THE ROLE OF THE MICROBIOME IN REGULATING T-CELL RESPONSE IN ASTHMA AND FOOD ALLERGY

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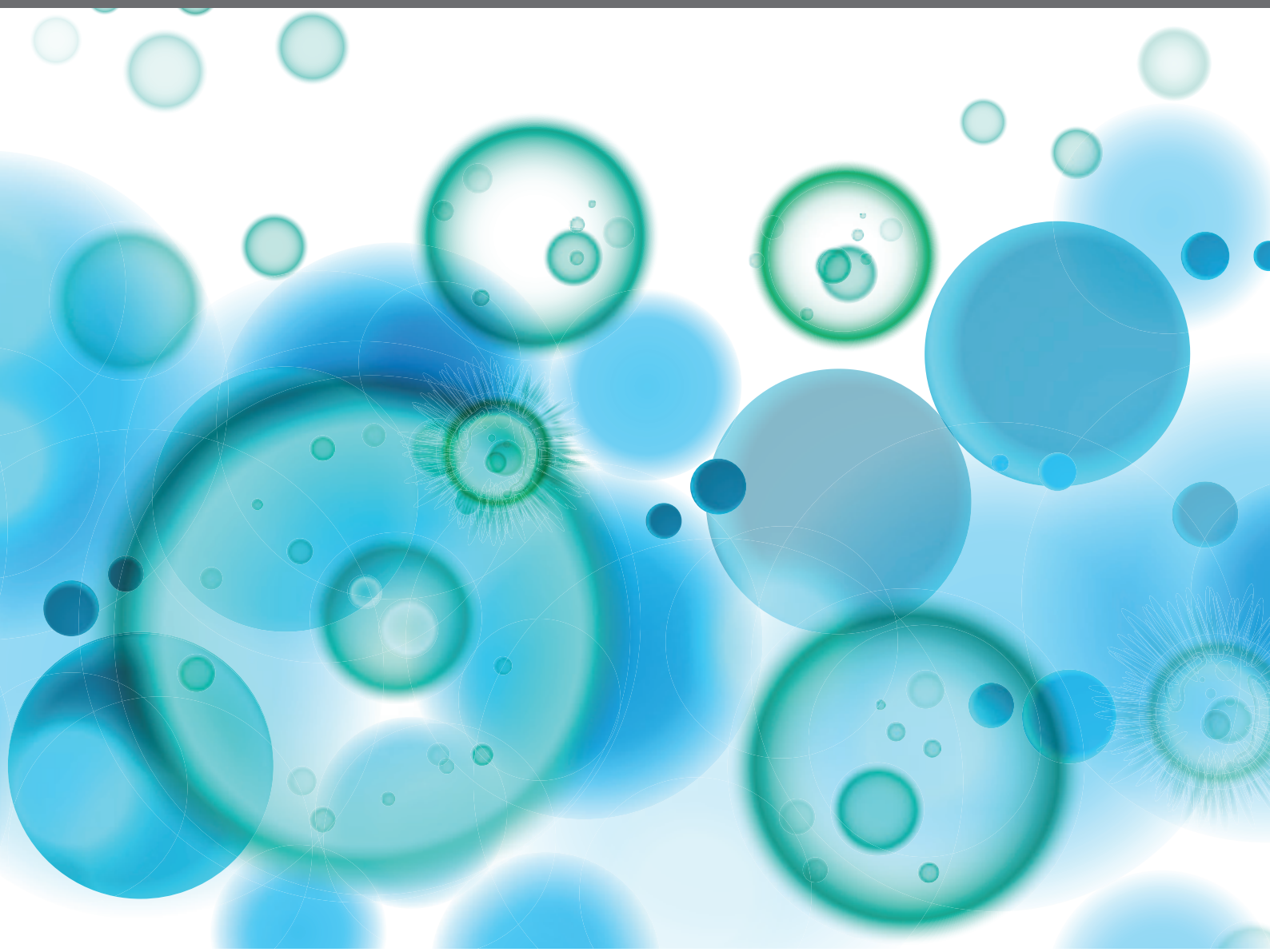

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THE ROLE OF THE MICROBIOME IN REGULATING T-CELL RESPONSE IN ASTHMA AND FOOD ALLERGY

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EDITORIAL
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Editorial: The Role of the Microbiome in Regulating T-Cell Response in Asthma and Food Allergy

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Editorial for the Research Topic

The Role of the Microbiome in Regulating T-Cell Response in Asthma and Food Allergy

The role and importance of the microbiome for human health has been investigated in recent years (1, 2). A dysbiosis of the gut microbiome has been shown to cause drastic changes in the immune system (3, 4) Leading to disbalance of immune homeostasis and consequently to the emergence of different diseases (5, 6). In this editorial, we investigate the role of microbiome in regulating immune response in Asthma and Food Allergy.

The interplay between gut and lung as two separate organs has been introduced previously (7–10). In their review, Di Gangi et al. have explored this concept and investigated the importance of gut microbiota in protection or augmentation of allergic asthma. Click or tap here to enter text.The authors shed light on the importance of *Lactobacillus spp* as an important part of the human gut microflora (11). Data from clinical cohorts hint towards a link between microbial dysbiosis and asthma risk in children. They review the findings from e.g. the PASTURE, EFRAIM and WHEALS cohorts. These studies show a protective effect of farm-exposure on asthma risk in children early in life with higher Treg numbers in peripheral blood of children consuming farm-milk (12, 13). Key findings of the WHEALS study include a delayed diversification of the gut microbiota and a relative difference in the composition with fewer *Lactobacillus, Bifidobacterium, Akkermisia* and *Faecalibacterium* and more *Candida spp* (14). The protective effect, however, was not lasting. The reasons for this phenomenon are not understood.

Collectively, there has been much research focusing on the role of microbiota in the development of asthma. The "Hygiene hypothesis" has put a lot of value on how bacterial species and bacterial metabolites are protective against asthma and allergy development. In that sense, Hagner and Harb et al. showed a protective effect of different microbial species, either isolated from cow shed or used as prebiotics, against the development of different hallmarks of allergic airway inflammation in mice (11, 15). These protective effects were transferred from mothers to offspring *via* TLR activation and signaling in the mothers exposed to these bacterial species (16). Mechanistically, these protective effects were related to epigenetic modification of the IFNG promoter in T-cells (17).

Bacterial fermentation in the lower gastrointestinal tract degrades indigestible complex carbohydrates from fiber, amino acids or mucus and gives rise to a variety of microbial metabolites. These metabolites include hydrogen; organic acids, such as lactate and succinate; alcohols, such as 1,2 propanediol; and short-chain fatty acids (SCFAs), such as acetate, butyrate,

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formate, propionate, and pentanoate [reviewed in (18)]. With highest concentrations in the gut (within millimolar range), SCFAs are transported *via* proton-coupled monocarboxylate transporter isoform 1 (MCT1, gene name SLC16A1) or the Na^+-coupled monocarboxylate transporter 1 (SMCT1, gene name SLC5A8) into colonocytes where a large part is metabolized locally for energy production (19, 20). Only ow levels reaching the blood circulation and therefore peripheral organs, it is assumed that SCFAs interact with gut resident immune cells, which then affect immune processes in peripheral tissues. In that regard, Yip et. al. review the expression of butyrate sensing cell-surface receptors of the G protein-coupled receptor (GPCR), namely GPR41, GPR43, and GPR109A, on leukocyte subsets and downstream regulated cellular mechanisms. The broad anti-inflammatory activity of SCFAs is achieved by either activating the Peroxisome proliferator-activated receptors, like PPARγ1, or inhibiting histone deacetylases (HDACs) activity and therefore promoting gene transcription from targeted chromatin.

While the beneficial effect of SFCAs in human disease is currently unclear, mouse models of ovalbumin- and house dust mite-induced allergic airway inflammation provide positive results. Oral administration of high fiber diet or SFCAs in mice, either nursing dams or uptake *ad libitum*, alleviated the symptoms in allergic airway inflammation, including airway reactivity, systemic immunization, and leukocyte infiltration into the lung. *Ex vivo* experiments, reviewed by Luu et al. and Yip et. al., in this Research Topic, highlight the diverse levels of regulation by SCFAs in general and only butyrate. Butyrate exposure reduces dendritic cell activation and migration to local lymph nodes, thereby limiting Th2 polarization of naive CD4+ T cells post allergen exposure. Under Th9 polarizing conditions, butyrate induces $Foxp3^+$ expression and therefore enforces a regulatory phenotype (21, 22). In ILC2 the secretion of IL-5 and IL-13, with pronounced downstream effects on eosinophils, was suppressed. Butyrate further reduces isotype/class switching in B cells reducing IgE production (23).

On the other hand, Zakzuk et al. explored how helminth infection shapes the landscape of the T-cell immunity in patients with asthma. In their original article, the authors collected peripheral blood mononuclear cells from helminth infected and healthy control subjects in the rural areas of Colombia. They report an inverse correlation between egg worm burden and histone 4 (H4) acetylation at the IL-13 gene. Additional results comprise a significant correlation between the same histone acetylation mark at the IL-4, CHI3L1 genes and IgE levels to *Ascaris lumbricoides*. In relation to asthma, there were significant associations between HDM specific IgE antibodies and H4-acetylation levels in the gene *TNFSF13B* encoding the B cell activating factor (BAFF).

Finally, in our proposal for this Research Topic, it was our intention to gather ideas, research, thoughts, and insights into the role of microbiota in regulating T-cell responses in different allergic diseases. Unfortunately, our topic stumbled by the beginning of the pandemic, closure of laboratories and stay at home orders in different countries. We believe that this topic carries an extensive potential between its pages and it would be great, if the future focus of allergy/microbiota research would still be on regulating different immune processes in the hosts.

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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IgE Levels to *Ascaris* and House Dust Mite Allergens Are Associated With Increased Histone Acetylation at Key Type-2 Immune Genes

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Background: Epigenetic changes in response to allergen exposure are still not well understood. The aim of this study was to evaluate histone acetylation levels in peripheral blood leukocytes from humans naturally infected by intestinal parasites and perennially exposed to house dust mites (HDM).

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation from 20 infected and 21 non-infected individuals living in a rural/village in Colombia. Histone 3 acetylation (H3Ac) and histone 4 acetylation (H4Ac) levels were measured in six immune genes previously associated with helminth immunity by chromatin immunoprecipitation (ChIP)-quantitative PCR. Then we analyzed the association between histone acetylation levels with total parasite egg burden and IgE levels.

Results: We found an inverse correlation between H4Ac levels in the *IL13* gene and egg worm burden that remained significant after adjustment by age [−0.20 (−0.32 to −0.09), $p < 0.0001$]. Moreover, we found significant associations between H4Ac levels in *IL4* [0.32 (0.05–0.60), $p = 0.02$] and *CHI3L1* [0.29 (0.08–0.51), $p = 0.008$] with the IgE levels to *Ascaris lumbricoides*. In addition, the levels of specific IgE antibodies to HDM were associated with H4Ac levels in the gene *TNFSF13B* encoding the B cell activating factor (BAFF) [0.51 (0.26–0.76), $p < 0.001$]. All values are presented as beta (95% CI).

Conclusion: Histone acetylation levels at key type-2 immune genes in humans were modified by nematode infection and HDM allergens and are associated with the intensity of the IgE response.

Keywords: histone acetylation, IgE levels, nematode infection, H3Ac, H4Ac, house dust mites, epigenetics

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INTRODUCTION

Epigenetic modifications and more specifically DNA methylation, have been associated with increased total IgE levels (1) and increased IgE sensitization to house dust mites (HDM) (2). In addition, allergen exposure induces epigenetic changes in immune cells affecting the inception and maintenance of type-2 skewed immune phenotypes (3). The IgE response to HDM allergens is very prominent in humans living in tropical environments, and even though

perennial exposure may explain this observation, the co-exposure with intestinal helminth infection provides a unique opportunity to dissect key molecular events implicated in type 2 immunity (4). Indeed, a study in a mice model revealed that chronic helminth infection also reprograms T cell differentiation via histone acetylation changes (5), by the addition of acetyl groups to lysine residues (K) at the N-terminal tail of histones. Acetylation neutralizes the positive charge of lysine reducing histone affinity for DNA and (this way) opens chromatin. Also, by providing a tag in histone tails for transcription factors and regulatory proteins, histone acetylation affects the accessibility of promoters to the transcriptional machinery (6, 7). Several studies support that H3 acetylation at K9 and K14 (H3Ac) are a hallmark of gene activation and exhibit remarkable correlation with active promoters and active enhancers. Also, H4 acetylation has been associated with transcriptional activation and maintenance of euchromatin. Therefore, increased acetylation of lysine residues at H3 and H4 is informative on active transcription of the marked gene (6, 7).

