods further shapes the gut microbiome and its development (13). We have unfortunately not investigated skin exposures, but these may also add to the complex interplay of farm exposure routes.

We are therefore left with the impression that key elements of protection within the farm environment affect a number of body compartments (upper and lower airways, gut and skin) in probably redundant and overlapping pathways, which may in turn confer protection for a large majority of exposed subjects with rather diverse genetic and immune response backgrounds.

If this notion was correct, then translation into novel asthma preventive approaches will have to be multifaceted. As discussed in a recent review (14), it seems unlikely that asthma phenotypes are distinct conditions with distinct underlying pathologies resulting in exclusive unambiguous disease categories. Complex diseases such as asthma are more likely to consist of combinations of various traits and underlying redundant mechanisms given the many weak genetic and environmental effects and their interactions on disease development. In such a scenario the one and only causal mechanism can neither be found nor successfully targeted for prevention. Then only some facets of the disease will be addressed which however results in weak effects on a population level. In a complex disease a combination of a multitude of involved mechanisms matters which should preferably all be targeted if all of asthma should be prevented. Given that the "farm effect" on asthma is strong across multiple populations it seems more likely that the multitude and diversity of exposures across several routes of exposures (upper and lower airways, gut, skin) contributes to the overall protective effect.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Excessive Unbalanced Meat Consumption in the First Year of Life Increases Asthma Risk in the PASTURE and LUKAS2 Birth Cohorts

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A higher diversity of food items introduced in the first year of life has been inversely related to subsequent development of asthma. In the current analysis, we applied latent class analysis (LCA) to systematically assess feeding patterns and to relate them to asthma risk at school age. PASTURE (N=1133) and LUKAS2 (N=228) are prospective birth cohort studies designed to evaluate protective and risk factors for atopic diseases, including dietary patterns. Feeding practices were reported by parents in monthly diaries between the 4th and 12th month of life. For 17 common food items parents indicated frequency of feeding during the last 4 weeks in 4 categories. The resulting 153 ordinal variables were entered in a LCA. The intestinal microbiome was assessed at the age of 12 months by 16S rRNA sequencing. Data on feeding practice with at least one reported time point was available in 1042 of the 1133 recruited children. Best LCA model fit was achieved by the 4-class solution. One class showed an elevated risk of asthma at age 6 as compared to the other classes (adjusted odds ratio (aOR): 8.47, 95% CI 2.52–28.56, p = 0.001) and was characterized by daily meat consumption and rare consumption of milk and yoghurt. A refined LCA restricted to meat, milk, and yoghurt confirmed the asthma risk effect of a

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particular class in PASTURE and independently in LUKAS2, which we thus termed unbalanced meat consumption (UMC). The effect of UMC was particularly strong for non-atopic asthma and asthma irrespectively of early bronchitis (aOR: 17.0, 95% CI 5.2–56.1, $p < 0.001$). UMC fostered growth of iron scavenging bacteria such as Acinetobacter (aOR: 1.28, 95% CI 1.00-1.63, $p = 0.048$), which was also related to asthma (aOR: 1.55, 95% CI 1.18-2.03, $p = 0.001$). When reconstructing bacterial metabolic pathways from 16S rRNA sequencing data, biosynthesis of siderophore group nonribosomal peptides emerged as top hit (aOR: 1.58, 95% CI 1.13-2.19, $p = 0.007$). By a data-driven approach we found a pattern of overly meat consumption at the expense of other protein sources to confer risk of asthma. Microbiome analysis of fecal samples pointed towards overgrowth of iron-dependent bacteria and bacterial iron metabolism as a potential explanation.

Keywords: asthma, Infancy, cow's milk, meat, introduction of solid foods, nutritional immunity, gut microbiome, latent class analysis

INTRODUCTION

The development of childhood-onset asthma is certainly affected by familial factors (1). The latter involve both genes and environment, two seemingly opposing principles, often paraphrased by the paronomasia of nature and nurture. Our take on this dichotomy, however, has been revolutionized by the recent advances of microbiome research. With a strong influence on our microbiome, nutrition shapes our metagenome. In other words, what we eat essentially determines how we feed our microbial patrons and which genes we borrow from them.

This is particularly true for the first year of life, when the microbiome undergoes profound changes during the transition from breastfeeding to supplemental foods (2, 3). In the PASTURE study we have already seen that this maturation process depends on environmental stimuli and can influence the development of asthma (4). One stimulus that has been inversely related to the development of allergic diseases is the diversity of foods introduced during the first year of life (5).

In the present work, we go a step beyond diversity and explore specific feeding patterns in the first year and their relation to asthma at school age. The PASTURE birth cohort provides a unique dataset on 19 food items and the intensity of feeding from monthly over weekly to daily feeding recorded on a monthly basis. We hypothesized that specific feeding patterns exist, which depend on distinct but unknown phenomena involving different cultural backgrounds. We applied latent class analysis (LCA) to this large dataset to handle the complexity of information and to estimate patterns. To complement nurture in its literal meaning with a flavor of nature in the sense of metagenome we related feeding patterns to the gut microbiome at 12 months.

METHODS

Study Design and Population

Both birth cohorts were set up to study the development of childhood asthma and allergies and pertinent early risk factors. PASTURE recruited 1133 children in 2002-2005 from rural areas in 5 European countries: Austria, Finland, France, Germany, and Switzerland (6). Children of mothers living on family-run livestock farms were assigned to the farm study group. The reference study group comprised children of mothers from the same rural areas but not living on a farm. The independent population-based LUKAS2 cohort was established in analogy to the Finnish arm of PASTURE. All pregnant women with scheduled delivery at Kuopio University Hospital between May 2004 and May 2005 were invited to the study without selection by area of living or occupation (7). Both studies were approved by the ethics committees of the participating institutions, and written informed consent was obtained from the children's parents or guardians.

Questionnaires

In both cohorts, the same questionnaires were administered at the end of pregnancy and when the children were 2, 12, 18, 24, 36, 48, 60, and 72 months of age to obtain information on frequencies of wheeze, parental atopic status, and environmental exposures with a focus on farming and nutrition (5, 8). Parents reported feeding practices by weekly and monthly diaries between month 2 and 12 with introduction of solid foods mainly from month 4 onwards. Consumption of the 19 food items (bread, cake or cookies, cereals with and without gluten, meat, fish, egg, milk, yoghurt, butter, other milk products, margarine, fruits, vegetables, nuts, chocolate, sugar and sweets, soy, cod-liver oil) was asked every four weeks in categories of at least daily, weekly, monthly or less than monthly. For most items, the questionnaire differentiated between home-made and finished products. The question on meat did not specifically focus on read meat; rather it included all sorts of meat but not fish. Asthma was defined as parental report of doctor-diagnosed asthma ever or recurrent doctor-diagnosed episodes of obstructive bronchitis between 3 and 6 years. The questionnaires were developed for the PASTURE and LUKAS2 studies based on questionnaires from the ALEX study, which were validated against a food frequency questionnaire (6, 9).

Lung Function Measurements

In PASTURE, at the age of six years in 799 children, forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), and FEV1/ FVC-ratio was measured and z-standardized (8, 10).

Genetics

Genotyping in the PASTURE study was performed at the Centre National de Génotypage, Evry, France, as previously described (11). For the interaction analysis, the SNPs were coded additively.

Fecal Samples

Fecal samples were collected at the age of around 12 months and processed as described previously (4). In brief, DNA was extracted from the fecal samples at a central laboratory (THL Kuopio, Finland). For sequencing, primers F515 (5′–NNNNNNNNGTGTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–GGACTACHVGGGTWTCTAAT–3′) were used to amplify the V4 region of the 16S rRNA gene (12). Demultiplexed data was imported into QIIME2-2018.661 and quality trimmed. Reads were denoised using DADA262 as implemented in QIIME2. Taxonomy was assigned to representative sequences using a naïve Bayes classifier pre-built from the 99% GreenGenes database65 specific to the 515F/806R region (13). The 16S rRNA amplicon sequence variants were used as input for prediction functional abundances using Phylogenetic Investigations of Communities by Reconstruction of Unobserved States 2 (PICRUST2) v.2.1.3-b software (14). The PICRUST2 pipeline was run with default parameters and a total of 170 KEGG pathways were inferred from the predicted KEGG ORTHOLOGY (KO) abundance table.

Assessment of mRNA Expression at Year 1 in Peripheral Blood

Assessment of mRNA was performed as described previously (15). In brief, peripheral blood samples were collected at the age of 1 year in a PAXgene® Blood RNA tubes and frozen to -80°C within 24 hours. At the central laboratory of the Children's Hospital of Zürich, RNA was isolated, and mRNA was reverse-transcribed into cDNA. Quantitative real-time PCR was performed on the 7900HT Fast Real-Time PCR System using the Micro fluidic card TaqMan Array system of Applied Biosystems. Data presented are normalized values for the endogenous controls (18S rRNA and beta-2-microglobulin) according to the manufacturer's instructions (Applied Biosystems).

Statistical Analysis

Calculations were performed with SAS 9.4 (The SAS Institute, Cary, NC), MPLUS 8.2 (Muthén & Muthén), and R (R Core Team, 2019). Calculation of food introduction patterns was based on all months with at least 25% of children consuming any solid foods, resulting in the period from month 4 to 12, altogether covering 9 months. Only food items with a frequency of at least 5% over this period were considered, thereby excluding soy and cod-liver oil and thus resulting 17 of the 19 asked food items. These were used as categorical variables or dichotomized at daily, weekly, or monthly consumption, respectively. In the main analyses, we used the highest frequency of the two respective production styles, i.e., home-made and finished products, whereas in sensitivity analyses we considered them individually.

Food introduction patterns were determined by a latent class analysis (LCA) based on 4-staged ordinal variables (SAS PROC LCA Version 1.3.1 and MPLUS for cross-validation). This approach considers food introduction as a latent phenomenon, which influences the time point of introduction and frequency of consumption of any food item. The LCA computes a predefined number of latent classes (LC) and models the posterior probability of belonging to each class for each individual, thereby handling missing values of up to 8 of the 9 assessed time points per food item. Individuals are then assigned to a single LC by their highest posterior probability. The appropriate number of LCs was determined by the entropy, i.e., the sum of all highest posterior probabilities over all individuals, and the sample size adjusted Bayesian information criterion (aBIC) to render the two studies with different sample sizes comparable. If both measures did not reveal the same optimal solution, a compromise was sought considering the solution with the pronounced increment in entropy at a relatively low aBIC. The resulting LCs were labeled according to their key features where necessary. To support the identification of the key features of the classes, random forest prediction models were established (R package ranger), and variable importance was used to compare the contribution of individual variables to the prediction model.

Associations of LCA classes with potential determinants, disease outcomes and composition or functional features of the microbiota were calculated by linear or logistic/multinomial regression. Effect estimates are given with 95%-confidence intervals as adjusted odds ratios (aOR) for dichotomous outcomes and β-estimates (aß) for continuous outcomes such as lung function parameters. All regression analyses were adjusted for the study group (farm versus reference), sex, and parental history of any atopic disease in both cohorts, and in PASTURE additionally for study center and atopy. The latter was defined as a combination of the "severe" and "symptomatic" atopy phenotypes as defined previously based on measurements of allergen-specific IgE (16).