Previous studies revealed that when naïve T cells differentiate into Th2 cells, the Th2 locus (chromosome 5q31) undergoes extensive epigenetic modifications that lead to a poised chromatin configuration (8), making chromatin accessible and promoting IL-4, IL-5, and IL-13 expression (9, 10). Regulatory regions, such as the *IL4* and *IL13* promoters, the Th2 locus control region (LCR), and enhancers are the primary targets of these modifications (11–13). The isotype class switching and specific IgE production resulting from these changes may be used as a proxy of Th2 locus activation. In the context of helminth infection, the magnitude of IgE production to parasite components depends on the individual predisposition toward type 2 immunity (14–17). Egg burden is also a marker of individual ability to resist parasite infection (18, 19). A quantitative trait locus (QTL) for *Ascaris* egg counts has been described in chromosome 13q33 in a region encoding for ligase IV (*LIG4*), abhydrolase domain containing 13 (*ABDH13*) and the B cell activating factor BAFF (*TNFSF13B*) (20). Genetic variants in this region are also associated with increased IgE against *Ascaris* and the HDM *Dermatophagoides pteronyssinus* (21), although the underlying mechanisms remain unclear. Since parasite immunity and allergic responses share several biological pathways, we hypothesized that the relative effects of these genes depend on environmental factors that could induce epigenetic modifications.

To date, no study has analyzed if exposure to *Ascaris lumbricoides* and HDM allergens can influence histone acetylation at these loci. In this study, we aimed to evaluate H3 and H4 acetylation levels in mononuclear leukocytes from humans living in a rural community exposed to *A. lumbricoides* and HDM, and to investigate the relationship of H3 and H4 acetylation with the specificity and intensity of the IgE response.

MATERIALS AND METHODS

Study Population

For this study we selected 41 subjects from a cohort of 739 well-characterized subjects living in Santa Catalina (Colombia) and previously described by Zakzuk et al. (20). This is a small tropical farming/fishing town in northern Colombia (10° 36′ 0″ N, 75° 18′ 0″ W) with a territorial extension of 153 km^2 and a population of approximately 12,500 inhabitants. Half of the people have at least one unsatisfied basic need, only 4.5% of the population has a sewage system and 56% has tap water. This study included 20 non-infected subjects and 21 infected with *A. lumbricoides* (**Table 1**). Criteria for non-infected subjects included having two negative results in stool examinations conducted in 2014 (22), and when resampled in two consecutive stool tests collected for this study during May–June 2016. Criteria for infected subjects include active parasite infection as detected by fresh fecal smear in at least one stool test collected for this study in 2016. Parasite burden was quantified as eggs per gram (e.p.g) of feces by the Kato Katz method using a commercial kit (Copro Kit, C&M Medical, Campinas, Brazil). Blood samples were taken on the same day or within 2 days after the stool test. Albendazole treatment was prescribed after blood sampling in all infected subjects. This study was approved by the Ethics Committee of the University of Cartagena (nr. 1705-2012) and was conducted following the guidelines of the Declaration of Helsinki. All the participants gave their written informed consent prior to their inclusion in the study.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood samples were collected in EDTA tubes by standard phlebotomy and 3 mL of blood were mixed with 3 mL of RPMI-1640 based-medium (RPMI-1640 supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 1% antibiotic/antimycotic solution and 1% L-Glutamine) and then, layered over 3 mL of Histopaque (Sigma, city, United States). The sample was centrifuged at 800 × *g* for 20 min without a break. The mononuclear cell layer was aspirated, transferred to a new tube, and resuspended in 10 mL of RPMI-1640 based-medium. Cells were washed at 800 *g* for 10 min and the cell pellet resuspended in 2 mL of FCS-DMSO freezing medium and stored at −80°C until analysis.

Histone Modifications

Seven candidate genes were selected based on previous genetic association with helminth immunity: *IL4* and *IL13* at chr. 5q31 for their well-known involvement in helminth immunity (23, 24); *LIG4*/*ABHD13* and *TNFSF13B* in the *A. lumbricoides* susceptibility locus at chr. 13q33 (20) and *CHIA* at chr. 1p13.2, and *CHI3L1* at chr. 1q32.1 for their genetic association with anti-*Ascaris* IgE levels and protective immunity to helminths (21, 25). We also analyzed histone levels in the housekeeping gene *RPL32* encoding the Ribosomal protein L32 as a non-immune related control. Chromatin immunoprecipitation (ChIP) followed by quantitative polymerase chain reaction (qPCR) using specific primers (**Table 2**) were used to assess histone H3 acetylation of K9 and K14 (H3Ac) and H4 acetylation of K5, K8, K12, and K16 (H4Ac) levels at the promoters of the selected loci, as described previously (26). H3Ac and H4Ac levels were

TABLE 1 | Descriptive features of the study sample according to *Ascaris* infection status.

	Infected (*n* = 21)	Non-infected (*n* = 20)	*p*-value
Age (mean ± SD)	23.8 ± 18.7	33.7 ± 19.2	0.053
Female [*n* (%)]	12 (57.1)	13 (65)	0.7
Trichuris epg. [median (IQR)]	2.244 (655–8150)	0 (0–0)	n/a
Ascaris epg. [median (IQR)]	8.020 (2015–8940)	0 (0–0)	n/a
Total egg burden [median (IQR)]	9030 (3689–16820)	0 (0–0)	n/a
IgE levels, kU/L [GM ± SD]			
Ascaris spp.	1.84 ± 5.9	0.31 ± 1.9	0.004
D. pteronyssinus	0.37 ± 13.7	0.24 ± 4.3	0.1
B. tropicalis	0.72 ± 26.3	0.29 ± 13.6	0.3
IgE ≥ 0.35 kU/l [*n* (%)]			
Ascaris spp.	17 (81)	10 (50)†	0.037
D. pteronyssinus	8 (38.1)	7 (35)	0.8
B. tropicalis	11 (52.4)	7 (35)	0.2
Total IgE, kU/L [GM ± SD]	736 ± 1207	176 ± 568	0.003

epg: eggs per gram of feces; GM: geometric mean; SD: standard deviation. †Due to immune memory, non-infected subjects can be found positive for IgE antibodies to Ascaris albeit not having active infection by the time of blood sampling.

TABLE 2 | Primers used for quantitative assessment of H3 and H4 histone acetylation by PCR following chromatin immunoprecipitation (ChIP).

Target	Forward primer	Reverse primer
Chitinase 3 like 1 gene (*CHI3L1*) promoter	AATTGTGCCCAGTTTCCACC	GGGCTTCTGGAGATGTGACT
Acidic chitinase gene (*CHIA*) promoter	CGGACACTGGACTTAAGTTGT	GAAGCTTTGGCACCGTCT
Interleukin 13 gene (*IL13*) promoter	TGTGGGAGATGCCGTGGG	TCTGACTCCCAGAAGTCTGC
Interleukin 4 gene (*IL4*) promoter	TGGGTAAGGACCTTATGGACC	GGTGGCATCTTGGAAACTGTC
TNF superfamily member 13b gene (*TNFSF13B*) promoter	TAAGGGTGGGCTTCTCAGAC	GGTTTGCTGGCATTTACCCT
DNA ligase 4 gene (*LIG4*) promoter 1/Abhydrolase domain containing 13 gene (*ABHD13*) promoter†	GGCTCCCACATAACCTGTTC	GGTACGGAACTGGAGGGAGT
Ribosomal protein L32 gene (*RPL32*, *L23*; control gene)	GGAAGTGCTTGCCTTTTTCC	GGATTGCCACGGATTAACAC

†*See Kuhmann et al. (44) for the location of the promoters.*

expressed as percentage of the input control and corrected for the isotype control.

IgE Levels

Total serum IgE levels, specific IgE levels to the nematode *Ascaris*, and specific IgE levels to *Blomia tropicalis* and *D. pteronyssinus* were measured by ImmunoCAP (Thermo Fisher, Uppsala, Sweden). Total IgE levels were reported in IU/mL. Specific IgE levels above 0.35 kU_A/L were considered positive for IgE sensitization. Specific IgE levels to the purified nematode specific marker ABA-1 were determined by indirect ELISA as described previously (27). ABA-1 is an allergen of *Ascaris* sp., and a member of the nematode polyprotein allergen/antigens with fatty acid-binding properties (28). It has been found only in nematodes (29) and has been used as a serological marker of Ascaris infection (14, 16).

Statistical Analysis

Demographic data were compared between study subgroups by either Fisher's exact test (binary variables) or Mann–Whitney *U* test (continuous variables). The correlation between acetylation levels with age, total egg burden, total IgE and specific IgE levels was calculated by Spearman correlation. Generalized linear models (GLM) were applied using the most appropriate function according to the distribution of the data to evaluate the relationship between histone acetylation and egg burden or IgE antibodies adjusting by the effect of age. Logistic regression was applied to model the relationship between histone acetylation and IgE sensitization to HDM as a categorical variable adjusting for age and gender. A p-value < 0.05 was considered significant. Model-based receiver operating characteristic (ROC) curves were drawn to test for the ability to predict sensitization to HDM allergens and the area under the curve (AUC) was calculated as a measure of performance using the logistic regression model described above.

RESULTS

Study Population

The descriptive characteristics of infected and non-infected subjects are presented in **Table 1**. Of the twenty-one subjects infected by *A. lumbricoides,* nineteen were also infected by *Trichuris trichiura*. Median total IgE levels in infected subjects [902 IU/ml (IQR: 246-2097)] were higher than in non-infected [170 IU/ml (50.8–486), Mann–Whitney $p = 0.003$]. Median

specific IgE to *Ascaris* spp. were significantly higher in the infected group [2.10 kU/l (0.57–8.72)] compared to non-infected [0.30 kU/l (0.08–1.14), Mann–Whitney $p = 0.004$], reflecting induction of the type 2 inflammation by the active helminth infection. Specific IgE levels to *Ascaris* significantly correlated with fecal egg counts of *A. lumbricoides* (rho 0.36, $p = 0.02$) and *T. trichiura* (rho 0.42, $p = 0.007$), thereby the sum of eggs of both helminths per individual was computed as *total egg burden* and this variable used in all subsequent analyses. This tropical population is also perennially exposed to HDM. A positive IgE response to *B. tropicalis* was detected in 43.9% of the individuals and to *D. pteronyssinus* in 36.6%. There was no difference in IgE levels to HDM between infected and non-infected subjects (**Table 1**).

Histone Acetylation and Nematode Infection

Age was inversely correlated with total egg burden (rho − 0.40, $p = 0.01$) and directly correlated with H4Ac (rho 0.38, $p = 0.014$) and H3Ac (rho 0.30, $p = 0.05$) levels in *LIG4/ABDH13*. Total egg burden was inversely correlated with H4Ac levels in *IL13* (rho − 0.32, $p = 0.03$) and in *LIG4/ABDH13* (rho − 0.31, $p = 0.04$). To further evaluate these relationships considering the effect of age as confounding factor we implemented multivariate GLM. These analyses confirmed that increased H4Ac levels in *IL13* were associated with reduced total egg burden even after adjusting by age (**Table 3**). We also detected significant differences in the H3Ac acetylation levels and total egg burden in these genes, which remained significant after adjustment for *IL13* [β −0.58 (−0.86 to −0.29), $p < 0.0001$] and *LIG4/ABDH13* [β −0.28 (−0.40 to −0.16), $p < 0.0001$].

Histone Acetylation and IgE Levels to Ascaris

Specific IgE levels to *A. lumbricoides* extract were directly correlated with H4Ac (rho 0.35, $p = 0.025$) and H3Ac (rho 0.33, $p = 0.03$) levels in *IL4*. In addition, specific IgE levels to *A. lumbricoides* correlated with H4Ac in *CHI3L1* (rho 0.31, $p = 0.049$). The associations of the increased H4Ac levels in *IL4* and *CHI3L1* with increased IgE levels to *A. lumbricoides* also remained significant after adjusting by age (**Table 3**). Moreover, specific IgE levels to the nematode specific marker ABA-1 were directly correlated with histone acetylation levels in *CHI3L1*, affecting H4Ac (rho 0.38, $p = 0.01$) and H3Ac (rho 0.33, $p = 0.03$), supporting that epigenetic changes in these loci were induced by the infection with this nematode.

H4 Acetylation and IgE Sensitization to HDM

We found significant correlations between H4Ac levels in *LIG4/ABHD13* and *TNFSF13B* with specific IgE levels to HDM (**Figure 1**). GLM adjusting by age, confirmed the association between H4Ac levels in *TNFSF13B* with specific IgE levels to *B. tropicalis* and *D. pteronyssinus* (**Table 3**). When sensitization was analyzed as a categorical variable, H4Ac levels in *TNFSF13B* were significantly higher in individuals sensitized to HDM (Mann–Whitney test $p < 0.05$, **Figure 2**). This association remained significant after adjustment by age and gender in a logistic regression model. Next, we computed model-based ROC curves to see how well the regression models can predict sensitization to HDM allergens and computed AUC as a measure of performance. These analyses suggested a good predictive value of H4 acetylation over HDM sensitization with an AUC = 0.76 (**Figure 3**). Interestingly, the differences in the H4Ac in *TNFSF13B* gene were only detected with the response to HDM extracts but not with the *Ascaris* extract (**Figures 2**, **3**).

DISCUSSION

The immune response to helminths and the allergic response share several biological pathways whose study have helped to understand the pathogenesis of both conditions. In the tropics, allergy and helminthiases are frequent, allowing the study of the mechanisms and clinical impact of their interactions (4). In addition to cellular and molecular mechanisms, genetic studies have provided evidence of genes involved in the same pathways activated in allergy and helminth immunity (19, 21). In contrast, epigenetic studies in this field are scarce, even considering the well-known importance of epigenetic mechanisms regulating gene expression. In this study, we present distinct epigenetic changes in *Ascaris* immunity and HDM IgE response. To our knowledge, this is the first report of association between differences in histone acetylation levels in the *IL13* gene and parasite egg burden, which is expected because the great importance of IL-13 in helminth immunity (30). Reduced egg burden can be explained because increased H4Ac in *IL13* may facilitate higher IL-13 production and suggests that this gene is sensitive to and modified by helminth infection. In our study asthmatic patients were not included; however, since IL-13 is also crucial for bronchial inflammation in asthma, this finding could help to explain why there is an increased severity of asthma in some Ascaris infected individuals (31, 32). Thus, our finding supports the traditional evolutionary hypothesis that Th2 allergic inflammation mechanisms are, at least partially explained, by helminth immunity legacy.

We also detected significant associations between H3 and H4 acetylation levels in the *LIG4/ABHD13* at the 13q33 locus with total egg burden, supporting previous association of this chromosomic region with Ascaris egg counts (20) and suggesting that susceptibility to this infection is not only mediated by genetic but also by epigenetic effects. The mechanisms how these genes participate in parasite immunity and influence egg burden remain to be elucidated. The role of IgE on protective immunity to Ascaris has not been sufficiently explored; however, considering previous associations between *LIG4/ABHD13* and the IgE responses to Ascaris (21), our results suggest that IgE may play a role in reducing egg burden during ascariasis (14, 16).

We also detected here a direct association between H4Ac in the *IL4* gene and IgE levels against *Ascaris* suggesting that increased acetylation at the *IL4* locus might influence IgE synthesis. H3 and H4 histone acetylation levels in *IL13* and *IL4* were directly correlated (**Figure 1**), however, the association

TABLE 3 | Generalized linear regressions on the relationship between H4 acetylation with indicators of parasite and HDM exposure (n = 41).

	β (95% CI), *p*-value (crude)	**β (95% CI), *p*-value (adjusted by age)**
Gene	*Total egg burden*	
IL13	−0.21 (−0.32 to −0.09), $p < 0.0001$	−0.20 (−0.32 to −0.09), $p < 0.0001$
LIG4/ABDH13	−0.17 (−0.29 to −0.04), $p = 0.009$	−0.12 (−0.29 to −0.05), $p = 0.17$
	IgE to *Ascaris*	
IL4	0.29 (0.03–0.55), $p = 0.03$	0.32 (0.05–0.60), $p = 0.02$
CHI3L1	0.28 (0.05–0.51), $p = 0.018$	0.29 (0.08–0.51), $p = 0.008$
	IgE to *B. tropicalis*	
TNFSF13B	0.57 (0.32–0.82), $p < 0.0001$	1.50 (0.67–2.32), $p < 0.0001$
LIG4/ABDH13	0.69 (0.14–1.25), $p = 0.014$	5.36 (−6.9 to 17.6), $p = 0.39$
	IgE to *D. pteronyssinus*	
TNFSF13B	0.52 (0.28–0.76), $p < 0.001$	0.51 (0.26–0.76), $p < 0.001$
LIG4/ABDH13	0.53 (0.30–0.77), $p < 0.001$	0.47 (0.24–0.70), $p < 0.001$

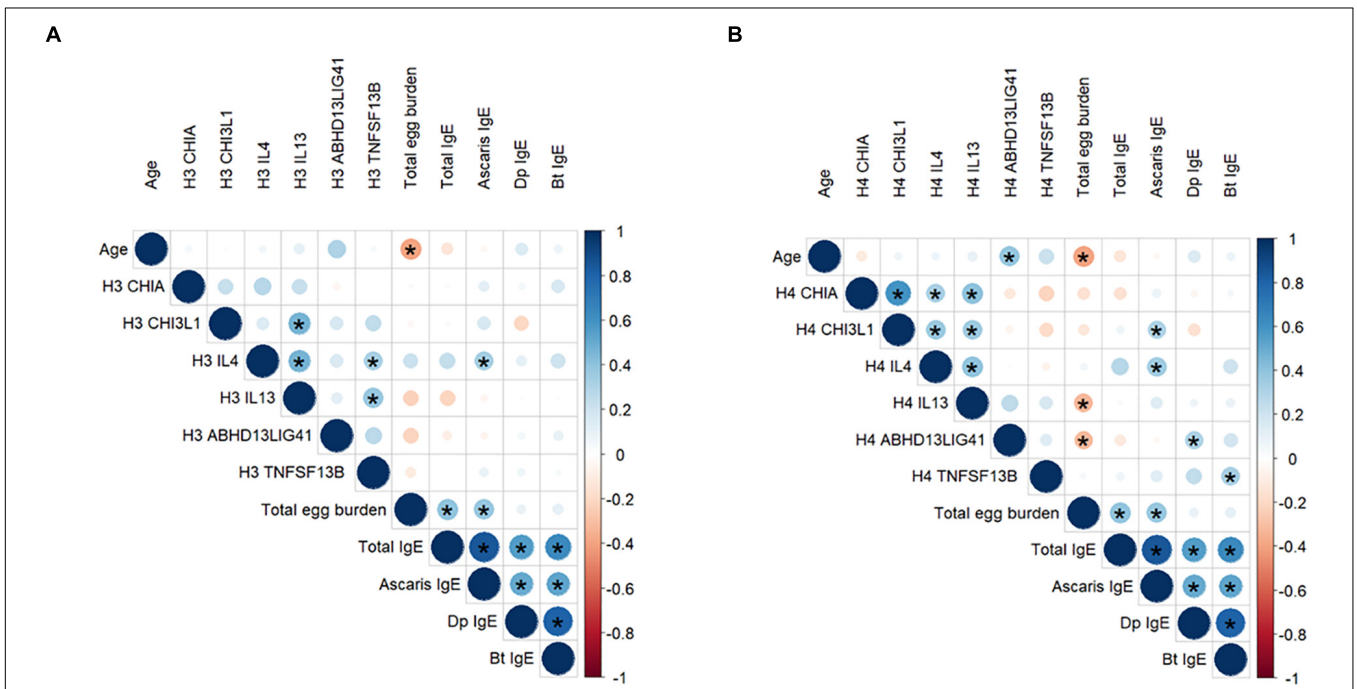

FIGURE 1 | Correlation of H3Ac **(A)** and H4Ac **(B)** levels at six gene promoters with age, total egg burden, and total and specific IgE levels to Ascaris and HDM. The scale indicates the Spearman coefficient (Rho) from –1 to 1. Direct correlations are indicated in the blue scale and inverse correlations are indicated in the orange scale. Significant correlations ($p < 0.05$) are indicated with an asterisk. Dp: *D. pteronyssinus*; Bt: *B. tropicalis*.

between *IL13* acetylation with egg burden was significant with both histone marks (H3 and H4) while the association between *IL4* acetylation with specific IgE levels to *Ascaris* was only significant with H4Ac. The reason why each cytokine is associated to each of these outcomes is unknown, but a mice model revealed that a deletion in a DNase I-hypersensitive site 2 (HS2) element in the second intron of the interleukin 4 locus (*Il4*) impaired the acetylation of histone H3 at Lys9 and Lys14 in the *Il4* locus and affected the production of IL-4 but not of other Th2 cytokines, suggesting that it may occur chromosomal modifications on *Il4* that are independent of the *Il5* and *Il13* loci (33). Also in agreement with our previous genetic study (21), here we confirmed the association of *CHI3L1* with the specific IgE levels to *Ascaris* and ABA-1, showing for the first time increased H4Ac levels in high IgE responders to *Ascaris*. Recent studies revealed that expression of *CHI3L1* is modified upon contact with the *Ascaris* larvae (34). Further research is needed to elucidate the role of H4 acetylation in *CHI3L1*

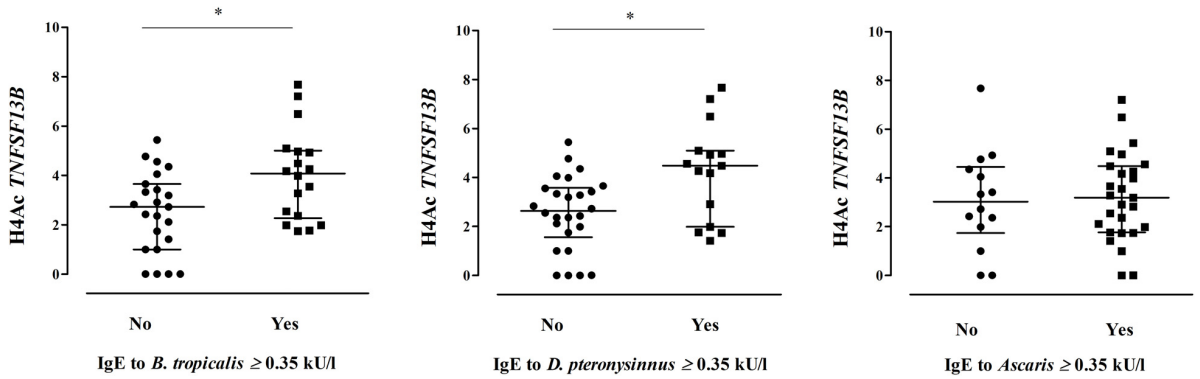

FIGURE 2 | Comparison of the H4Ac levels in *TNFSF13B* according to HDM and *Ascaris* sensitization (*Mann–Whitney test $p < 0.05$). Each dot represents an individual, lines indicate median and IQR.

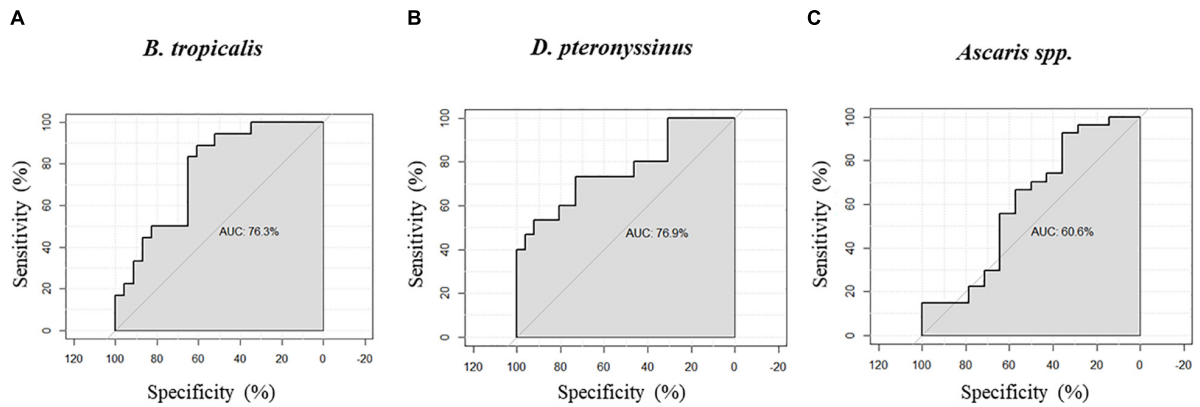

FIGURE 3 | Receiver operating characteristic (ROC) curves and area under the curve (AUC) obtained from logistic regression models to predict the sensitization to HDM allergens. **(A)** H4 acetylation at the *TNFSF13B* gene versus IgE sensitization to *B. tropicalis*. **(B)** H4 acetylation at the *TNFSF13B* gene versus IgE sensitization to *D. pteronyssinus*. **(C)** H4 acetylation at the *TNFSF13B* gene versus IgE sensitization to *Ascaris* spp.

expression and its contribution to boosting IgE synthesis upon *A. lumbricoides* infection.

Epigenetic changes leading to bronchial inflammation and hyperresponsiveness have been induced by HDM under experimental conditions in mice (35, 36) suggesting that those epigenetic mechanisms may also contribute to asthma pathogenesis. Performing this kind of research in humans is more difficult, however indirect analyses can be done using specific phenotypes in natural exposed individuals (37). In this study we found that increased H4Ac at the gene *TNFSF13B* encoding B-cell activating factor was associated with elevated IgE levels to HDM allergens, suggesting that perennial exposure to HDM might affect histone acetylation at this locus in those predisposed to IgE sensitization. The B cell activating factor plays a critical role in B cell development and immunoglobulins production (38, 39). We found no association between H4Ac at this gene and IgE to Ascaris, which is in contrast with a previous study suggesting that this gene is associated with the humoral responses to Ascaris extract (40). However, in a more detailed study we found that among 13q33.3 region-genes enriched in high responders to Ascaris, *TNFSF13B* was not associated with specificity but rather the strength of IgE levels (21). These findings indicate that more studies are needed to dissect the control of the IgE response by the Ascaris susceptibility locus chr. 13q33.3.