RESULTS

The analysis population, as defined by available data on feeding behavior at least once per month, consisted of 1042 children (92%) from the PASTURE birth cohort (N=1133) and 204 children (89%) from the LUKAS2 cohort (N=228, **Figure 1**). Nearly 40% of the children did not have any missing values for feeding patterns, and about 90% of children had complete values for at least 50% of the variables used for calculating feeding patterns.

The LCA on introduction of 17 food items at 4 frequency categories over 9 months in the PASTURE study revealed

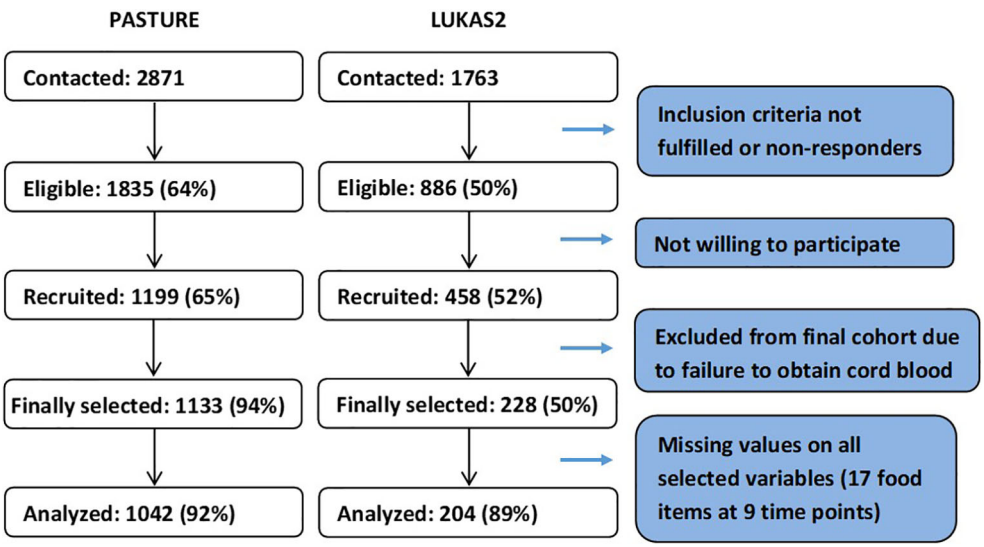

FIGURE 1 | Selection of study populations.

solutions with 2 to 6 classes with the 4-class solution scoring best for entropy and sample size adjusted BIC (**Table 1**). **Figure 2** illustrates the 4 resulting food introduction patterns in relation to age, frequency, and food items. The classes LC1 (n=165) and LC2 (n=173) showed a rather early introduction pattern with common daily consumption of fruits and vegetables. LC1 was additionally characterized by daily consumption of milk and milk products, which were considerably less frequent in LC2. The hallmark of LC2 was daily consumption of meat and cereals. In contrast, LC3 (n=414) and LC4 (n=290) revealed a moderate but diverse increase of food items, with daily feeding of fruits and vegetables in less than half of the infants. Monthly and weekly consumption revealed delayed introduction in LC3 as compared to LC4. The asthma prevalence in LC2 (14.7%) was much higher as compared to the other LCs (aOR for LC2 vs. the pooled other LCs: 8.47, 95% CI 2.52–28.56, p = 0.001).

To better understand this phenomenon, we reduced the complexity of **Figure 2** by contrasting the asthma risk class LC2 against all other LCs and by grouping the food items by key macronutrients, i.e. carbohydrates, fat, proteins, and the group of fruits/vegetables (**Figure 3**). For fruits/vegetables and fats no relevant differences were noted, whereas among carbohydrates, consumption of cereals occurred earlier and was more common in the asthma risk class LC2. The most striking difference between LC2 and the other LCs, however, was the common daily consumption of meat with hardly any other protein source in LC2.

For determining a potential impact of these discrepancies on the asthma risk, we predicted asthma by a random forest model including contrasts between meat and all other protein sources, as well as contrasts between cereals and the other carbohydrate sources. The resulting most important variables for the prediction of asthma were "daily meat but no daily milk intake in month 11", and "daily meat but no daily yogurt intake in month 11", followed by variables mostly dealing with meat and milk products between month 7 and 11 (**Figure 4**). In a logistic regression model, the variables "daily meat but no daily milk intake in month 11" and "daily meat but no daily yogurt intake in month 11" were strongly associated with asthma (aOR: 2.59, 95% CI 1.31-5.13, p = 0.006 and aOR: 2.59, 95% CI 1.22-5.48, p = 0.013, respectively). On the opposite, the contrast between cereals and bread was not significantly associated with asthma (aOR: 2.04, 95% CI 0.77-5.43, p = 0.152), which led us to focus the subsequent analyses on the protein variables.

Therefore we repeated the LCA restricting food variables to the three items meat, milk, and yoghurt, which yielded a 7-class solution with the best model fit (**Table 1**). A smaller asthma risk

TABLE 1 | Model fit criteria of the latent class analyses.

Study	Food items	Number of classes	Entropy	Sample size adjusted BIC
PASTURE	All 17 food items	2	0.964	211771.13
		3	0.976	206688.52
		4	**0.985**	**203558.50**
		5	0.979	206923.63
		6	**0.986**	204405.74
PASTURE	Meat, milk, and yoghurt	2	0.921	27813.77
		3	0.909	26555.80
		4	0.916	25621.61
		5	0.924	25103.03
		6	0.924	24679.64
		7	**0.935**	24374.79
		8	0.931	**24171.74**
LUKAS2	Meat, milk, and yoghurt	2	0.878	4324.76
		3	0.914	4204.42
		4	0.914	**4201.71**
		5	0.953	4232.09
		6	**0.960**	4310.01

The best solutions are underlined and local extrema are marked in bold. BIC, Bayesian information criterion.

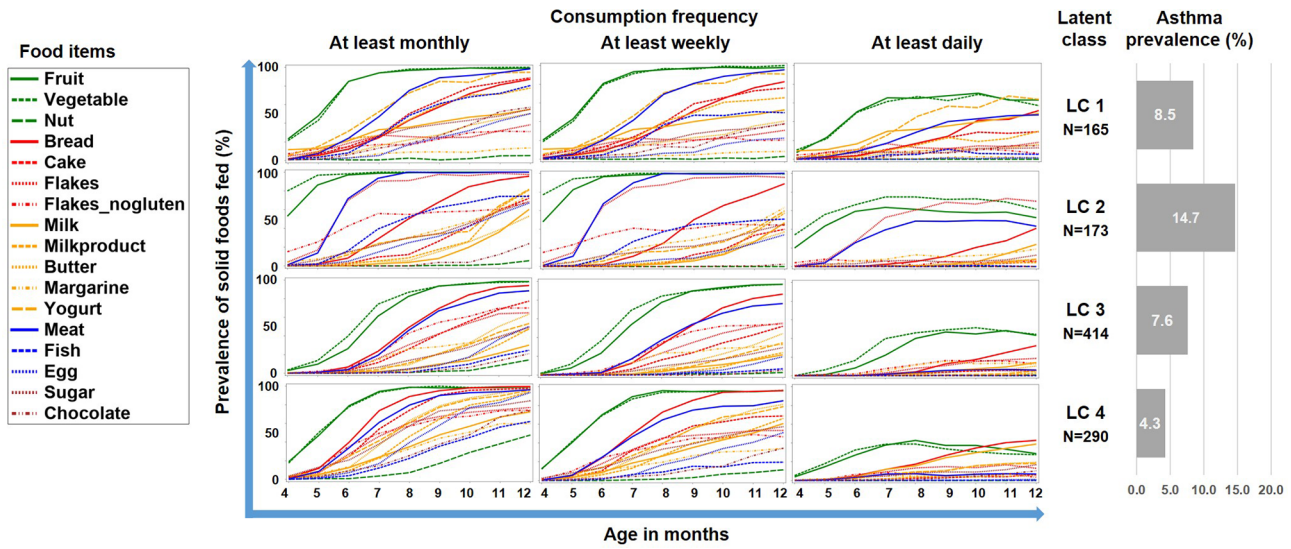

FIGURE 2 | Food introduction styles in PASTURE as defined by a latent class analysis of consumption of 17 food items over 9 months.

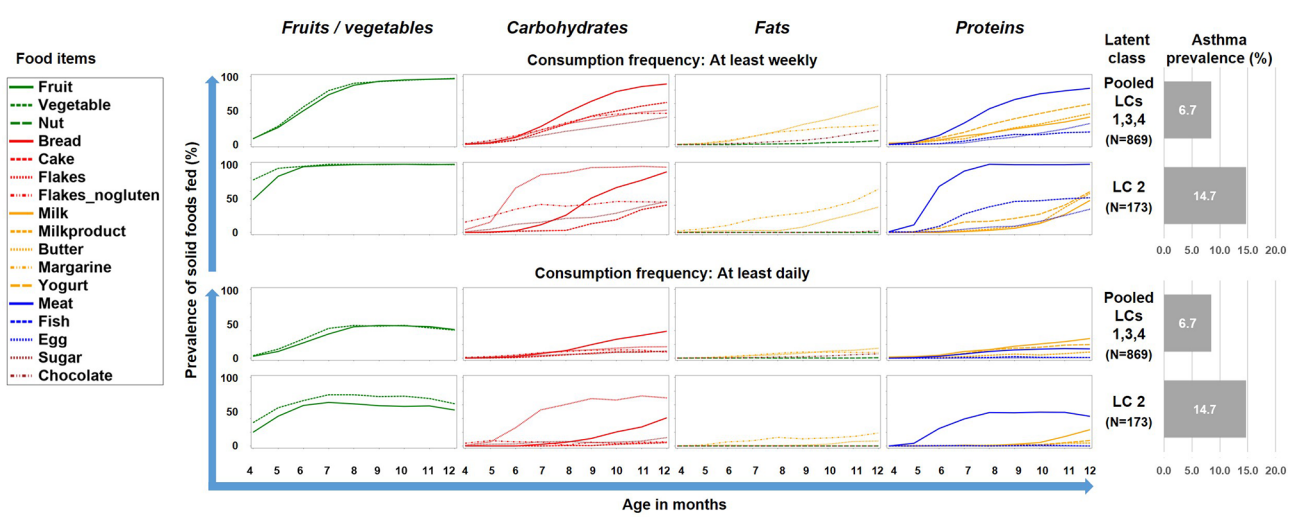

FIGURE 3 | Food introduction styles stratified by macronutrients and fruits/vegetables. The asthma risk class LC2 is contrasted with a pool of the all other class (LC1, LC3, and LC4).

class (LC7, n=120) emerged with a more pronounced asthma risk (20.2% asthma cases, aOR: 6.73, 95% CI 2.77-16.34, p < 0.001) as compared to all other LCs (6.4% cases, **Figure 5**). At any consumption frequency from monthly to daily, the contrast between excessive meat and low milk / yoghurt consumption was obvious in the asthma risk class LC7. We therefore termed LC7 "unbalanced meat consumption" (UMC). The pool of all other LCs showed a rather balanced pattern of meat and milk / yoghurt intake (**Figure 5**). The hallmark of the class with the lowest asthma risk (LC1, 2.6%) was less frequent meat consumption as compared to milk consumption (aOR: 1.28, 95% CI 1.18-2.03, p = 0.001).