In this study we evaluated acetylation changes in amino acid residues of H3 and H4; both implicated in the regulation of cytokine gene expression (41, 42). Whether histone tails act independently or have synergistic effects is still disputed. Acetylation of histone H4 is often found to be anticorrelated with acetylation of H3 or the other histones in binding of transcription factors, expressing genes or remodeling the chromatin (42, 43). In our study we found significant direct correlations of H3Ac and H4Ac levels in *IL-4, CHIA* and *CHI3L1* genes, while there were no significant correlations in the acetylation levels of these histones in *IL-13* and the two regions in 13q33 (**Supplementary Figure S1**). Interestingly, the associations with total egg burden were detected with both H3 and H4 histones while the associations with IgE levels only remained significant with H4Ac levels, suggesting that H4 marks might be more informative for epigenetic effects associated to the allergen exposure at these genes. We also showed for the very first time the

significant correlations between the H4 acetylation levels in gene regions that albeit being in different chromosomes seem to be related (**Figure 1B**). Still, how acetylation levels at chitinase related genes in chromosome 1 are mechanistically connected to acetylation levels in *IL13* and *IL4* at the chromosome 5q31 remains to be investigated (**Figure 1B**), but the results suggest that nematode infection may induce coordinated histone changes in type 2 immunity pathways. However, since our study is cross-sectional, the effects of other environmental factors on the acetylation patterns cannot be ruled out.

In conclusion, this study provides evidence that allergen exposure alters the patterns of epigenetic modifications in human mononuclear leukocytes. Increased H4 acetylation in key immune genes is reflected by increased IgE levels to nematode and HDM allergens, suggesting an additional explanation to the similarities between helminth immunity and the allergic response. Further studies are needed to elucidate the functional effects of these acetylation marks on gene expression and the mechanisms promoting the type-2 immune response.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the University of Cartagena (nr. 1705-2012) and was conducted following the guidelines of the Declaration of Helsinki. All the participants gave their written informed consent prior to their inclusion in the study. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LC and DP conceived and designed the experiments. JZ, HH, and LE performed the experiments. JZ and NA analyzed the data. JZ, NA, HH, DP, HR, and LC contributed the reagents, materials, and analysis tools. NA, LC, and JZ wrote the manuscript. All authors involved in writing, reviewing, and editing. LC, JZ, HR, and DP involved in funding acquisition. LC overall responsibility of the project.

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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.2020.00756/full#supplementary-material

FIGURE S1 | Correlations between H3 and H4 acetylation levels in the six promoter regions analyzed in this study.

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Exploring the Molecular Mechanisms Underlying the Protective Effects of Microbial SCFAs on Intestinal Tolerance and Food Allergy

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A body of evidence suggests that food allergy (FA) has increased in prevalence over the past few decades. Novel findings support the hypothesis that some commensal bacteria and particularly microbial metabolites might contribute to development of oral tolerance and prevention from FA. Recently, beneficial effects of short-chain fatty acids (SCFAs), the main class of gut microbiota-derived metabolites, on FA have been proposed. The intestinal SCFAs are major end products during bacterial fermentation of complex and non-digestible carbohydrates such as dietary fiber. The multifaceted mechanisms underlying beneficial effects of SCFAs on the mucosal immune system comprise the regulation of diverse cellular pathways in epithelial, dendritic, and T cells, as well as the impact on the immunometabolism and epigenetic status of regulatory lymphocytes. Of note, SCFAs are effective inhibitors of histone deacetylases (HDACs). As a consequence, SCFAs appear to be implicated in attenuation of intestinal inflammation and autoimmune diseases. In this review, we will discuss the recent development in this research area by highlighting the role of the individual SCFAs acetate, propionate, butyrate, and pentanoate in promoting the differentiation of regulatory T and B cells and their potential beneficial effects on the prevention of FA. In this context, targeted alterations in the gut microbiota in favor of SCFA producers or supplementation of medicinal food enriched in SCFAs could be a novel therapeutic concept for FA.

Keywords: commensal bacteria, microbial metabolites, short-chain fatty acids, gut homeostasis, food allergy

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INTRODUCTION

The human gut harbors one of the densest microbial habitats on the planet Earth containing thousands of uncharacterized metabolites. Intestinal microbiota synthesize diverse small molecules that play an important role in the communication between the host immune system and commensals (1, 2). Such soluble messengers may affect various physiological processes such as inhibition of colonization of pathogenic bacteria, supporting metabolic and immunological functions of the host, and even the modulation of host behavioral processes (3, 4). Bacterial fermentation of dietary fiber results in the generation of the main class of gut-microbiota derived metabolites, short chain fatty acids (SCFAs). SCFAs, including acetate, propionate, butyrate, and pentanoate, regulate multiple aspects of human health including beneficial effects on autoimmune and inflammatory disorders (5, 6). While host digestive enzymes in the oral cavity, stomach, and upper intestine lack the ability to digest complex carbohydrates such as pectin and inulin,

those water-soluble dietary fiber are readily fermented in the gut lumen by various members of the human microbiota. Amounts of SCFAs vary along the gastrointestinal tract reaching the highest concentrations within millimolar range in the proximal colon and cecum (7). Specific bacterial species implicated in the synthesis of individual SCFAs have been recently identified (2). The most dominant commensal butyrate producers belong to the phylum Firmicutes, whereof Clostridia from the human gut microbiota are the major butyrate-producing class (8). Particularly, *Faecalibacterium prausnitzii*, *Eubacterium rectale,* and several *Roseburia* species are able to synthesize high amounts of this SCFA (9, 10). In contrast to conventionally raised mice that have high levels of acetate, propionate and butyrate, germ-free animals are completely devoid of SCFAs. There is substantial evidence that SCFAs have various effects on host physiology not only in the gut, but also in the distal organs such as brain and lung (11–13). This review summarizes recent work carried out over the past several years illustrating diverse impacts of SCFAs and dietary fiber on host immune system, microbial, and oral tolerance, as well as their beneficial effects on food allergy.

MECHANISMS OF SCFA-MEDIATED REGULATION OF THE HOST IMMUNE SYSTEM

Proposed mechanisms underlying SCFAs-mediated modulation of the gut epithelium and mucosal immune system comprise at least three different modes of action. SCFAs act as diffusible signaling molecules that have substantial effects on eukaryotic cells expressing G protein-coupled receptors (GPRs) such as GPR41, GPR43, and GPR109a (14). Although the preferential binding of individual SCFAs to various GPRs has not yet been completely elucidated, diverse signaling cascades can be activated following ligation of microbial SCFAs to metabolite-sensing molecules. In colonic epithelial cells, propionate and acetate have been shown to induce p38 and ERK MAPK activation through GPR41 and GPR43 (15). These cell surface SCFA-receptors are expressed not only on the gut epithelium, but also on intestinal immune cells such as dendritic cells (DCs) and regulatory T cells (Tregs). The GPR109a expression on DCs supports the proliferation of Tregs and thus promotes tolerogenic effects in the gut (16). In addition, colonic Tregs express high levels of the SCFA-sensing receptor GPR43, which enables them to protect mice against colitis (17). Moreover, SCFA-derived atoms serve as carbon source for epithelial cells, thus directly fueling host metabolism (2). Finally, as strong histone deacetylase (HDAC) and lysine deacetylase (KDAC) inhibitors, butyrate and propionate elicit most of their effects by modulating the expression of various genes involved in several biological processes such as cell proliferation and differentiation, antimicrobial immunity, integrity of epithelial barrier, and intestinal tolerance to bacterial antigens and dietary proteins (18–20). Although some controversies remain, recent findings have revealed that SCFAs enhance the glycolytic rate of immune cells and increase acetyl-CoA concentrations, thus connecting the cellular metabolism and chromatin modifications (13, 21). The SCFA-mediated increase in glucose-derived pyruvate and acetyl-CoA levels in eukaryotic cells leads to the accumulation of citrate, its transport to the cytosol and subsequent conversion into cytosolic acetyl-CoA by ATP citrate lyase (ACLY). ACLY is the key cytosolic enzyme that converts citrate to acetyl-CoA, which is needed for histone acetyltransferase (HAT)-dependent histone acetylation (22). There is a substantial body of evidence that SCFAs are not only HDAC inhibitors, but they are also able to promote histone modifications in immune cells by acting as acyl-CoA precursors. Thus, the carbon atoms derived from SCFAs can directly be transferred to histones via a metabolic-epigenetic link leading to HAT-mediated histone acetylation and recently described histone propionylation and butyrylation (23). Remarkably, SCFAs seem to be unique molecules able to regulate the gene expression at the epigenetic levels by modulating the activity of both, HATs and HDACs. Although further studies are still required to better understand interactions between microbial metabolites, HAT activity and histone acylations, current data suggest that SCFAs provide a pool of acyl groups for generation of acetyl-CoA and other endogenous metabolites in gut epithelial and immune cells, which can be used for various cellular activities (24).

SCFAs ACTIVELY SUPPORT THE TOLERANCE TO FOOD ANTIGENS AND COMMENSAL BACTERIA

Metabolomic analysis of the gut microbial community has shown that SCFAs, a major group of bacterial molecules in the gut lumen, are potent modulators of the mucosal immune system (2, 25). Recent studies have demonstrated that SCFAs are not only locally protective in the intestinal environment, but they can even act in remote tissues such as pancreas, lung, and brain (11, 26, 27). Although it is well-appreciated that SCFAs impact on the colonic epithelial cells, Tregs and DCs, less is known about the complex mechanisms underlying bidirectional interactions between intestinal cellular networks and individual members of SCFA-producing microbiota. Moreover, despite some promising results obtained in experimental murine models, a possible protective effect of SCFAs and dietary fiber on the onset of human gastrointestinal disorders such as inflammatory bowel disease (IBD), celiac disease, and food allergy is relatively poorly characterized. Among SCFAs, butyrate has been specifically associated with the expansion of mucosal Tregs and it also acts as a preferred carbon source for colonocytes (2). During gut homeostasis, the metabolism of colonic epithelial cells is profoundly dependent on oxidative phosphorylation, which leads to high oxygen consumption. Interestingly, microbiota-derived butyrate utilized by the gut epithelium affects the O_2 levels in these cells resulting in activation of the oxygen sensor hypoxia-inducible factor (HIF), a transcription factor that is crucial for coordinating gut integrity and barrier protection (28). In addition, butyrate and other SCFAs have a strong influence on tight junctions (TJ) and production of mucin (29, 30). Furthermore, SCFAs seem to maintain intestinal barrier function by stimulating the synthesis of

antimicrobial peptides and the cytokine IL-18, which strengthens the tolerance to commensal bacteria and promotes intestinal homeostasis (31, 32). Butyrate influences intestinal $CD103^+$ DCs by stimulating the GPR109a cell surface receptor, which enables this tolerogenic DC subpopulation to trigger proliferation and expansion of regulatory T cells (Tregs) in mesenteric lymph nodes (16). DCs treated with butyrate, propionate, and pentanoate exhibit a lower capacity to stimulate effector $CD4^+$ T cells (33). Small intestinal DCs display a selective capability to induce retinoic acid (RA)-dependent increase in the activity of aldehyde dehydrogenase (ALDH) that strongly supports the tolerance to food antigens due to concomitant expansion of food antigen-reactive Tregs (34). Recently, Surh and colleagues were the first to show that small intestinal Tregs recognize dietary antigens and limit undesired and adverse reaction to food by promoting dominant immunosuppressive response (35). Dietary antigens derived from solid food share the space inside the small intestinal lumen with various dietary components and microbial metabolites. Collectively, diet-derived RA and microbiota-derived SCFAs seem to act synergistically on intestinal DCs to control immune response to food antigens by dampening induction of inflammatory cytokines as well as by inducing Tregs that play a pivotal role in controlling the tolerance to food and commensal antigens (36).

Recent findings have revealed a broad heterogeneity of mucosal Tregs (37, 38), however, there is still no evidence that SCFAs and SCFA-producing bacteria might preferentially support the generation of a particular Treg subpopulation under certain environmental conditions. Butyrate has been suggested to potentiate the expansion of intestinal Tregs by promoting the acetylation of histones at the Foxp3 gene, but also by protecting the Foxp3 protein from degradation through enhancing its acetylation (39). Thus, by acting within Tregs as a KDAC inhibitor to enhance acetylation of Foxp3 protein and as a HDAC inhibitor at the Foxp3 gene locus, butyrate suppresses inflammation and adverse immune responses in intestinal tissues. Beyond modulating the epigenetic status of Tregs, butyrate and other SCFAs have been shown to influence the function of B cells in Peyer's patches (PPs) and the small intestine. SCFAs appear to be capable of increasing the number of IgA-secreting lamina propria plasma cells and B cells in PPs (21, 40). These effects of SCFAs on B cells seem to be mediated by enhancing their metabolic activity. It has also been suggested that particularly pentanoate and butyrate are able to induce IL-10 production in B cells, which promotes the differentiation of regulatory B cells (Bregs) (13). These unexpected results suggest that microbiota-derived SCFAs are not only important for the maintenance and expansion of mucosal Tregs and their function, but also for promotion of the Breg cell phenotype. Gaining a better understanding of the anatomic sites at which SCFAs-mediated effects on T and B lymphocytes occur under physiological conditions could be of importance for the future. There is some evidence that not only surface molecules of commensal bacteria, but also soluble microbial metabolites such as SCFAs support the synthesis of protective IgA and IgG antibodies during the intestinal infection with *Citrobacter rodentium* by increasing activity of mTOR and glycolysis in B cells (21). This suggests that SCFAs do not only promote the tolerance to food antigens and microbiota by modulating IgA antibody responses, but they also may help eliminating intestinal pathogenic infections.

EFFECTS OF SCFAs AND DIETARY FIBER ON MAST CELLS AND FOOD ALLERGY

A better understanding of the influence of gut-microbiota derived molecules on the maturation and function of the immune system in the small intestine may open novel important therapeutic options in a variety of gastrointestinal disorders ranging from IBD to food allergy. In the last decades, a significant increase in the prevalence of food allergies that is characterized by adverse immune responses to food antigens, which are mainly derived from peanuts, milk, eggs, tree nuts, strawberries, or shellfish, has been observed (41). The most characteristic form of food allergy is mediated by IgE-dependent pathways (42). Human IgE-triggered peanut allergy is associated with a high cell number of somatically mutated and clonally expanded gastrointestinal allergen-specific IgE^+ B cells suggesting a local isotype switching, which likely includes the transition between IgA and IgE antibody isotypes (43). Recent data suggest that some dietary components such as RA (an active derivative of dietary vitamin A) and dietary peptides, as well as microbial SCFAs may act together to promote intestinal homeostasis and suppress food allergy. Interestingly, dietary proteins induce the expansion of food protein-reactive Tregs in the small intestine, as well as the production of IgA and generation of follicular helper T (Tfh) cells in the PPs, thus strengthening intestinal homeostasis (35, 44, 45). In a mouse model of peanut allergy, SCFAs and RA have been shown to shape local immune responses and oral tolerance by increasing the function of tolerogenic $CD103^+$ DCs that are essential for generation of mucosal Tregs. Moreover, high-fiber diet and SCFA supplementation protected mice from food allergy by promoting production of IgA in small intestinal lamina propria and by enhancing the frequency of follicular T (Tfh) cells in the PPs. Particularly, mice orally treated with the SCFAs acetate and butyrate displayed a reduction in anaphylactic clinical scores and diminished serum IgE levels as compared to control animals following induction of peanut allergy (34). In human food allergy, individuals exposed to food allergens have a high amount of intestinal Th2 cells as well as type 2 innate lymphoid cells (ILC2) that produce cytokines such as IL-4, IL-5, and IL-13 (46). IL-4 is known to strongly support the differentiation of B cells into IgE-synthesizing plasma cells (47). The subsequent exposure of those individuals to food allergens mediates the cross-linking of allergen-specific IgE via FcεRI on mast cells. This induces degranulation and release of histamine and several other effector mediators, which results in immediate allergic reaction (41). Interestingly, recent studies suggest that the SCFA butyrate exhibits a direct effect on mast cells by epigenetically regulating the FcεRI-mediated signaling molecules (48–50). Thus, by directly inhibiting the IgE-mediated mast cell degranulation and allergen-induced

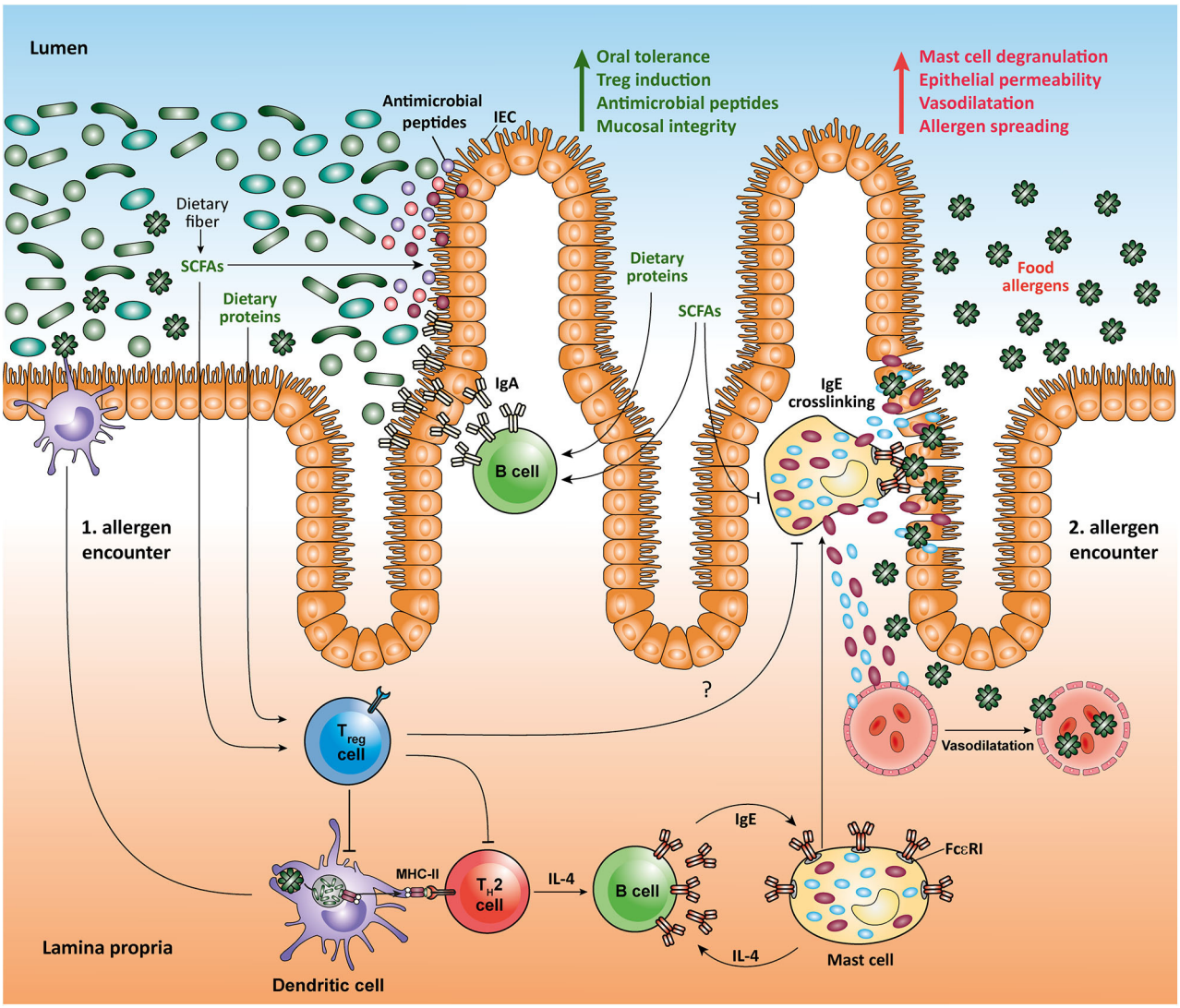

FIGURE 1 | Impact of microbial SCFAs on intestinal homeostasis and food allergy.

histamine release, microbial SCFAs such as butyrate could have therapeutic benefits in human food allergies (**Figure 1**). Of note, high levels of SCFAs butyrate and propionate in feces in early life of children are associated with protection against food allergy and asthma (51). Furthermore, children with cow's milk allergy were shown to have reduced fecal levels of butyrate compared to healthy controls (52). Together, novel results establish an important role for dietary fiber and SCFAs in promoting the integrity of epithelial barrier, oral tolerance and protection against food allergies. These observation could, at least in part, be explained by inhibitory effects of SCFAs on HDACs in several immune cells such as Tregs, B cells, and mast cells, as well as via stimulation of SCFA-receptors such as GPR41, GPR43, and GPR109a on epithelial cells and $CD103^+$ DCs.

CONCLUSIONS

Although some controversies remain, accumulating evidence supports the role of microbiota-derived metabolites in promoting tolerogenic immune responses in the healthy intestine. In the last decade, a better understanding of microbiota-interactions that influence many aspects of human health including protection against pathogens, strengthening epithelial barrier function and promotion of tolerance to food antigens and commensals has led to the idea that a healthy core microbiome and its main metabolites SCFAs may be of high therapeutic interest. Such low-cost and potent small molecules might not only help maintaining intestinal homeostasis in healthy individuals, but they could be also applied to a variety of gastrointestinal disorders ranging from IBD and celiac disease to pathogenic conditions such as food

allergies and irritable bowel syndrome (IBS), which are often associated with altered gut microbiota. We suggest that designing medicinal food enriched in SCFAs may lead to development of novel therapeutic approaches in food allergy.

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

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Go With Your Gut: The Shaping of T-Cell Response by Gut Microbiota in Allergic Asthma

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Novel methods in immunological research and microbiome evaluation have dramatically changed several paradigms associated with the pathogenesis of allergic asthma (AAS). Ovalbumin and house dust mite-induced AAS in germ-free or specific pathogen-free mice are the two leading experimental platforms that significantly contribute to elucidate the relationship between AAS and gut microbiota. Beyond the exacerbation of T helper (Th) 2 responses, a complex network of immunological interaction driven by gut microbiota could modulate the final effector phase. Regulatory T cells are abundant in gastrointestinal mucosa and have been shown to be pivotal in AAS. The gut microbiota could also influence the activity of other T cell subsets such as Th9, Th17, and populations of effector/memory T lymphocytes. Furthermore, gut microbiota metabolites drive the hematopoietic pattern of dendritic cells and ameliorate lung Th2 immunity in AAS models. The administration of probiotics has shown conflicting results in AAS, and limited evidence is available on the immunological pathways beyond their activity. Moreover, the impact of early-life gut dysbiosis on AAS is well-known both experimentally and clinically, but discrepancies are observed between preclinical and clinical settings. Herein, our aim is to elucidate the most relevant preclinical and clinical scenarios to enlighten the potential role of the gut microbiota in modulating T lymphocytes activity in AAS.

Keywords: gut microbiota, asthma, children, T lymphocytes, microbiome

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T CELL PLASTICITY AND GUT MICROBIOTA

During homeostatic periods, gut-microbiota and T-cells within the gut mucosa engaged profitable crosstalk capable of shaping the systemic immune response of the individual. T-cell-receptors are reactive to microbiota-derived antigens that are necessary for an adequate maturation of the immune system and to ensure proper colonization of the gut lumen. Heterogeneous microbiota-derived signals could drive the polarization of Th cells into four different categories: while Th1 (1) and Th17 (2) dysregulation are relevant in inflammatory and autoimmune diseases, $GATA3^{+}$ Th2 responses (3) and T regulatory lymphocytes (Tregs) (4) are pivotal in allergic responses. Some bacteria metabolites, such as short-chain fatty acids (SCFAs), could regulate the tolerogenic effect of Tregs and they are directly sensed by G-protein-coupled-receptors (GPCRs) (5). Alternatively, Tregs polarization could be promoted by an IL-10 dependent pathway from microbiota-derived antigen presentation by dendritic cells (DCs) (6). Several other Th subsets such as Th9 (7) and Tregs subtypes (8) are implicated in allergic responses while their relationship with the gut microbiota is not entirely defined. Furthermore, the role of Th17 in AAS was heavily investigated (9) and they are currently recognized as one of the crucial mediators of AAS.

WHAT WE HAVE LEARNED FROM GUT MICROBIOTA MANIPULATION IN MURINE MODELS OF ALLERGIC ASTHMA

More than a decade ago, evidence in murine models rose in support of the hygiene hypothesis, when Th2 response was associated with a state of gut dysbiosis (10). Nowadays, the paradigm has evolved, and the complex immunological network beyond the gut-lung axis is under active research.

The Role of *Clostridium spp* Emphasize Regulatory T-Cell Activity

A number of studies identified *Clostridium spp* as crucial modulators of AAS. First, insights come from the investigation on the impact of antibiotic therapies on T cell populations. It was demonstrated that $CD4^+CD25^+FoxP3^+$ Tregs are slightly reduced when mice are treated with polymixin B or vancomycin but not streptomycin withing the intestinal wall, while lung Tregs frequencies are comparable among vancomycin or streptomycin-treated animals or controls (11, 12). Interestingly, the ovalbumin (OVA) challenge exacerbates AAS in vancomycin-treated animals but not within the streptomycin group (12). It is well-known that vancomycin preferentially targets *Clostridium spp*. In order to further elucidate the role of these strains in AAS, stool transfer experiments between mice were conducted. Murine models colonized with fecal water derived from mice supplemented with a mixture of *Clostridium* strains have demonstrated a higher percentage of $IL10^+CTLA\text{-}4^{high}IKZF2^-$ colonic Tregs when compared to controls while a similar approach with *Lactobacillus spp* and *Bacteroides spp* failed to show any significant variation (11). Of note, in this context, Tregs does not express IKZF2, an essential transcription factor that is necessary for the stabilization of their suppressive activity in autoinflammatory models (13). A similar approach with fecal material from human volunteers has demonstrated that clusters of Clostridia IV/XIV and XVIII are able to increase the frequency of $IL10^+ICOS^+CTLA4^+CD25^+$ Tregs in colon mucosa and they are implicated in protection against OVA-induced Th2 colitis (14). However, their relevance in AAS is under debate. One of the significant limitations of these studies is represented by the absence of a detailed functional characterization of T-cells; for instance, IL-10 and CTLA-4 are essential for the immunosuppressive activity of Tregs, while ICOS is crucial for their effector activity rather than their induction (15) thus, the contribution of different T effector/memory (Tem) populations should be addressed.