As illustrated by **Figure 6**, LC7/UMC was most common in Finland though it was present in all study centers. The effect of UMC on asthma in the entire PASTURE population (aOR: 6.73, 95% CI 2.77–16.34, p < 0.001) was also present within the Finnish arm (aOR: 13.42, 95% CI 3.17–56.89, p < 0.001), and with borderline significance in the other centers (aOR: 3.99, 95% CI 0.96–16.63, p = 0.057). To exclude residual confounding by center, we replicated the LCA in a purely Finnish cohort, i.e. LUKAS2. In this smaller sample, the 5-class solution provided the best compromise of entropy and sample size adjusted BIC (**Table 1**). The LUKAS2 LCA identified a class (LC5) with a similar pattern of unbalanced meat consumption and elevated

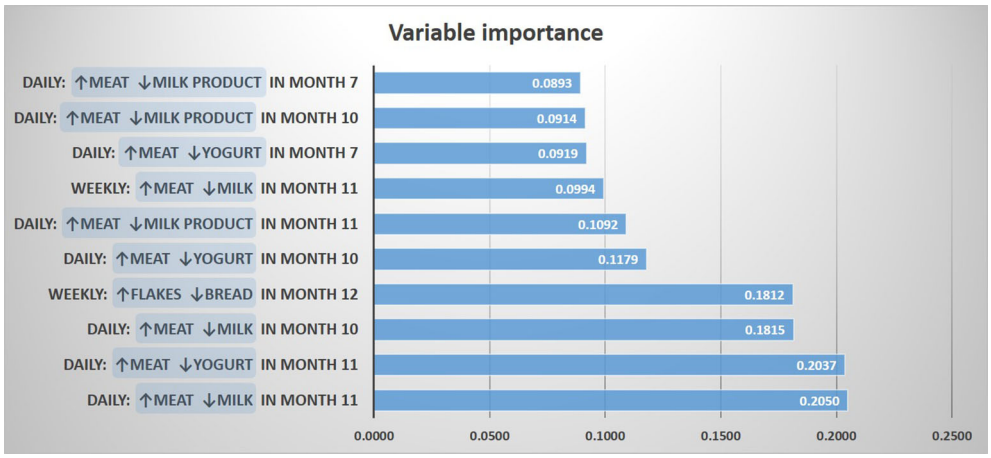

FIGURE 4 | Prediction of asthma by protein and carbohydrate sources. The 10 most important prediction variables representing contrasts between food items are shown.

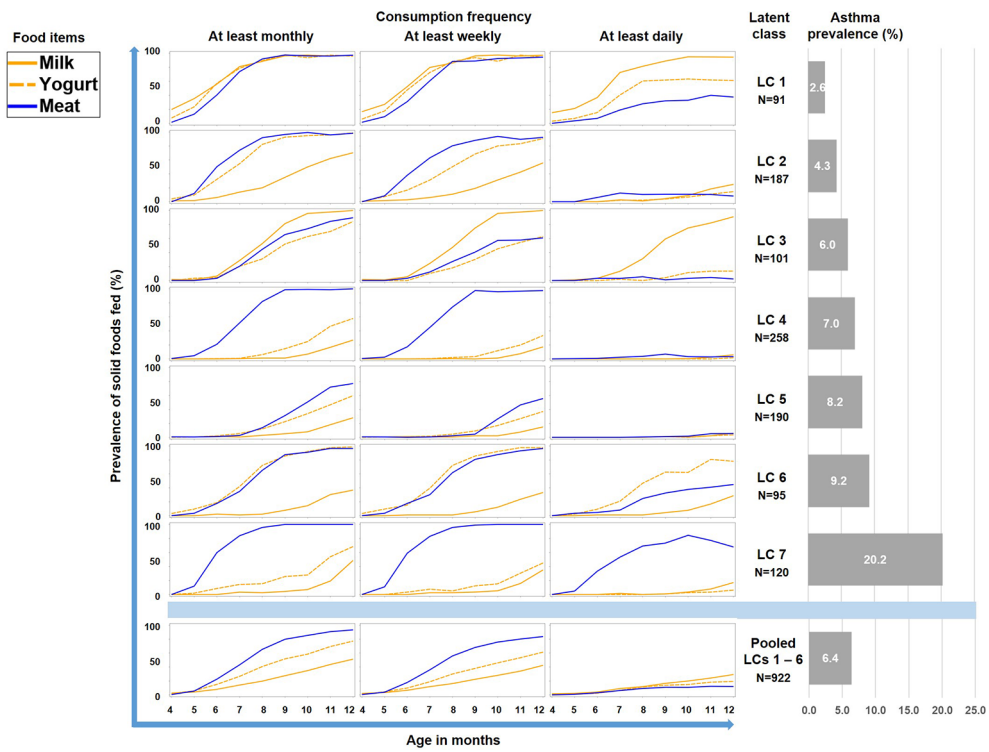

FIGURE 5 | Food introduction styles in PASTURE as defined by a latent class analysis of consumption of meat, milk, and yoghurt over 9 months from month 4 to 12.

asthma risk (aOR=2.32, 95% CI 1.06–5.08, p = 0.035, **Figure 7**), thereby replicating the findings from PASTURE.

In PASTURE, the effect of UMC on asthma at age 6 (aOR=6.73, 95% CI 2.77–16.34, P < 0.001) persisted until age 10 (aOR=4.35, 95% CI 1.91–9.93, P < 0.001, **Figure 8A**). The effect on atopic asthma was much weaker as compared to non-atopic asthma. When defining an asthma diagnosis irrespectively of recurrent bronchitis, the risk effect was stronger (aOR=17.0, 95% CI 5.2–56.1, p < 0.001). UMC was also significantly associated with current wheeze until age 10 with few exceptions (**Figure 8B**). For wheeze at month 18, we found significant associations of UMC only in the asthma risk strata of the single-nucleotide polymorphisms at the 17q21 locus related to ORMDL3 (rs8076131) and GSDMB (rs7216389 and rs2290400, all p-values for interaction < 0.001).

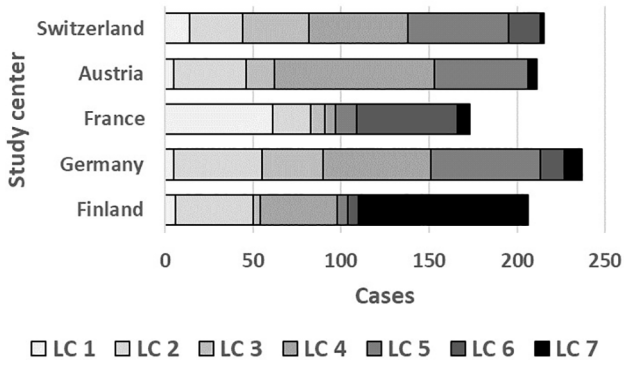

FIGURE 6 | Distribution of latent classes across study centers in PASTURE.

Furthermore, UMC was associated with parameters indicating poor lung function at age 6 such as FEV1 (aβ=-0.49, 95% CI -0.82; -0.16, p=0.0040) and FVC (aβ=-0.54, 95% CI -0.88;-0.19, p = 0.0023).

A sensitivity analysis demonstrated that the effect of UMC on asthma (aOR=7.57, 95% CI 3.03-18.93, p < 0.001) did not change when adjusting for duration of breastfeeding (aOR=7.58, 95% CI 3.03-18.98, p < 0.001) or formula feeding (aOR=7.62, 95% CI 3.04-19.09, p < 0.001). UMC, however, significantly interacted with the effects of duration of breastfeeding and formula feeding on asthma (**Figure 9**). In children with shorter breastfeeding or prolonged formula feeding , UMC increased the risk of asthma, impairment of lung function, and weight gain until year 2 (**Table 2**). Furthermore, UMC was related to a biomarker of intestinal inflammation: CD40L expression was increased in children with UMC, particularly when duration of breastfeeding was shorter (aβ=0.89, 95% CI 0.14-1.64, p = 0.0198, **Table 2**).

The asthma risk effect was particularly strong with UMC at 10 to 11 months of age (**Figure 10**). When zooming in on this vulnerable window, the effect showed a threshold phenomenon with a risk only at daily meat consumption over both months, whereas the beneficial counterbalancing effect of milk consumption tended to increase continuously with amount of consumption (**Figures 11A, B**, respectively). The effect of UMC was hardly related to industrial processing of meat (finished products versus homemade meat meals), whereas for farm milk the protective effect was stronger as compared to industrially processed milk (**Figure 11C**).

As we could not measure direct effects of dietary patterns on the gut physiology, we used the available gut microbiome data as a proxy for any impact of the feeding patterns. UMC influenced the composition of the gut microbiome at 12 months and fostered genera such as Lactococcus (aOR=1.88, 95% CI 1.01-3.5, p = 0.046), Granulicatella (aOR=1.74, 95% CI 1.01-2.98, p = 0.045), and Acinetobacter (aOR=1.28, 95% CI 1.00-1.63, p =

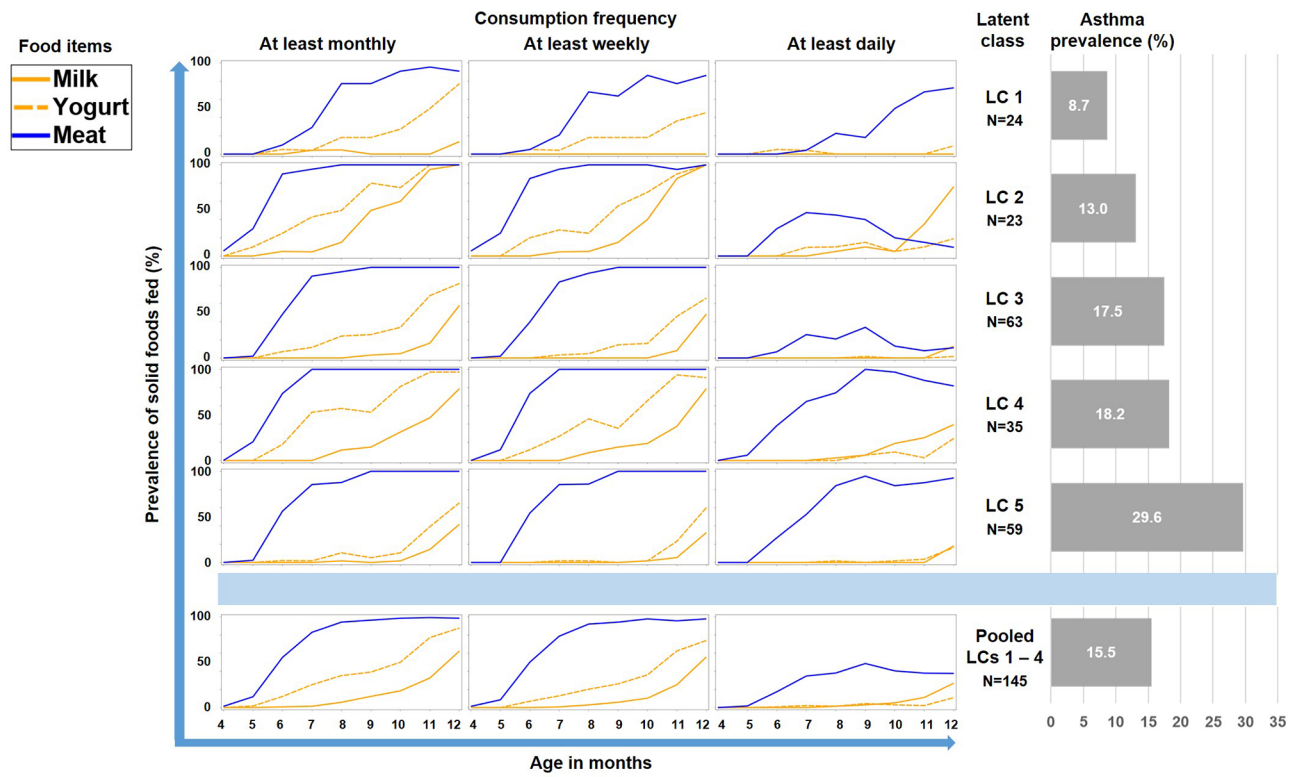

FIGURE 7 | Food introduction styles in LUKAS2 as defined by a latent class analysis of consumption of meat, milk, and yoghurt over 9 months.