The Importance of Effector/Memory T Cells Revealed by *Bacteroides spp*

Observations in AAS models enhance the role of *Bacteroides spp*. Even if they are not a direct target of vancomycin, perinatal treatment in mice reduces *Bacteroides spp* and relatively increases *Lactobacillus spp* (16). Importantly, the effect of vancomycin on AAS is evident only during early-life and not during adulthood, identifying a specific "window of opportunity" (16). Mechanistic studies on *Bacteroides fragilis* in AAS provide further insights into the regulation of T cell response. In other settings, it was shown that the polysaccharide A (PSA) is a non-protein antigen presented by MHC-II (17) capable of activating $CD4^+$ cells (18). The oral exposure to PSA protects murine models against AAS trough a T-cell mediated pathway (19). PSA usually elicits a $FoxP3^+$ peripheral Tregs response in mice and $FoxP3^-$ Tregs cells in humans (20). However, in mice primed with PSA and sensitized to OVA, the PSA-responding population is not composed of $FoxP3^+$ Tregs but relies on IL-10 to exert its protective effect (19). Thus, it seems that PSA could protect against AAS through an IL-10 dependent mechanism, but the source of IL-10 was unclear. The adoptive transfer approach of PSA-responding $CD4^+$ T lymphocytes between $IL10^{-/-}$ and wild type animals allows the recognition that PSA-responding T cells are a population of $FoxP3^-CD45Rb^{low}CD44^+CD62L^-$ Tem that support the production of IL-10 by lung resident $FoxP3^+$ Tregs (21). To date, it is not known if other gut-derived antigens could act indirectly on lungs T response in a similar way.

Modulation of the Gut-Lung Axis Through Antibiotics and Probiotics Impacts the T-Cell Response

Mice treated with a combined intermittent antibiotic regimen early after weaning and subsequently challenged with house dust mite (HDM) show a significant reduction of the $FoxP3^+/CD4^+$ ratio in mediastinal lymph nodes (MLNs), that is proportional to the Simpson diversity index variation of the fecal microbiome (22). Therefore, a direct link between Tregs homeostasis and gut microbiota in AAS exists, and a state of dysbiosis could strongly influence the severity of Th2-mediated inflammation. Among the limitations of this approach, the use of aggressive and intensive antibiotic regimens is one of the most relevant obstacles for translation into clinical practice. Although direct evidence of its implication in AAS is currently lacking, other works address the long-lasting immunomodulatory effects of an early-life single macrolide course vs. a three pulses course (23). In this context, intestinal $CD4^+IL17A^+$ T lymphocytes are decreased while $CD4^+$IFN-γ T cells are not affected, and $CD4^+FoxP3^+$ Tregs percentage is only slightly increased among treated animals (23). Of note, only slight differences are noted in germ-free mice, and the degree of systemic immune perturbance of a single macrolide pulse is relatively modest, but it is strong enough to induce an imbalance in local Th17 immunity, leading to long-lasting microbiome alterations (23). It would be of great interest to explore T-cell development in a similar model challenged with HDM or OVA.

Since transient gut dysbiosis is relatively common in children due to the widespread use of antibiotics (24), it is possible that an early probiotic administration could recover this state and drive the Th1/Th2 balance. Supplementation with *Lactobacillus Ramnosus* or *Bifidobacterium lactis* is proven to be an efficient AAS suppressor and a robust inductor of $FoxP3^+$ Tregs in MLNs of newborn OVA-sensitized mice (25). Moreover, the proliferative response to probiotic administration enhances the production of TGF-β secreting $CD4^+/CD3^+$ T

lymphocytes (25), thereby contributing to the establishment of a tolerogenic environment. Probiotic treatment has been shown to be effective in protecting newborn mice against AAS while it has been proven to be ineffective in adult mice. Interestingly, both adult and newborn mice are able to induce a $CD4^+FoxP3^+$ Treg response in the lungs after probiotic administration, but when splenocytes from tolerant wild-type mice were adoptively transferred into adult mice, only newborn's Tregs are able to control pulmonary inflammation (26). Therefore, the generation of Tregs itself is not sufficient to confer protection to AAS, but an intrinsic feature of neonatal Tregs is necessary (26), and it would be of great interest for future research.

Diet Modulation of T Cell Response Is Only Partially Driven by Gut Microbiota Alterations

Environmental factors play a crucial role in the development of AAS, and specific maternal diets could be beneficial in preventing the onset of AAS. Bacteria metabolize fermentable dietary fiber into SCFAs, small soluble molecules that could trigger strong immunomodulatory effects (27). SCFAs diet content is capable of driving the gut microbiota composition, and this shift could lead to significant protection against AAS (5). Interestingly, vancomycin treatment in mice mainly delates bacteria capable of producing SCFAs (28).

After a high-fiber diet, the gut microbiota is enriched with *Bacteoidaceae* and *Bifidobacteriaceae,* while SCFAs, particularly acetate and propionate, increased (5). However, SCFAs are not detected in lung tissue after HDM challenges; thus, they exert their action through an indirect mechanism (5). Furthermore, butyrate enhances the extrathymic generation of Tregs (29), but a high-fiber diet was not associated with an increase in $CD25^+FoxP3^+$ Tregs (5). Therefore, other mechanisms should underline this effect. Bone-marrow derived DCs were investigated as possible metabolite-specific mediators. It was demonstrated that butyrate significantly alters DCs gene expression, reduces costimulatory molecules and impairs CCL19-dependent DCs migration (28). On the other hand, propionate acts via GPCRs in a context-dependent manner, enhancing the hematopoietic activity of DCs precursors that could impair Th2 activity in the lungs (**Figure 1**) (27). Therefore, gut microbiota diet perturbations could indirectly affect the Th2 response through the modulation of the hematopoietic activity in the bone marrow through a metabolite-specific pathway. In addition to this mechanism, SCFAs can establish an anti-inflammatory activity by the direct interaction with the histone deacetylase (HDAC) protein family (30). Of note, HDAC proteins confer a permissive chromatin structure that enhances the transcription of involved regions (27). Propionate, but not acetate, could inhibit HDACs, enhancing the extrathymic Treg generation promoted by butyrate (29). According to this finding, dramatic protection against AAS after HDM exposure in progeny was related to the downregulation of *Nppa* that inhibits HDAC-9 and increases the acetylation rates of the FoxP3 promoter (30). Furthermore, this action was reported as independent from the microbiota shift observed in treated mice (30).

Other T-cell subsets could be involved in SCFAs-mediated protection against AAS. In newborn mice treated with a commercially available mixture of probiotics, increased levels in butyrate, but not other metabolites, are able to induce the proliferation of Tregs and negatively modulate OVA-induced AAS (31). Since Tregs are potent inhibitors of Th9 (32), their capacity to reduce IL-9 expression and Th9 differentiation was assessed. Butyrate-treated mice show lower frequencies of Th9 cells in the lungs, while no difference is observed for Th2 cells (31). Moreover, adoptive transfer of Th9 or IL-9 administration could revert the protective effect of butyrate, indicating that the mechanisms are partially related to this Th subclass (31). Lastly, while the link between obesity and non-allergic bronchial hyperresponsiveness is widely accepted, a high-fat diet has little effect in modulating Tregs responses by an impairment of DCs activation in OVA-induced AAS (33). Therefore, diet could have a significant role during early developmental stages, and it is capable of shaping the T-cell effector phase through a variety of mechanisms.

The Hidden World: Fungi and Viruses

Fungal dysbiosis was recently recognized as one of the foremost promoters of AAS in experimental models, but the mechanisms are only partially understood. Fluconazole can exacerbate AAS in mice and increase $IL\text{-}4^+CD4^+$ and $IL\text{-}13^+CD4^+$ T-cells (34). In another study, *Wallemia mellicola* colonizes the intestine efficiently after depletion of resident microbiota by wide-spectrum antibiotics and promotes the accumulation of $IL\text{-}13^+CD4^+$ T-cells in MLNs and AAS exacerbations (35). Therefore, the induction of Th2 responses seems to be cardinal during mycobiota dysbiosis. However, *C. albicans spp.* were able to increase AAS susceptibility through $ROR\delta T^+$ T-cells (36) and recent findings in mice treated with fluconazole and then "recovered" with oral gavage of three dysbiotic fungal species demonstrate that AAS susceptibility is mediated by a mixed $GATA3^+$ and $ROR\delta T^+$ T-cell response (37). Therefore, both Th2 and Th17 are involved in AAS during mycobiota dysbiosis, but further studies are needed to enlighten the mechanisms and possible therapeutic opportunities. Of note, Th17 are induced under the presence of both IL-6 and TGF-β while the absence of IL-6 promotes the differentiation into Tregs (4). To our knowledge, there is a lack of evidence on mechanisms that could link intestinal viruses and T lymphocytes response in AAS; it would be of great interest to address this topic in the near future.

WHAT HAVE WE LEARNED FROM BIRTH COHORTS AND OTHER EPIDEMIOLOGICAL OR CLINICAL EVIDENCE?

Alterations within the gut microenvironment are linked to AAS in children (38). Several studies suggest a negative correlation between farming lifestyle during early life and the risk of AAS (39, 40) while others identify a specific role for farm-milk

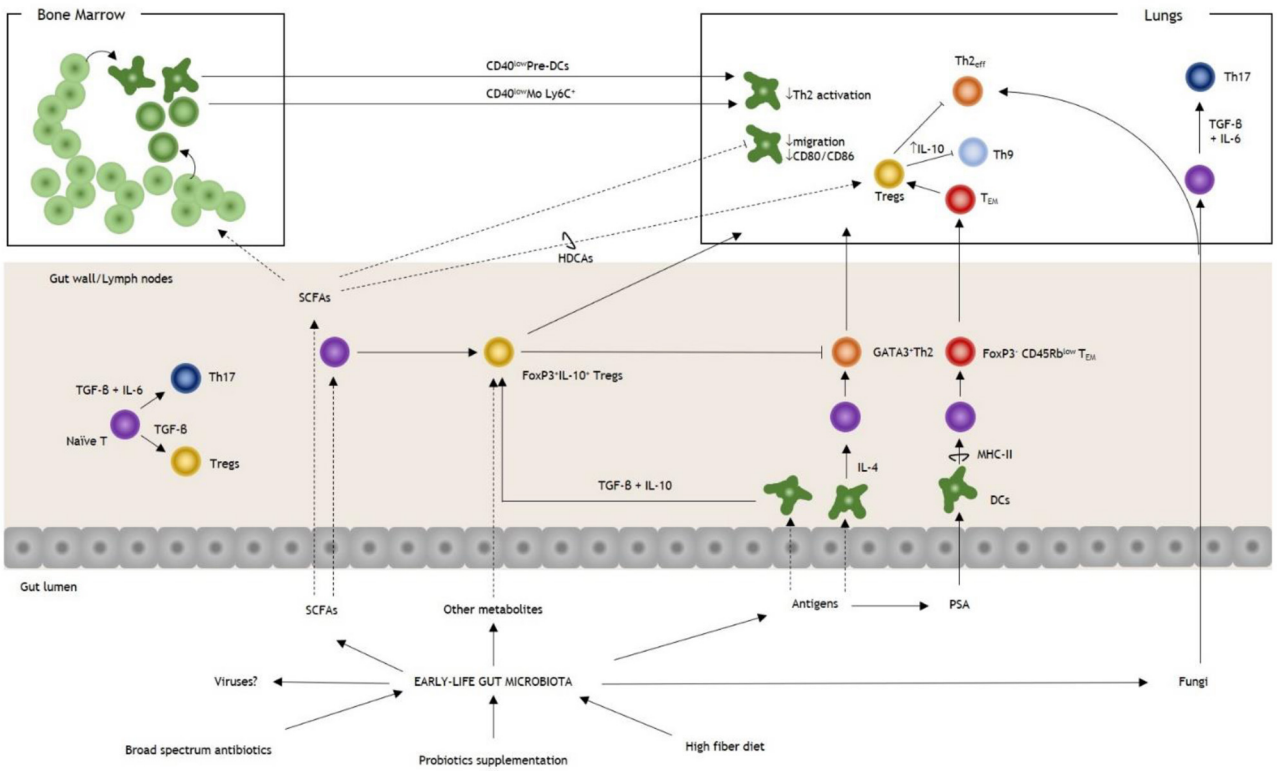

FIGURE 1 | Overview of gut microbiota and T cell modulation in allergic asthma. Early-life perturbations, such as broad-spectrum antibiotics, diet, and probiotics, are essential in determining the risk of allergic asthma. Antigens derived from the gut microbiota stimulate the proliferation of DCs, and they could trigger both Th2 or Tregs responses under the influence of IL-4 or TGF-β+IL-10, respectively. PSA of *Bacteroides fragilis* stimulates the expansion of a FoxP3$^-$ Tem population that regulates lung immunity through the induction of FoxP3$^+$ Tregs. SCFAs modulate T cell response, both priming naïve T cells and upregulating the FoxP3 promoter in lung Tregs. SCFAs influence the hematopoietic pattern in a context-dependent way. In AAS, they increase the production of DCs and monocytes progenitors with the generation of pre-DCs and inflammatory Mo Ly6C$^+$. During AAS inflammation, after their homing to the lungs, they express reduced levels of CD40 (CD40^{low} DCs and CD40^{low} Mo, respectively), impairing the action of Th2$_{eff}$. Among SCFAs, butyrate could promote the induction of pulmonary Tregs trough HDCAs leading to the inhibition of Th9 activity and ameliorating AAS. Fungal dysbiosis could trigger both a Th2 and a Th17 response, thereby increasing the susceptibility to AAS. Note that under the influence of TGF-β+IL-6 T cells are polarized to Th17 while in the absence of IL-6 Tregs are induced. It is not known if intestinal viruses could have a direct effect on T-cell response in AAS. AAS, allergic asthma; DCs, dendritic cells; IL, interleukin; Mo, monocytes; PSA, polysaccharide antigen; SCFAs, small chain fatty acids; Tem, T effector memory; TGF-β, tumor growth-factor β; Th, T helper cell; Th2$_{eff}$, T helper 2 effector; Treg, T regulatory cell.

consumption (41). Since "*correlation does not imply causation*," evidence on the underlying immune mechanisms is needed.

T Regulatory Populations: Tricks or Treat?

Recent findings show that the neonatal gut microbiota is different among children concerning AAS risk, and prove a different propensity to induce specific T cell responses. The analysis of gut microbiota of the WHEAL cohort, in which neonates and infants are clustered for AAS risk, demonstrates that neonates at higher risk exhibit a delayed diversification of the gut microbiota and a relative difference in the composition with fewer *Lactobacillus*, *Bifidobacterium*, *Akkermisia* and *Faecalicaterium* and more *Candida spp* (42). Sterile fecal water from these subjects impairs *in vitro* Tregs differentiation, while a reduction of CD4$^+$CD25$^+$FoxP3$^+$ Tregs and an increase in IL-4$^+$CD4$^+$ T cells is observed (42).

The analysis of peripheral blood cells in PASTURE and EFRAIM children cohorts display an increase in CD4$^+$CD25$^+$FoxP3$^+$ Tregs at 4.5 years (43) and a subsequent reduction at 6 years of age (44). Farm-milk is associated with a protective effect on AAS at 4.5 years of age, and this protection is partially dependent on Tregs. Beyond the number, the demethylation pattern of the FoxP3 promoter increases in farm-milk children but not in children with farm-exposure only (43). However, the longitudinal assessment of this cohort at 6 years of age finds no differences in functional assays nor Tregs frequencies among high or low farm milk intake groups, but an increased expansion of Tregs after LPS stimulation was demonstrated in children affected by AAS (44). Together, these studies support the hypothesis that the number of Tregs in peripheral blood is not a hallmark of tolerogenic responses.

The Impact of Antibiotics: A Difficult Assessment in "Real-Life"

Some studies address the role of early antimicrobials administration and AAS. Early exposure to beta-lactams or

macrolides has a differential impact on the development of the microbial community in children (45). Infants who are exposed to antibiotics early in life are more prone to develop AAS in a variety of cohort studies, but it is difficult to extract some conclusions on the shaping action on T cell response. Since oral intake is one of the most common routes of administration, it is rational to hypothesize a connection between antibiotics induced gut-dysbiosis and a Th2 polarized response. However, to our knowledge, no clustered analysis is available to date and evidence that link antibiotics induced gut dysbiosis and T cell response in AAS are currently lacking in these clinical scenarios.

Effects of Preventive Probiotics Administration on T-Cell in Children

Interestingly, *Lactobacillus spp* is a constituent of genitourinary microflora and is an essential element of the microbiota in vaginally delivered-infants (46). Several shreds of evidence remark the role of delivery mode in determining the AAS risk in children, but other studies find no differences between elective cesarean and vaginal delivery (47). *Lactobacillus* is one of the strains depleted in high-risk toddlers; thus, it is hypothesized that supplementation could prevent the onset of AAS in children through a persistent modification of the microbiota. Since the therapeutic window is probably confined within the first year of life, the longitudinal comparison of stool samples collected from infants at high risk of AAS, treated, respectively, with *Lactobacillus rhamnosus GG* or placebo for 6 months was performed (48). While children at high risk show a distinct meconium microbiota and an impaired gut diversification, the treatment with *Lactobacillus rhamnosus* is able to restore this alteration at 6 months of life, but this effect is lost at 1 year of age (48). Moreover, sterile fecal water from infants at high risk treated with *Lactobacillus rhamnosus* at 6 months, but not at 12 months, promotes $CD4^{+}CD25^{+}FoxP3^{+}$ T regs expansion and IL-10 production in DC/T-cell assays (48). Therefore, *Lactobacillus rhamnosus GG* could promote a tolerogenic environment, but it does not persist. Of note, a randomized, double-blind controlled trial of *Lactobacillus rhamnosus* supplementation during the first six months of life failed to show any significant difference in AAS at 5 years of age (49). Significant limitations of these studies include the intrinsic difficulty in controlling confounding factors and the heterogeneous definitions of atopy risk and AAS. A deeper immunological characterization in infants treated with probiotics in relation to the risk of AAS is needed.

CONCLUSIONS

The complex interactions between the gut microbiota and the T-cell response in AAS are only partially uncovered. Further pathways should be outlined such as the relationship with lungs resident memory T cells (50), the induction of differential response in effector or central memory Th2 (51) or if an immune shift similar to those observed during immunotherapy (52) could be achieved by microbiome manipulation. Clinical trials are difficult and sometimes tainted by several confounding factors and rarely emphasize aspects related to the T cell responses. Moreover, when perinatal interventions are considered, the result should be clustered into pre, post, and combinatory ones in order to understand the priming effect of the delivery mode (49). The clarification of the mechanisms beyond the gut-lung axis strongly encourages further efforts to explore the potential therapeutic roles of microbiota-based primary prevention of AAS during early infancy.

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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REVIEW
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Butyrate Shapes Immune Cell Fate and Function in Allergic Asthma

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The microbiome plays a fundamental role in how the immune system develops and how inflammatory responses are shaped and regulated. The "gut-lung axis" is a relatively new term that highlights a crucial biological crosstalk between the intestinal microbiome and lung. A growing body of literature suggests that dysbiosis, perturbation of the gut microbiome, is a driving force behind the development, and severity of allergic asthma. Animal models have given researchers new insights into how gut microbe-derived components and metabolites, such as short-chain fatty acids (SCFAs), influence the development of asthma. While the full understanding of how SCFAs influence allergic airway disease remains obscure, a recurring theme of epigenetic regulation of gene expression in several immune cell compartments is emerging. This review will address our current understanding of how SCFAs, and specifically butyrate, orchestrates cell behavior, and epigenetic changes and will provide a detailed overview of the effects of these modifications on immune cells in the context of allergic airway disease.

Keywords: butyrate, SCFA (short chain fatty acids), allergic asthma, epigenetics, microbiome, inflammation, cell fate and differentiation, HDAC inhibitor (histone deacetylase inhibitor)

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INTRODUCTION

The gut microbiome is an intricate community composed of microorganisms from diverse groups of bacteria, fungi, protists, archaea, and viruses. Decades of research have revealed the significance of the gut microbiome in physiological processes, initially in regulating nutrition and metabolism (1) and, more recently, in the pathogenesis of respiratory, gastrointestinal, and neurological disease (2–7). These studies have revealed that perturbation of the microbiome can have acute and chronic effects on disease course and outcome. The intestinal microbiome collectively produces a wide variety of metabolites that may be involved in a spectrum of biological processes, ranging from immune defense and host immune cell interactions to inhibition of colonization by pathogenic bacteria (8). Short-chain fatty acids (SCFAs) are metabolites produced from the bacterial fermentation of indigestible fiber and amino acids in the intestinal lumen. In the human and murine gut, the three most abundant SCFAs are acetic acid (2 carbons), propionic acid (3 carbons), and butyric acid (4 carbons). The highest levels of SCFAs are found in the proximal colon, where they are either consumed locally as an energy source by intestinal epithelial cells (colonocytes) or transported across the gut epithelium and absorbed into the bloodstream (9, 10).

Here we highlight the dynamic role of one SCFA, butyrate, in inflammatory immune cell responses in allergic airway disease.

SOURCE, ABSORPTION, AND BIOAVAILABILITY OF BUTYRATE

There are very few endogenously generated sources of butyrate and essentially all butyrate comes from the diet directly or via fermentation by commensal bacteria. Dairy products, especially butter, contain butyrate but these sources of the metabolite are paltry compared to the butyrate produced by intestinal commensal bacteria from non-digestible dietary fiber (11). In humans, this fermentation primarily takes place in the proximal large intestine by butyrate-producing Firmicutes phylum, including *Ruminococcaceae*, *Lachnospiraceaes*, *Erysipelotrichaeceae* and *Clostridiaceae* [reviewed in (11)].

The literature-reported concentrations of SCFAs (including butyrate) in blood circulation and tissues varies, likely owing to differences in diet and disease state as well as methods of tissue/fluid collection, processing, and assay for these volatile SCFA. Nevertheless, the measured order of magnitude of butyrate concentrations in tissues are consistent (**Figure 1**). The physiological concentration of butyrate in humans is highest in the large intestinal lumen (∼100 mmol/kg chyme) (12) and intestinal tissue (∼25 mmol/kg tissue in cecum, ascending, and transverse colon) (13). Microbially-produced butyrate in the mammalian gut lumen is transported across the apical mucosal surface of colonocytes via the proton-coupled monocarboxylate transporter isoform 1 (MCT1, gene name *SLC16A1*) or the Na^+-coupled monocarboxylate transporter 1 (SMCT1, gene name *SLC5A8*) (9, 14). The efflux of butyrate into blood circulation is accomplished by monocarboxylate transporters (MCT3-5) located at the basolateral surface of colonocytes (14) However, as butyrate is an important source of fuel for colonocytes, much of the absorbed butyrate is metabolized for energy within colonocytes (14). As such, the systemic butyrate concentration rapidly declines with increasing distance from the liver portal system (10–50 μM in portal vein plasma) so that, in circulation and peripheral tissues, butyrate concentrations in human blood circulations are ∼1–10 μM (13, 15–19). Butyrate concentration in mouse circulation is typically reported to be up to 100 μM (18, 20, 21). Non-metabolized butyrate is then transported to the liver through the blood where it is absorbed again via hepatocyte MCT1 and SMCT1 and largely metabolized. Absent a concentrating mechanism, butyrate levels in organs other than the colon and liver are likely in the low μM range as well. MCT1 and SMCT1 are widely expressed transporters and therefore also allow uptake of butyrate directly into most cell lineages including immune cells (9). Although passive diffusion of non-ionic butyrate (protonated form) has been proposed as an alternative method for butyrate uptake, it is unlikely to contribute significantly to intracellular butyrate concentrations (22) (**Figure 2**).

In large part because of the availability of butyrate, most evidence for its effects on inflammatory cell function, and the study of the underlying molecular mechanisms, have focused on tissues that are exposed to mM-range concentrations of butyrate *in vivo* (i.e., intestinal epithelia) or purified cells exposed to these concentrations *in vitro*. In normobiotic humans and mice, direct oral dietary supplementation of butyrate will only marginally increase circulating or, presumably, peripheral tissue butyrate concentration (18, 20, 21, 23). A single large dose (maximum toleration) of butyrate will transiently spike circulating butyrate levels in mice to ∼10 mM, but levels return to baseline (∼100 μM) in 30 min (intravenous administration) or ∼3 h (enteral administration) (24). Nevertheless, evidence for the effect of butyrate on inflammatory disease pathophysiology outside of the liver and intestine is mounting.

BUTYRATE SENSING MECHANISMS

G Protein-Coupled Receptors

Butyrate is sensed by cells via three known cell-surface receptors of the G protein-coupled receptor (GPCR) class: GPR41/FFAR3, GPR43/FFAR2, and GPR109A/HCAR2 [reviewed in (9)]. These receptors vary in their sensitivity and response to butyrate, they bind other ligands (including other SCFAs), and they have both overlapping and distinct tissue expression patterns (9). GPR41 and GPR43 are expressed on immune cell lineages and are also activated in response to acetate, propionate, butyrate, and other SCFAs. Conversely, GPR109A, which is also expressed on immune cells, responds strongly to both butyrate and nicotinate but not acetate and propionate. Critically important for the consideration of butyrate's role in immune cell function, the half maximal effective concentration (EC_{50}) of butyrate for GPR41, GPR43, and GPR109A is in the high μM to low millimolar (mM) range [reviewed in (25)]. Thus, one would predict the circulating concentration of butyrate in most peripheral tissues other than liver and intestinal lumen may be too low to ***potently*** activate these receptors.

PPARγ Agonist

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors that are activated by fatty acids and eicosanoids (26). PPARγ1 is expressed highly in adipose tissue, the large intestine and immune cells (27, 28). PPARγ can be activated by butyrate but not acetate and propionate (29). Activation of PPARγ has broad anti-inflammatory effects in many cell types. In intestinal epithelial cells, at concentrations of 0.01–1 mM, butyrate induces activation of PPARγ, and promotes epithelial barrier integrity (30). Thus, although immune cells do express PPARγ, effective activation of PPARγ may be restricted to the intestinal epithelium and liver where butyrate concentrations provide adequate exposure.