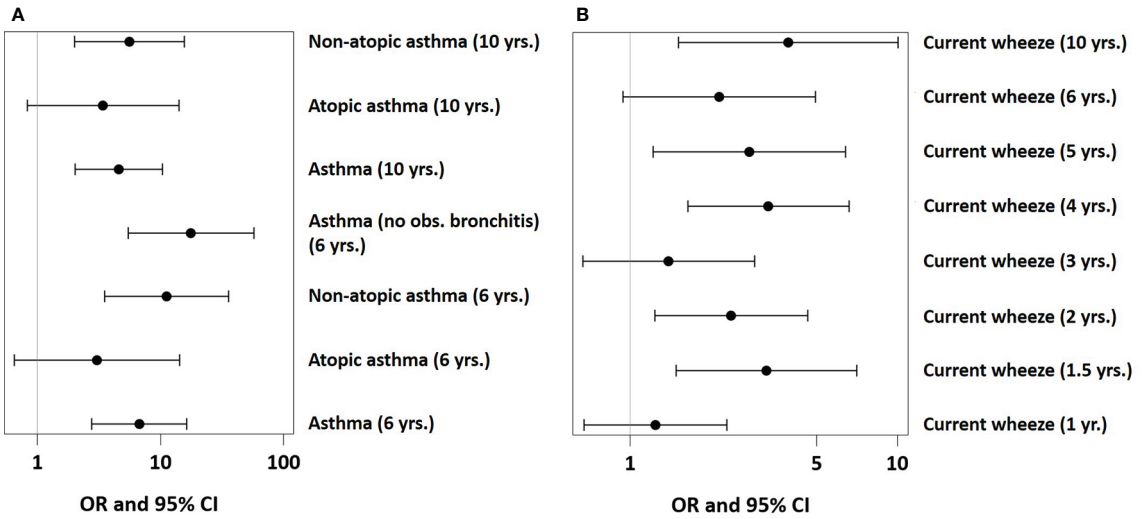

FIGURE 8 | Effects of UMC on asthma (panel **A**) and wheeze (panel **B**) phenotypes in PASTURE.

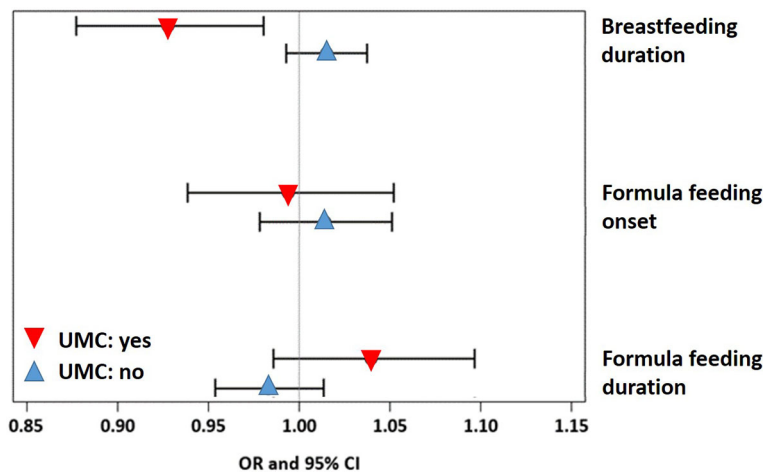

FIGURE 9 | Associations of asthma with breastfeeding and formula feeding stratified by UMC.

0.048, **Figure 12A**), of whom the latter genus was also related to asthma (aOR=1.55, 95% CI 1.18-2.03, p = 0.001). Most of the Acinetobacter amplicon sequence variants (ASVs) were compatible with the species johnsonii and calcoaceticus / pittii (**Table 3**). For the ASV compatible with calcoaceticus / pittii, the association was particularly strong at borderline significance (aOR=2.62, 95% CI 0.93-7.39, p = 0.068). To explore potential influences of dietary patterns on functional properties of the microbiota, we explored metabolic pathways of the microbiota and their relations to UMC. Biosynthesis of siderophore group nonribosomal peptides showed the strongest association with UMC (aOR=1.58, 95% CI 1.13-2.19, p = 0.007, **Figure 12B**).

DISCUSSION

By systematically assessing feeding patterns in the first year of life we discovered a feeding type associated with high risk of asthma at school age. We termed the pattern 'unbalanced meat consumption' (UMC) because daily intake of meat was by far the predominant protein source. This pattern was rather common in Finnish children; the association with asthma, however, was independent of center in the multicenter PASTURE birth cohort and found similarly within the independent Finnish LUKAS2 population. Prolonged formula feeding potentiated the detrimental effect of UMC on asthma,

TABLE 2 | Effects of UMC stratified by duration of formula feeding and breastfeeding.

	All children	Formula feeding up to 28 weeks of life	Formula feeding more than 28 weeks of life	Breastfeeding up to 19 weeks of life	Breastfeeding more than 19 weeks of life
Asthma 6 years	aOR=7.57 [3.03-18.93] **p< 0.0001**	aOR=5.34 [0.89-32.01] p= 0.0666	aOR=12.1 [3.9-37.8] **p< 0.0001**	aOR=11.61 [3.95-34.17] **p< 0.0001**	aOR=4.14 [0.66- 26.19] p= 0.1309
FEV1 z-score	aβ=-0.54 [-0.89; -0.19] **p= 0.0025**	aβ=-0.35 [-1.02; - 0.32] p= 0.3000	aβ=-0.65 [-1.06; -0.25] **p= 0.0015**	aβ=-0.79 [-1.25; -0.34] **p= 0.0007**	aβ=-0.20 [-0.75; +0.35] p= 0.4666
Weight change (g) birth – 2 years	aβ=286 [-116; +689] p= 0.1627	aβ=-207 [-867; -452] p= 0.5375	aβ=+504 [-6; +1015] p= 0.0527	aβ=+678 [+144; +1212] **p= 0.0128**	aβ=-257 [-877; +363] p= 0.4160
mRNA expression CD40L 1 year	aβ=+0.43 [-0.06; +0.93] p= 0.0847	aβ=+0.11 [-0.51; +0.72] p= 0.7329	aβ=+0.65 [-0.07; +1.37] p= 0.0776	aβ=+0.89 [+0.14; +1.64] **p= 0.0198**	aβ=-0.15 [-0.73; +0.43] p= 0.6020

Duration of formula feeding and breastfeeding was dichotomized at the cut-off where the interaction for the effect on asthma was maximized. Significant associations are printed in bold. All effects were calculated in the subset of children with data for formula feeding weeks (N=972) and breastfeeding weeks (N=1069).

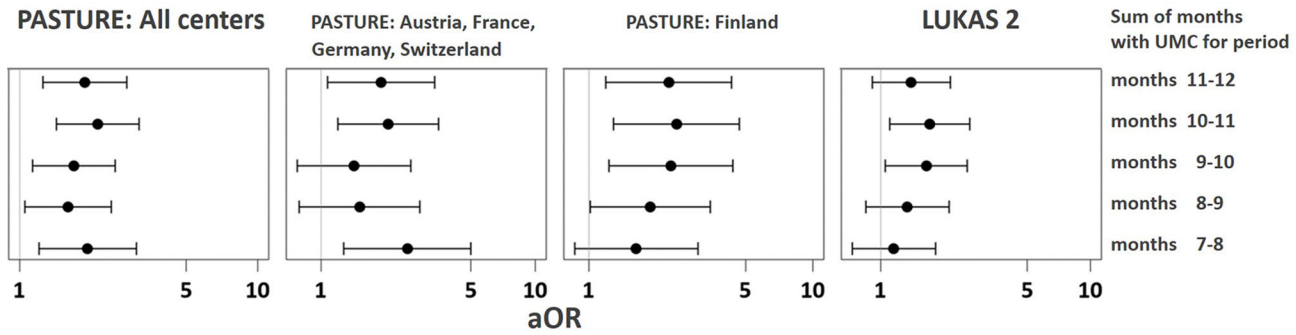

FIGURE 10 | Vulnerable window for the effect of UMC on asthma at age 6. UMC is coded as daily meat consumption without milk or yoghurt consumption at least on a weekly level.

lung function, weight gain, and induced inflammation as measured by expression of CD40L. UMC boosted growth of *Acinetobacter* and *Christensenella*, which have previously been found asthma risk genera (4, 17). In addition, UMC fostered growth of bacteria with the capability to synthesize siderophore peptides.

Latent class analysis (LCA) offers a data-driven approach to unveil latent factors behind measurable variables (18). Therefore, we used this approach to perform an unbiased analysis of the complex phenomenon of introduction of solid foods during the first year of life. Though the diaries did not provide information on serving size or the precise weight of foods, they covered information on frequency of feeding from monthly over weekly to daily. This information was particularly helpful as it allowed differentiation of feeding patterns by intensity and ultimately revealed the asthma risk class with daily meat consumption. Furthermore, the usage of LCA discovered specific combinations of foods such as the balanced combination of meat and milk / yoghurt consumption (LC1 in **Figure 5**) with its low asthma risk. This created a strong contrast to the asthma risk class (UMC, i.e. LC7 in **Figure 5**), in which consumption of milk or yoghurt was very rare. We considered the strong preference of meat over other protein sources an obvious misbalance and thus termed the risk phenomenon 'unbalanced meat consumption' (UMC).

Though being aware of the disease relevance of the diversity of foods introduced during the first year (5), we were surprised by the clear-cut risk class emerging from the primary LCA. To understand better this phenomenon we first stratified the 17 food items by macronutrients and found protein and carbohydrate sources to be relevant. When predicting asthma directly from the food items using a random forest model, we realized that the characteristic intensive consumption of flakes in the asthma risk group was only an epiphenomenon to the essential contrast between meat and milk / yoghurt consumption. Milk products other than yoghurt (e.g. butter) seemed to be less important.

Under the assumption that other food items might have increased statistical noise, we refined the LCA by restricting the food items entered in the model to meat, milk, and yoghurt. This advanced LCA sharpened the contrast between meat and milk / yoghurt consumption and its effect on asthma. Since the selection of entry variables was based on the previous random forest for asthma, the stronger association was expected and possibly an effect of circular reasoning. Therefore, we replicated this finding in an independent population. The latter consisted only of Finnish

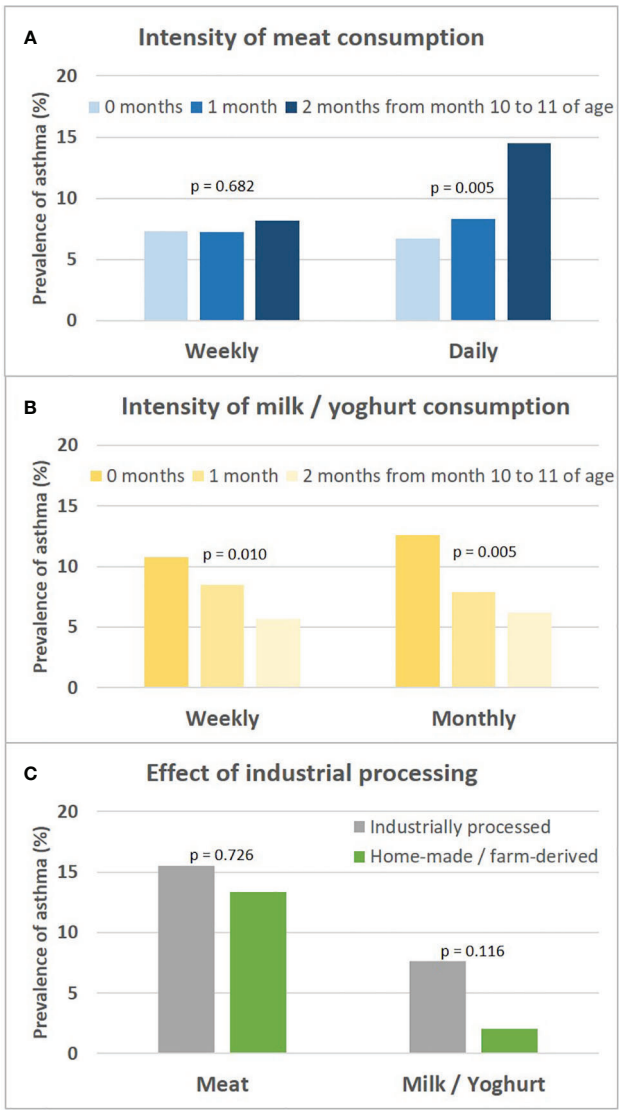

FIGURE 11 | Sensitivity analyses on intensity of meat and milk or yoghurt consumption and the role of industrial processing. Shown is the prevalence of asthma in relation to the intensity of meat and milk/yoghurt consumption during month 10 and 11 (**A, B**, respectively). **(C)** presents prevalence of asthma in relation to industrial food processing; for meat, excessive consumption (daily over both months 10 and 11) and for milk, moderate consumption (weekly either during month 10 or 11) is displayed. For bivariate comparisons, we used the Chi-square or Fisher's exact test, for trends we used the Cochran-Armitage trend test.

children, which at the same time excluded confounding by center, country, or possibly by genetic background.

The effect of UMC in the first year was even detectable at 10 years, when asthma risk was still positively associated with UMC. Consequently, we assume that the feeding pattern resulted in fundamental, potentially epigenetic, changes persisting over at least 10 years and impacting on objective lung function parameters. Since the effect was stronger on non-atopic asthma, an allergic component in the pathomechanism seems less probable. The stronger effect on asthma irrespectively of early recurrent bronchitis also renders involvement of viral infections in the pathogenesis unlikely. Though UMC was also associated with early wheeze, this association was restricted to the asthma risk genotype. Early wheeze in individuals with genetic asthma risk encoded on chromosome 17q21 is highly suggestive of subsequent asthma rather than just transient viral wheeze (19).

To better characterize UMC we performed various sensitivity analyses and found the effect of UMC particularly pronounced at 10 to 11 months of age thereby suggesting a susceptible window. Moreover, the asthma risk was only present when daily consumption was maintained over both month 10 and 11 supporting the notion that quantity and duration of unbalanced meat consumption matters. Whereas for milk and yoghurt the well-known effect of unprocessed milk on asthma was detected (9), there was hardly any influence of industrial processing on the effect of UMC, pointing towards meat itself as the culprit irrespectively of artificial additives. However, we have not asked for the processing status of meat in full detail, thus we might have missed a detrimental effect by industrially processing as described for meat consumption and impaired lung function in adults (20).

Since concomitant milk consumption seemed to abrogate the detrimental effects of excessive meat, we explored also other types of milk that were excluded from the LCA of supplemental foods by definition, i.e. breast milk and formula milk. We found short duration of breastfeeding and prolonged formula feeding to enhance the effects of UMC. Initiation of formula feeding may be a proxy for duration of breastfeeding in the PASTURE study (21); alternatively, duration of formula feeding may point towards an independent source of milk and thereby to delayed introduction of normal cow's milk and yoghurt. In contrast, breastfeeding may counterbalance the detrimental effects of excessive meat similarly to (mildly processed) cow's milk.

The interaction of UMC with breastfeeding for the expression of CD40L, a marker of both allergic and intestinal inflammation (22), is highly interesting. In the absence of the anti-inflammatory properties of maternal milk (23, 24), UMC might induce (low-grade) inflammation of the intestinal wall possibly leading to systemic T-cell activation (22). Admittedly, the evidence provided by the data available now is weak but the postulated link between feeding patterns and intestinal inflammation might be a promising direction for future research.

Beyond local mucosal inflammation, the question remains how UMC may influence a rather distant organ system and affect the pathogenesis of asthma. Because we have previously seen an effect of consumption of milk and eggs on the gut microbiome at 12 months and its effect on asthma (4), we again followed this route. The particularly pronounced effects of UMC at 10 to 11 months rendered an assessment of the gut microbiome at 12 months an ideal situation. Given the exploratory approach, the associations with microbiota were not corrected for multiple testing. With this caveat in mind, we found several taxa to be possibly fostered by UMC. By a Picrust analysis of functional

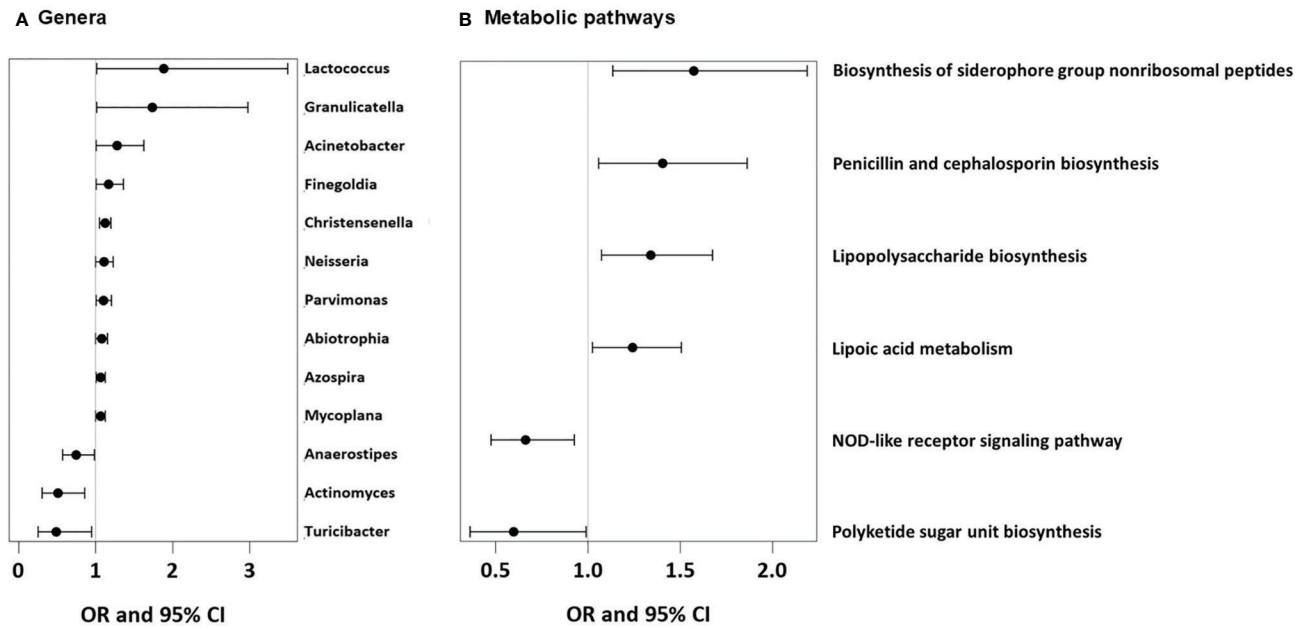

FIGURE 12 | Microbial genera and microbial metabolic pathways associated with UMC. **(A)** Odds ratios (OR) with 95%-confidence intervals for the associations of UMC with relative abundance of bacterial genera in fecal samples. **(B)** Odds ratios (OR) with 95%-confidence intervals for the associations of UMC with relative abundance of metabolic pathways in fecal samples.

TABLE 3 | Sequences of the genus Acinetobacter.

Amplicon sequence variants (ASVs)	Reads	Species	Identity	Identity with A. baumannii
c6357b5b5bb8c067c51faad5180b4e99	402	johnsonii	99.6%	98.0%
50a3b56e0b7db75ee9daccf7a751ba41	339	johnsonii	100%	97.6%
588b5ccdde9b0d39d61568731d7e223f	173	calcoaceticus, lactucae, nosocomialis, pittii, oleivorans	100%	98.0%
107aa7e6b56274803bffd1ccff5b2ee6	150	colistiniresistens, gerneri, proteolyticus, courvalinii, wuhouensis, gyllenbergii, junii, tandoii, guillouiae, bereziniae Nakamurella silvestris	100%	96.8%
2f41b4b15416f046360ecaf1cfeb4b28	24	schindlerijohnsonii	100% 99.6%	
5c96ec70d2839b27eb48f2226c96e2ca	16	idrijaensis, pseudolwoffii, lwoffii	100%	
262dc7bf78e937b0c7065aef82e18d08	13	parvus, tjernbergiae, beijerinckii,disperses, haemolyticus	100%	

properties, the common denominator of these taxa was unraveled: The top hit of the biosynthesis pathways pointed towards siderophore group nonribosomal proteins and thus to bacterial iron metabolism. This notion is supported by a study on iron supplementation in pregnant women, which revealed an association of high supplementation with the very same pathway in the gut microbiome (25). Siderophores are employed by intestinal bacteria in their battle for nutritional iron. More than 80% of the dietary iron is not resorbed and passed to the colon, where adverse gut bacteria may overgrow and lead to inflammation (26). At a closer look, most of the genera associated with UMC were actually well-known iron scavengers or even pirates. This is particularly documented for *Neisseria*, a known pathogen (27). Likewise, *Acinetobacter baumanii* is a human pathogen closely related to the here identified species *calcoaceticus / pitti* (28). The mentioned *Acinetobacter* species form together the phenotypically homogenous *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex (29), which plays a role in airway infections such as pneumonia (30, 31).

As a countermeasure, the host organism can launch a number of defense mechanisms and establish "nutritional immunity" by limiting bacterial acquisition of necessary nutrients such as metals (32, 33). Bacteria can be kept at bay by the innate bactericide function of lipocalins (34), which are secreted by gut epithelial cells and possess the ability to neutralize siderophores (35). In addition, cow's milk contains a molecule of the lipocalin family: β-lactoglobulin. This molecule has previously been found at high levels in unprocessed cow's milk and has been directly related to lower asthma risk (36). Likewise, lactoferrin, another defense molecule secreted to the gut lumen and to milk, can sequester siderophores (37, 38). Remarkably,

both molecules emerged as candidates for the beneficial effect of farm milk, when not inactivated by high heat treatment (9, 36, 39). Since formula milk is produced from ultra-heat treated milk, these molecules are no longer active, which might explain the detected potentiation of the unfavorable UMC effect by prolonged formula feeding. In contrast, the intact compounds of breastmilk might have alleviated the disadvantages of UMC.

We have previously postulated a gut-lung axis in humans mediated by maturation of the gut microbiome with involvement of short chain fatty acids (4). Though associated with some asthma risk bacteria including *Acinetobacter* (4) and *Christensenella* (17), UMC exerted an effect on asthma independently from gut maturation (data not shown), thereby suggesting a second component to the gut-lung axis. A parallel finding might be seen in a recent gut metabolome study suggesting consumption of processed meat and lack of breastfeeding to induce a metabolic profile associated with subsequent development of asthma (17).

The above mentioned mechanisms of nutritional immunity, which may be compromised by UMC, may affect the entire organism since competition for iron and other metals is a systemic effect as illustrated by the well-known phenomenon of infectious anemia (40). Interestingly, the airway pathogens *Moraxella catharrhalis* and *Haemophilus influenzae*, which are specifically associated with wheeze and asthma (41, 42), are known to command potent strategies of iron acquisition involving lactoferrin and transferrin binding proteins (33).

The opposite health effects of milk consumption and excessive meat intake may be explained by a number of other known biological mechanisms, such as discrepant fatty acid patterns (43, 44), different impact on oxidative stress (45), or involvement of non-microbial, non-human antigens such as Neu5Gc (46), and Alpha-Gal (47), which are related to milk and red meat and influence human health in different directions. In the present analysis, the UMC finding could not be attributed to the effects of fatty acid patterns, Neu5Gc and Alpha-Gal (data not shown), and measurements of oxidative stress were not available in our cohorts. Besides, the interaction itself would not be explained by the above-mentioned alternatives.

The interplay between nutritional iron and overgrowth of potentially adverse bacteria on one side and defense mechanisms possibly supported by dietary milk-derived proteins on the other side may eventually explain the detected interaction of meat and milk intake (UMC) for asthma. This would provide a coherent and simple rationale compatible with the principle of simplicity, which is inherently more attractive (48). Future, more sophisticated studies may prove the hypothesized mechanism on a molecular level, while currently we only describe statistical associations. These limitations might be compensated by the specific strengths of this analysis, i.e. the replication in two independent cohorts, the detailed nutritional diary, and the long follow-up of children until school age.

Whether infants, particularly in Finland, are nowadays fed on excessive meat may be doubted, given the rapidly evolving evidence-based adjustments to Public Health measures (https://thl.fi/en/web/handbook-for-child-welfare-clinics) (49). If corroborated by other studies, it will be advisable to avoid excessive daily meat intake at home and in day care facilities, particularly during the vulnerable window of introduction of solid foods. Rather the consumption of mildly processed cow's milk may contribute to a healthy diet beyond the already known beneficial effects (9).

Beyond the Public Health dimensions, the here presented finding might be important for a deeper understanding of asthma pathogenesis. The postulated effect of microbial imbalance towards iron scavenging gut bacteria may result in long−term immune dysfunction and low−grade inflammation as involved in asthma, obesity, inflammatory bowel disease, and adverse metabolic conditions (49).

Taken together, we performed an unbiased analysis of feeding patterns in the first year of life and discovered a constellation of excessive meat consumption at the expense of other protein sources, which was characterized by a substantially elevated asthma risk. With the help of microbiome analyses we identified bacterial iron metabolism as a likely culprit for the adverse health phenomenon. Though not revealing final proof for this hypothesis, the current work may stimulate research into the effects of nutritional iron and other metals on the development of asthma and lead to novel preventive approaches.

DATA AVAILABILITY STATEMENT

PASTURE and LUKAS2 are two ongoing birth cohorts with fieldwork still being executed and biosamples not yet used up. As long as the studies are not yet anonymized and as long as the biosamples are not used up, European data protection legislation prohibits sharing of individual data (also when pseudonymized) to guarantee participant privacy.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local ethics committees. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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AUTHOR CONTRIBUTIONS

EvM, JR, and JP obtained funds, set up the PASTURE birth cohort and had responsibility for data collection and management of the study. CR, RF, RL, PK, AK, AC, BS, and OF were involved in acquisition, and interpretation of data. JG and EH were involved in data management. GP performed bioinformatics. AH and MD performed statistical analyses; ME supervised statistical analyses. AH and ME drafted the manuscript. All authors contributed to the article and approved the submitted version. The PASTURE study group were involved in acquisition, management and interpretation of data in Austria, Finland, France, Germany, and Switzerland.

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The Role of Lectin Receptors and Their Ligands in Controlling Allergic Inflammation

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More than fifty c-type lectin receptors (CLR) are known and have been identified so far. Moreover, we know the group of galectins and sialic acid-binding immunoglobulin-type lectins that also belong to the carbohydrate-binding receptors of the immune system. Thus, the lectin receptors form the largest receptor family among the pathogen recognition receptors. Similar to the toll-like receptors (TLRs), the CLR do not only recognize foreign but also endogenous molecules. In contrast to TLRs, which have a predominantly activating effect on the immune system, lectin receptors also mediate inhibitory signals. They play an important role in innate and adaptive immunity for the induction, regulation and shaping of the immune response. The hygiene hypothesis links enhanced infection to protection from allergic disease. Yet, the microbial substances that are responsible for mediating this allergy-protective activity still have to be identified. Microbes contain both ligands binding to TLRs and carbohydrates that are recognized by CLR and other lectin receptors. In the current literature, the CLR are often recognized as the 'bad guys' in allergic inflammation, because some glycoepitopes of allergens have been shown to bind to CLR, facilitating their uptake and presentation. On the other hand, there are many reports revealing that sugar moieties are involved in immune regulation. In this review, we will summarize what is known about the role of carbohydrate interaction with c-type lectins and other sugar-recognizing receptors in anti-inflammation, with a special focus on the regulation of the allergic immune response.

Keywords: carbohydrate, immunomodulation, allergic inflammation, asthma, C-type lectic receptor

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INTRODUCTION

There are more than 180,000 entries in Pubmed containing the search terms carbohydrate and immunomodulation. Moreover, there are many patents proposing carbohydrates as immunomodulatory substances. However, some polysaccharides known to bind to CLR, such as β-glucans and arabinogalactans, are already on the market as consumer healthcare products. However, the mode of action of these carbohydrates is predominantly unclear. It is likely that many of these effects can be explained by the interaction of sugar motifs with lectin receptors of the immune system. One function of lectins is to facilitate the uptake of antigens, even though these

molecules are more than simple uptake receptors. Several of these lectin receptors mediate signals since they possess distinct signaling motifs. In particular, CLR expressed on myeloid cells play a central role in innate immunity. The CLR are distinguished between those that have an 'immunoreceptor tyrosine-based activation motif' (ITAM) and those with an 'immunoreceptor tyrosine-based inhibition motif' (ITIM). Thus, they can mediate activating or inhibiting signals to the immune system depending on the ligand. In addition to CLR, we also know the group of galectins and the group of Siglecs (sialic acid-binding immunoglobulin-type lectins). Most receptors of the Siglec family also transduce signals *via* ITAM/ITIM domains, whereas galectins are somewhat different in that they are secreted proteins which bind to carbohydrates on the surface of other cells.

In this review, we will focus on the modulation of the allergic immune response of the airways by carbohydrate/lectin receptor interaction. There are several studies that focused on the role of lectin receptors and discussed carbohydrate structures on allergens as crucial factors in allergic sensitization (1, 2). On the other hand, there are known cases where signaling *via* c-type lectins is exploited by pathogens to suppress the immune response (3). Thus, knowing these interactions could pave the way for therapeutics that could be used for the suppression of allergic inflammation.

One initial event during allergic sensitization *via* the airways is that epithelial cells become activated and start to release cytokines, which attract and activate dendritic cells (4). After taking up and processing allergens, they migrate to the draining lymph nodes to activate T lymphocytes. Moreover, B cells produce allergen-specific immunoglobulin E (IgE), which binds to the surface of mast cells. During the effector phase, mast cells become activated to release histamine and pro-inflammatory mediators, eosinophilic granulocytes degranulate their toxic content and other innate immune cells contribute to inflammation. This type of allergic airway inflammation is the most common one that depends on the activity of T helper cells from the Th2 type (5). However, other endotypes have been described in the last few years that depend on Th17 cells, which attract neutrophils and promote severe disease courses (6). Many steps of this process could be modulated by carbohydrate/lectin interaction. Dendritic cells (DCs), mast cells, granulocytes and epithelial cells, for example, are known to express several different lectin receptors and could, therefore, be modulated by the appropriate ligands (**Table 1**). Thus, several of the important players of the allergic immune response could be modified by carbohydrates. There are many papers describing a potential benefit of treatment with different types of carbohydrates for protection against allergic sensitization and inflammation. The carbohydrates described range from synthetic oligosaccharides to support the growth of intestinal microflora (27) to polysaccharides isolated from plants (28), bacteria (29) or fungal (30) sources. However, the mode of action is clear only in a minority of examples. In this review, we will focus on these examples. We will discuss how stimulation of these receptors can modulate allergic inflammation and could hopefully be exploited in the future for allergy prevention.

DECTIN 1 – A PATTERN RECOGNITION RECEPTOR BINDING TO B-GLUCANS

The CRD of Dectin-1 recognizes 1,3-β-glucans, which are frequently found in the cell walls of fungi, bacteria and some plants. Dectin-1 is expressed in both human and mice on DCs, monocytes, macrophages and neutrophil granulocytes, and exclusively in human also on B cells, mast cells and eosinophilic granulocytes (7, 31). In addition to its endocytotic activity, Dectin-1 has its own signaling cascade. It does not have a complete ITAM, which would contain two tyrosines. Nevertheless, after ligand-binding and phosphorylation of the tyrosine, the 'spleen tyrosine kinase' (SYK) is activated. Subsequently, the formation of the CARD9/Bcl10/Malt-1 complex leads to the activation of NF-κB and, thus, to the production of pro-inflammatory cytokines (32, 33). In the case of DCs, the activation of Dectin-1 leads to their maturation and, thus, to a strong T-cell stimulatory capacity. Dectin-1 activated-DCs initiate mainly a Th1 and Th17 immune response (34).

Despite this immunostimulatory role of Dectin-1, there are some reports revealing a beneficial role of Dectin-1 stimulation in the prevention or control of allergic asthma. Invertebrate tropomyosin, for example, a ubiquitous arthropod-derived molecule, was shown to be a dectin-1 ligand that serves to restrain IL-33 release, thus, dampening type 2 immunity in healthy individuals (35). In this context, the immune modulation is likely to be mediated *via* an influence on the airway epithelium and not by a direct effect on DCs (**Figure 1A**). However, stimulation with a well-known Dectin ligand, namely Curdlan, also led to reduced allergy (36). In this paper, the authors showed that the activation of antigen-presenting cells is involved, which lead to the generation of the interleukin (IL)-10-producing T-helper *via* ICOS interaction.

By contrast, other reports showed that β-glucans may worsen allergic asthma by acting through Dectin-1 (37). In line with what is known about the polarizing activity of β-glucan-stimulated DCs, the authors observed that Curdlan induced a Th17 response with increased neutrophilic inflammation and exacerbation of the disease.

DECTIN 2 – A PATTERN RECOGNITION RECEPTOR BINDING TO HIGH MANNOSE STRUCTURES

Dectin-2 is described as representative for CLR, whose signaling pathway runs *via* a coupled ITAM that belongs to a neighbored Fc-receptor gamma chain (9). Dectin-2 is present on macrophages, monocytes and DCs (10). Its ligands are structures with a high mannose content and α-mannans, which are found in fungi (11). The activation of Dectin-2 leads to the recruitment and activation of SYK, which follows a similar signaling pathway as Dectin-1.

Dectin-2 is associated with allergic diseases mainly because house dust mite extracts are able to activate SYK *via* Dectin-2

TABLE 1 | Carbohydrate recognition receptors (CRR).

CRR	Expression in immune cells	Ligands	General functions	Impact on allergic immune response	Ref.
Dectin-1	Neutrophils, monocytes, myeloid DCs, in humans also B cells and mast cells and eosinophils	β1,3glucan	Recognition of fungal and mycobacterial infections, pro-inflammation	The ligand curdlan lowers TH2 allergic immune response	(7, 8)
Dectin-2	Monocytes, dendritic cells, macrophages	High mannose, alpha-mannans	Recognition of fungal and mycobacterial infections, pro-inflammation	Induces cysteinyl leukotriene secretion, which attracts eosinophils and neutrophils, increases TH2 response	(9–11) (8)
DCIR	Monocytes, DCs, granulocytes, B cells, macrophages	Glycoepitopes on HIV, carbohydrates of endogenous proteins such as antibodies	Virus capture and transmission	IVIgs can reduce allergic airway disease *via* interaction of their carbohydrates with DCIR	(8, 12)
DC-SIGN	Monocytes, myeloid DCs	High mannose or fucose containing carbohydrates, e. g. Lipoarabinomannan of mycobacteria	Endocytotic activity, immunomodulatory role	Induces IL-10 producing dendritic cells, the ligand arabinogalactan reduces the activation of NFkb and T-cell stimulatory capacity	(8, 13)
Macrophage Mannose receptor	Macrophages, myeloid DCs, Langerhans cells	Mannose containing carbohydrates	Endocytotic receptor, recognition of microorganism, cross presentation on MHCI	unknown	(8)
DEC-205	Granulocytes, monocytes,T-cells, B-cells, dendritic cells, NK cells	n.d.	Endocytosis and antigen presentation on MHCII	Endocytotic receptor, when OVA is fused to the receptor by an antibody, the allergic response is supressed	(8, 14)
Siglec F (m)/Siglec 8 (h)	Eosinophils mast cells	sialosides that contain both sialic acid and sulphate, with the position of the sulphate being an important determinant of specificity	Mediate cell-cell interactions and signaling functions in the immune system	Suppresses eosinophilic inflammation and mast cell activation	(15–17)
Siglec E (m)/Siglec 9 (h)	Myeloid cells			Suppresses neutrophilic inflammation	(18, 19)
Galectin 1 (secreted)	Mainly dendritic cells and monocytes	Galactoside containing glycans	Play a role in inflammation, adaptive immune response, cell migration, autophagy and signaling	Protects from allergic asthma, limits eosinophil recruitment and promotes apoptosis of eosinophils	(20–22)
Galectin 3 (secreted)	Mainly Monocytes, myeloid DCs			Induces Tregs, dampens mucus production and subepithelial fibrosis in allergic asthma, lowers airway hyperresponsiveness to metacholine	(23–25)
Galectin 9 (secreted)	Monocytes, myeloid DCs, granulocytes			It can bind to glycoepitopes present on IgE, inhibiting the activation of mast cells by specific allergens	(26)

The expression of CRR in this table is given for immune cells only. Many of these receptors are also expressed on other cell types, as listed in www.proteinatlas.org. The general functions of these receptors are described for immune cells, moreover, examples for the modulation of the allergic immune response by these receptors is given in the table. h, human; m, mous; n.d., not determined.

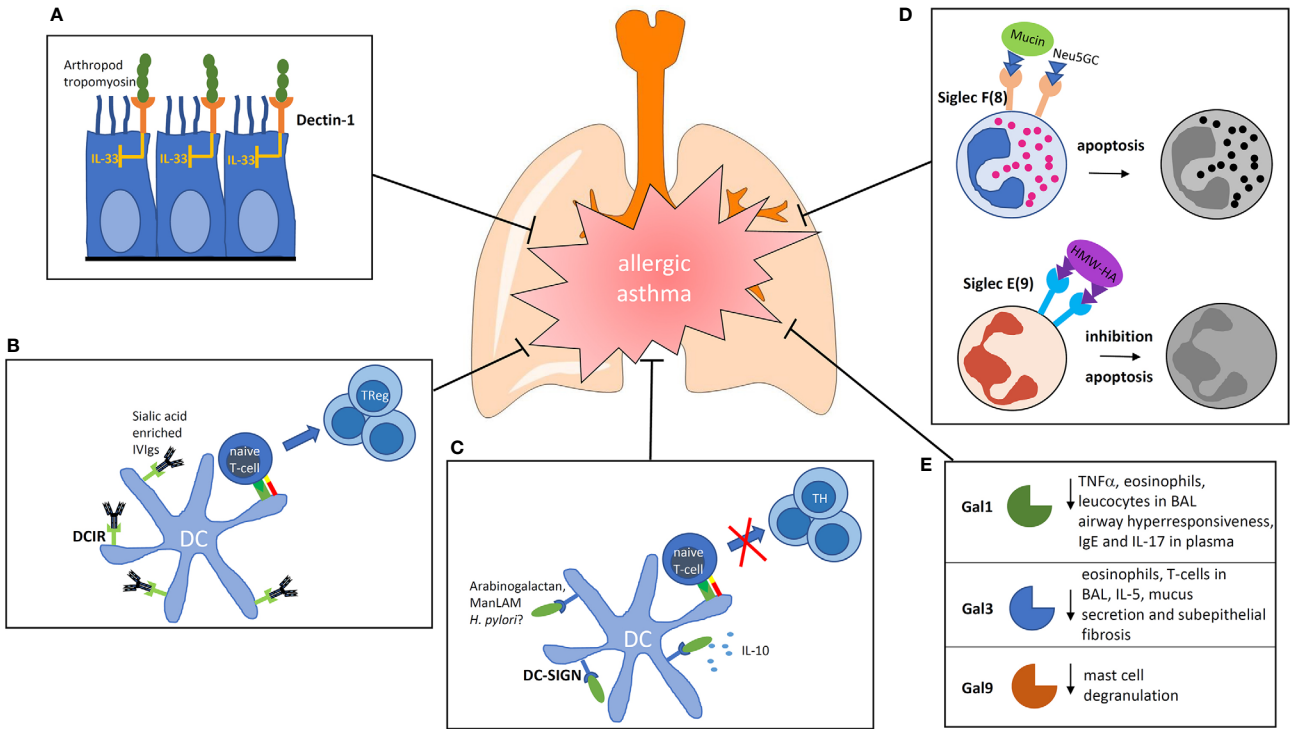

FIGURE 1 | Impact of carbohydrate recognition receptors and their ligands on the modulation of allergic inflammation. **(A)** Binding of arthropod tropomyosin to Dectin-1 on airway epithelial cells inhibits IL-33 release. **(B)** DCIR is known for its inhibiting signaling properties. If sialic acid-rich antibodies are bound by DCIR on DCs they are able to induce regulatory T cells. **(C)** Ligation of DC-SIGN can induce prolonged IL-10 production. The ligand arabinogalactan reduces the activation of NFκb and T-cell stimulatory capacity of human DCs. **(D)** Siglecs are membrane spanning receptors with signaling properties. Siglec F signaling induces apoptosis of eosinophils, Siglec E inhibits neutrophils. **(E)** Galectins are secreted proteins which bind to the glycocalyx of cells. In the group of galectins, three members are known to lower the allergic response by different mechanisms. BAL, broncho alveolar lavage; Gal, Galectin; ManLAM, mannosylated Lipoarabinomannan; IVIg, intravenous Immunoglobulins; HMW-HA, high molecular weight hyaluronan; Neu5GC, N-Glycolylneuraminic acid.

and, thus, cause a rapid release of cysteinyl leukotrienes. This leads to a recruitment of eosinophilic and neutrophilic granulocytes and an amplification of the Th2-dependent allergic immune response (38).

In fact, there are no reports in the literature arguing for an anti-inflammatory role of Dectin-2 stimulation. Therefore it seems as if Dectin-2 stimulation is predominantly acting in a pro-inflammatory way and is, thus, triggering an allergic immune response.

DCIR – IMMUNE INHIBITING RECEPTORS BINDING TO ENDOGENOUS FUCOSE OR MANNOSE CONTAINING LIGANDS

There is also a group of receptors which show inhibitory activity in addition to activating CLR. Dendritic cell immunoreceptors (DCIRs) have an ITIM instead of an ITAM domain. Two paralogs of this receptor exist in mice. The 'dendritic cell immunoreceptor 1' (DCIR1), which is expressed on monocytes, granulocytes, macrophages, DCs and B cells, and DCIR2, that seems to be more restricted to expression in cDCs, are both representative of this group. Fucose and mannose-containing glycans are ligands for DCIR (12).

Only one DCIR is described in human. No exogenous ligands other than HIV-1 have been described for human DCIR so far (39). The intracellular domain of DCIR is associated with the non-receptor tyrosine kinases SHP-1 and SHP-2 *via* ITIM (40).

DCIR2-deficient mice were shown to be more prone to autoimmunity and have a higher number of DCs underlining the immunoregulatory role of the receptor (41). It is known that intravenous injections of immunoglobulin (IVIg) of healthy donors leads to an alleviation of the disease in different inflammatory conditions in human (42). It was thought earlier that IVIg would act in the sense of passive immunization. However, it became increasingly clear that the glycosylation pattern of the Fc part of the antibodies injected plays an important role in immunomodulation (43). Interestingly, IVIg alleviates allergic airway disease through the interaction of its carbohydrates with DCIR in a mouse model (**Figure 1B**). The authors concluded from their study that IVIg interaction with DCIR induces a tolerogenic response (44). Thus, DCIR seems to be a valuable target for modulation of the allergic immune response.

DC-SIGN – PATTERN RECOGNITION RECEPTOR BINDING TO FUCOSE OR MANNOSE CONTAINING ANTIGENS

Another well-known representative of the ITAM/ITIM independent CLR is DC-SIGN. This receptor has a CRD that recognizes mannose- and fucose-containing motifs. Accordingly, ligands for DC-SIGN are found in a variety of pathogens, such as Mycobacterium tuberculosis, HIV, measles virus, Helicobacter pylori, Candida albicans and Salp15 from tick saliva (45). In addition to its endocytotic activity, DC-SIGN has a distinct signaling cascade. After the binding of mannosylated lipoarabinomannan to DC-SIGN, Raf-1 becomes activated, which, in turn, initiates the phosphorylation and acetylation of NF-κB leading to a prolonged production of IL-10 (46). The IL-10-producing DCs are known for their tolerogenic phenotype, in accordance, it was shown that mannosylated lipoarabinomannan-stimulated DCs led to the generation of regulatory T-lymphocytes (47). However, different ligands for DC-SIGN seem to have different effects on DCs. Salp15, for instance, was shown to activate the 'dual specificity mitogen-activated protein kinase,' which, in turn, leads to the degradation of IL-6 and TNF-α mRNA (48). Different paralogs of the receptor exist in mice.

Although DC-SIGN seems to play an immunoregulatory role, there are only a few reports that studied the ability of DC-SIGN ligands to suppress unwanted immune reactions.

There are many reports from mouse models on the protective activity of Mycobacteria and their extracts on allergy protection. However, it is not clear whether murine homologues of DC-SIGN are involved in this protection. One homologue of human DC-SIGN in mice is SIGNR1. This receptor was shown to be involved in the induction of oral tolerance to mannosylated antigens in mice (49). Furthermore, it was shown that extracts of H. pylori protect against allergic asthma in a mouse model by the induction of IL-10-producing DCs (50). Therefore, it is interesting to speculate that the protective effect is mediated *via* a murine DC-SIGN homologue, but, to date, there is no supporting data to prove this hypothesis.

In addition to the ligands discussed above, it was shown that plant arabinogalactan binds to DC-SIGN on the surface of human DCs. The binding resulted in a reduced activation of the transcription factor NFκB after TLR-stimulation (**Figure 1C**). This led to DCs with a reduced T-cell stimulatory capacity (13). Interestingly, arabinogalactan is a molecule that is found abundantly in extracts of cowshed dust and can inhibit allergic sensitization in a mouse model of asthma, suggesting that it might be also involved in the "farming effect" (51).

MACROPHAGE MANNOSE RECEPTOR (MR) AND DEC205 – PATTERN RECOGNITION RECEPTORS FOR ENDOCYTOSIS

Both receptors are involved in endocytosis, however, they do not contain an intracellular signaling motif. In addition to macrophages, MR is also found on some DC subpopulations. DEC205 has a broad expression pattern and it expressed on immature DCs, but it is now known to be expressed on endothelium and selected macrophage subpopulations (52). Endocytosis *via* MR leads to the cross-presentation of antigens *via* the 'major histocompatibility complex' I (53), whereas endocytosis *via* DEC205 leads to an enhanced presentation of the ingested antigen *via* major histocompatibility complex II (54). Regarding MR, it is not clear whether it is able to trigger an intracellular signaling cascade itself or this happens in cooperation with another CLR. However, it could be shown that the crosslinking of MR *via* antibodies (AK) led to cytokine production in DCs involving the anti-inflammatory cytokine IL-10 (55). No signaling pathways have been described for DEC205 so far.

After the discovery of DEC205 as an antigen delivery receptor, it was attempted to exploit DEC205 as a potential target to be used in vaccination approaches. Surprisingly, it was observed that antigens targeted into the endocytotic pathway *via* DEC205 induced a regulatory immune response that might inhibit the induction of immunity (56). Subsequently, this finding was exploited for the delivery of allergens by gene-based immunization using an adenoviral delivery of single chain antibodies directed against DEC205 fused to OVA to target the allergen towards DCs. This treatment resulted in the efficient suppression of an allergic immune response (14).

SIGLEC – GLYCORECEPTORS BINDING TO SIALYLATED ANTIGENS

The Siglecs are transmembrane receptors that bind structures containing sialic acid. They are mainly expressed on immune cells and can be divided into three functional groups. The first group contains Siglec 1 and 4, without any known intracellular signal motifs. The second and largest group includes Siglec 2, 3 and 5 – 12 that contain an ITIM or ITIM-like motif intracellularly. Similar to CLR that contain an ITIM, the signaling cascade involves the recruitment of SHP-1 and SHP-2 and leads to an inhibition of other immunomodulatory signals, which are, for example, induced by the activation of TLRs (57). The third group of Siglecs consists of Siglec 14, 15 and 16 and is characterized by a positively charged residue in their transmembrane domain, through which they can associate with the negatively charged transmembrane domain of the DNAX-associated protein of 12 kDa (DAP12). The DAP12 carries an intracellular ITAM and can trigger a signaling cascade *via* SYK, similar to the CLR Dectin-1 or Dectin-2. Siglec H is transducing its signal in rodents *via* DAP12 (58).

Siglecs recognize both exo- and endogenous ligands. Their primary function is probably the regulation of immune responses. However, unlike CLR, this is not done *via* pathogen recognition since most pathogens are not sialinized.

The function of Siglec F (human functional paralog Siglec 8) has been explored *in vivo* using antibodies, knockout mice and models where the expression of specific ligands has been altered. Administration of agonistic anti Siglec-F antibodies, for example,

reduced the number of eosinophilic granulocytes in blood by reducing the viability of the cells (15). Moreover, antibodies binding to Siglec F abrogate eosinophilic pulmonary inflammation and virtually eliminates lung remodeling in mouse models of chronic allergic asthma (16). Muc5b and Muc4 were identified as endogenous ligands for Siglec F because they carry sialylated glycan ligands (59). Purified mucin preparations carried sialylated and sulfated glycans that were able to induce apoptosis in mouse eosinophils. Muc5b-deficient mice displayed exaggerated eosinophilic inflammation in response to the intratracheal installation of IL-13.

Regarding Siglec E (human ortholog = Siglec 9), it was shown that Siglec E-deficient mice in an LPS-induced lung inflammation model exhibited exaggerated neutrophil recruitment (18). The authors concluded that signaling *via* Siglec E may control neutrophilic inflammation. Since severe neutrophilic asthma is an endotype that is difficult to treat, using agonistic antibodies or glycan ligands as new treatment options was discussed (19). One natural ligand that was identified is high molecular weight hyaluronan from the capsule polysaccharides of the pathogen group A of *Streptococcus*. Their binding to Siglec 9 suppresses the activation of neutrophilic granulocytes (60).

N-glycolylneuraminic acid (Neu5Gc) is a sialic acid that is expressed on nonhuman mammalian cells and glycoproteins and is not present in bacteria. It was proposed to be partially responsible for the "farming effect" because there is a relationship between the exposure toward Neu5Gc in a rural environment and protection against allergy. The immunomodulatory activity of Neu5Gc was confirmed by suppression of allergic airway inflammation in a murine model (61). An involvement of Siglec 8 as the binding receptor was discussed by the authors (**Figure 1D**) (62).

GALECTINS – SOLUBLE GLYCORECEPTORS RECOGNIZING GALACTOSE-CONTAINING MOLECULES

In addition to the group of CLR, there is a large number of other glycoreceptors that play a role in the immune response but are not considered PRRs. Galectins are small, soluble proteins that are expressed and secreted by different cells. Fifteen members of this protein family have been discovered so far (20). Functionally, they are able to influence the differentiation and survival of T cells and their interaction with DCs (63).

Gal-1-deficient mice exhibit an increased recruitment of eosinophils and T lymphocytes in the airways as well as elevated peripheral blood and bone marrow eosinophils relative to corresponding WT mice (21). Moreover, mice had an increased airway hyperresponsiveness and displayed significantly elevated levels of TNF-α in the lung tissue. The authors suggested from their results that Gal-1 can limit eosinophilic airway inflammation by inhibiting the migration and promoting apoptosis of eosinophilic granulocytes.

In order to evaluate Gal 1 as a potential therapeutic protein, Ly et al. have shown that the recombinant Gal 1 protects from allergic asthma in a mouse model (22). The immunomodulatory effects in the allergic lung were correlated with the activation of the extracellular signal-regulated kinase signaling pathway and downregulation of endogenous Gal-1. rGal-1 reduced the plasma concentrations of anti-OVA IgE and IL-17, therefore, it can be hypothesized that it may also act on the Th17 response involved in severe asthma.

Gal-3 gene-deficient mice also showed enhanced disease activity in a mouse model of asthma by Zuberi et al., arguing for a regulatory role in asthma (23). Intratracheal instillation of plasmid DNA encoding Gal-3 led to the normalization of the eosinophil and T-cell count in BALF and a strong inhibition of IL-5 mRNA in the lungs in a rat asthma model (24). It was shown in a chronic asthma model in mice that twelve weeks after the first intranasal allergen instillation, treatment with the Gal-3 gene led to an improvement in the eosinophil count and the normalization of hyperresponsiveness to methacholine. In addition, this treatment resulted in a reduced mucus secretion and subepithelial fibrosis (25), showing that Gal-3 also seems to have some therapeutic merit, although the mode of action in the asthma model was unclear. Regarding the mechanism, Tsai et al. showed that Gal-3 gene-deficient mice have more severe disease activity in a colitis model, indicating that Gal-3 may protect from inflammation (64). Moreover, the authors showed that mucosal inflammation was reduced in the colitis model by treating with Gal-3. There was strong evidence that regulatory T cells were induced by Gal-3.

Regarding Gal-9, it was shown that it can bind to glycoepitopes present on IgE, therefore, inhibiting the activation of mast cells by specific allergens (26). Moreover, the authors showed that Gal-9 attenuated asthmatic reaction in guinea pigs and suppressed passive-cutaneous anaphylaxis in mice, showing that Gal-9 may also be a potent modulator useful for the treatment of allergy (**Figure 1E**).

CONCLUSION

We know several different lectin receptors playing a role in not only innate but also adaptive immunity. Some of them serve predominantly as PRR, others rather recognize the glycosylation pattern of self-molecules. Knowing these interactions may open the way to new therapeutics for immunostimulation in, for example, vaccination and for the regulation of exaggerated immune responses, such as allergic inflammation. The focus of this review was to sum up what is known about the carbohydrate/lectin interaction that could be exploited for immunomodulation to prevent or treat respiratory allergies.

We are still at the beginning of this exciting field of research that may contribute to the development of new allergy preventive drugs. However, there are many issues still to be addressed. Although we focused in this review on literature which showed allergy protection by carbohydrates, it cannot be generalized that treatment with carbohydrates always acts in a preventive manner. Many examples are known where carbohydrate/lectin receptor interaction was linked to allergic sensitization. Similar observations were described for experiments with TLR receptor ligands where contradictory results were also obtained, depending on the dose

of ligand used and how the experiment was conducted. Therefore, it is very important to consider the quality of the carbohydrate ligand used, the experimental design and the ligand receptor interaction that is involved in all experiments performed.

Furthermore, the majority of the results summarized in this review come from experiments with mice. However, it is important to bear in mind that there are several differences between lectin receptors found in mice and their human homologues. The binding behavior and the signal transduction often differs between the receptors of the two species. Furthermore, several paralogous exist in mice which do not have comparable receptors in human. Thus, when immunomodulatory carbohydrates were identified in murine model systems, it is of particular importance to know the binding receptor to get an idea whether the substance would also act in human in a similar manner.

Due to the increased hygienic measures accompanying the recent virus pandemic, it can be expected that allergic diseases will continue to rise. Effective treatments to prevent allergic disease are urgently needed. There is still a long way until the first carbohydrates will enter into clinical trials, however, it is worth it. There are many examples of how the allergic immune response is modified by carbohydrates and many of them seem to have no pro-inflammatory properties. Therefore, this substance class would be ideally suited for prophylaxis.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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