HDAC Inhibitors

Butyrate is a non-selective and potent endogenous inhibitor of "classical," Zn^{2+}-dependent class I, II, and IV histone deacetylases (HDACs) [reviewed in refs (31–33)]. HDACs are enzymes that hydrolyze *N*-acetyl groups from lysine residues of protein substates, in particular, acetylated side-chain lysines in the histones of chromatin nucleosome complexes. In general, acetylation of histones by histone acetyltransferases (HATs)

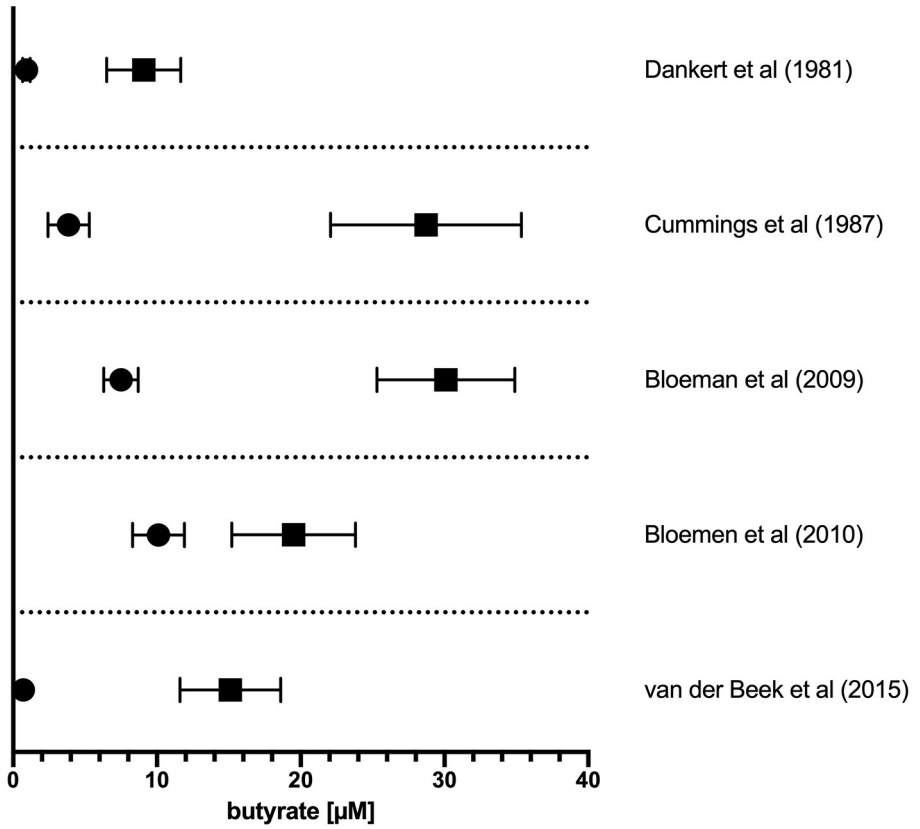


FIGURE 1 | Concentration of butyrate in portal vein and peripheral circulation in humans. The reported concentration of butyrate in plasma sampled from the portal vein plasma (solid squares) or peripheral circulation (solid circles). Values from original references were plotted and are displayed as means ± SEM as reported in original articles.

enhances chromatin accessibility and facilitates transcription. HDACs reverse the process by driving a return to a silent state typical of more condensed chromatin. The specific regulation of gene activation is much more complicated of course, and HAT and HDACs are only part of the control mechanisms. Overall, inhibition of HDACs by butyrate is expected to promote gene transcription from targeted chromatin. HDACs also have non-histone targets including the transcription factors forkhead box P3 (FoxP3) and several others (33–36). Acetylation of these transcription factors has been reported to regulate their stability, alter protein-protein interactions, affect subcellular localization, and modify transcriptional activating functions. Thus, HDAC modification of these non-histone targets would also be expected to alter cell activity independently (or in conjunction) with regulation of gene expression (32, 33). It is not always clear which HDAC isoforms are responsible for direct deacetylation of these targets. Finally, many HDAC isoforms participate in multi-protein regulatory complexes and thus may have activity in regulation of cell processes independent from their deacetylase catalytic domain (33). HDAC inhibitors (HDACi) have a varied effect on cell cycle, differentiation, and cell death (apoptosis, necrosis and autophagy) (33). The class I isoforms (HDAC1, 2, 3, & 8) are ubiquitously expressed whereas class II isoforms (HDAC4, 5, 6, 7, 9, & 10) and the sole class IV isoform (HDAC11) have more restricted tissue distribution including expression in some immune cell subsets (32, 33). Although the activity of class I isoforms is largely (but not completely) restricted to the nucleus, class II and IV isoforms shuttle between the nucleus and cytoplasm. Butyrate inhibits the activity of the class I HDACS (1, 2, 3, & 8) and class IIa HDACs (4, 5, 7, & 9). HDAC11 expression itself is also potently induced in human myeloid cells in response to butyrate exposure (37). The half-maximal inhibitory concentration (IC_{50}) of butyrate for HDACs *in vivo* is reported to be <1 mM depending on the type of assay and substrate (33, 38). The potency of butyrate is likely different for each of the responsive HDAC isoforms. Indeed, in a cell-free enzymatic assay using recombinant HDACs (1–4, 6–8, & 10), butyrate most potently inhibited class I HDACs 1, 2, 3, & 8 with IC_{50} values ∼10–20 μM (39). Notably, although butyrate is a potent natural inhibitor of HDAC enzymatic activity, it is 10^3-10^6-fold less potent than known pharmacological inhibitors including Entinostat and Panobinostat (27, 40).

EFFECTS OF SCFA ON ALLERGIC LUNG DISEASE AND ASTHMA

Analysis of SCFA fecal concentrations of 1-year-old infants in the European PASTURE study shows that children with the highest

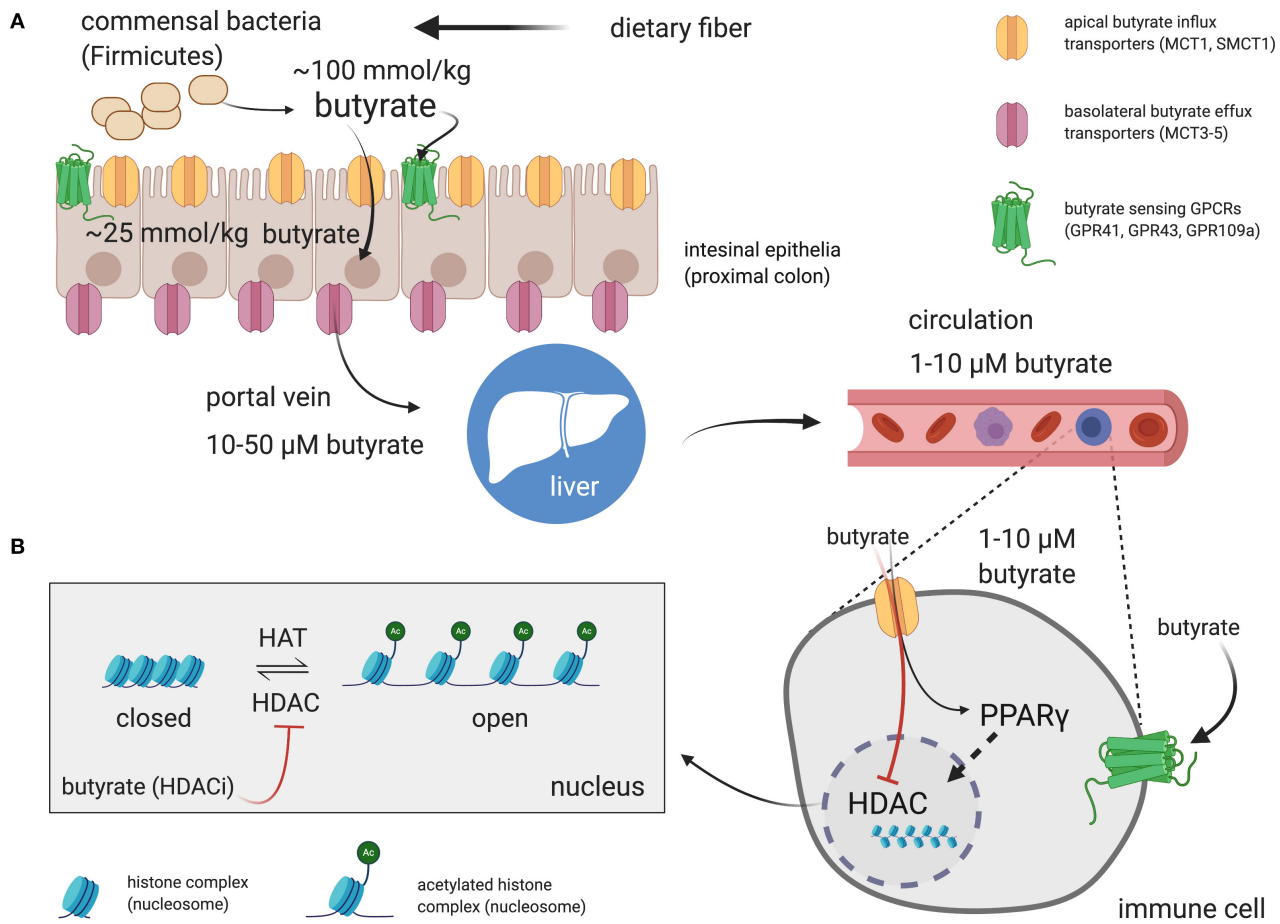


FIGURE 2 | Butyrate production in the intestinal lumen, absorption in the gut, and peripheral distribution. **(A)** Butyrate is produced from the fermentation of dietary fiber by commensal bacteria (mainly Firmicutes phylum) in the gut lumen. Butyrate can stimulate butyrate-sensitive G protein coupled receptors (GPCRs) expressed on luminal epithelia. However, most of the butyrate is efficiently absorbed by colonocytes through H^+- or Na^+-coupled monocarboxylate transporters (MCT1 and SMCT1, respectively) expressed on the apical surface of intestinal epithelia. Most of the butyrate is consumed by colonocytes for energy. The remainder is passed through the basolateral membrane into the liver portal system via monocarboxylate transports (MCT3-5). Butyrate then transits to the liver and is absorbed by hepatocytes. Any remaining butyrate not used as an energy source by hepatocytes is then distributed through the circulation to peripheral tissues. The effects of butyrate on immune cells, and most other cell types are mediated through direct activation of surface GPCRs or, following influx into the cell by activation of peroxisome proliferator-activated receptor gamma (PPARγ) or inhibition of butyrate-sensitive histone deacetylase (HDAC) isoforms. **(B)** Most of the reported effects of butyrate on immune cells discussed in the review are dependent on HDAC inhibitory activity of butyrate. Side chain lysine in histone complexes (nucleosomes) in condensed, closed, chromatin are acetylated by histone acetyltransferases (HATs) to provide access for transcription machinery. HDACs remove acetyl groups from histone lysines to promote chromatin condensation and, in general, attenuate gene transcription at targeted loci. Butyrate is the most potent "endogenous" HDAC inhibitor (HDACi) and thereby promotes open chromatin and encourages active transcription. The shown concentrations are those reported for butyrate in the indicated compartments in humans. Created with https://biorender.com/.

butyrate and propionate concentrations (≥95-percentile) were about half as likely to be sensitive to allergens at age 6 (41). Higher acetate concentrations were not correlated with lower incidence of atopy in this study. However, in another study, higher serum acetate in pregnant women during late phase pregnancy was associated with fewer doctor visits for cough/wheeze or parent-reported wheeze in the first year after delivery (42).

In animal model studies, mice fed SCFA (butyrate, propionate, or acetate) exhibit less severe disease in a model of allergic airway disease than those raised on a normal diet (43). Exogenous butyrate administered orally to adult BALB/c mice prior to disease induction attenuates severity measures of ovalbumin (OVA)-induced asthma including airway hyperresponsiveness (AHR), infiltration of eosinophils into the bronchoalveolar fluid and the frequency of CD25+FoxP3+ T regulatory cells (Tregs) in the lung tissue (41). Importantly, oral administration of these same SCFA to pregnant and nursing BALB/c dams also attenuated some symptoms of allergic inflammation in weaned adult offspring including eosinophil BAL-infiltrates and an elevated frequency of CD25+FoxP3+ Tregs. This treatment approach failed to attenuate AHR, however (41). Similarly, C57Bl/6 mice fed a high-fiber diet or regular diet supplemented

with acetate displayed attenuated disease severity in a house-dust mite (HDM) model of allergic airway disease (AAD) (42). This included attenuated AHR, BAL, and lung immune cell infiltrate (including eosinophils), goblet cell hyperplasia, and serum IgE concentration. Many of these same benefits were observed in adult C57Bl/6 that had been delivered by Cesarean section and cross fostered with mothers on a regular diet from dams that had received high fiber diet or acetate supplementation during pregnancy (E13-E18) (42). In these experiments, the high fiber diet significantly increased acetate concentrations in the feces and serum but did not alter fecal or serum butyrate concentration (42). Conversely, treatment of mice with vancomycin, an antibiotic that depletes butyrate-producing intestinal bacteria, exacerbates AAD (44). Oral supplementation of vancomycin-treated mice with SCFA (mixture of butyrate, acetate, propionate) reverses this effect and ameliorates disease severity (43). We have found that both, the vancomycin-induced severe AAD, and the restorative effects of SCFA-supplementation require early life application to alter the subsequent adult allergic responses. Similarly, there is likely a limited window of opportunity to alter later-life allergic responses in humans associated with early-life (or prenatal) exposure to microbial metabolites including SCFAs (44). These long-lasting effects would, accordingly, suggest SCFAs function by altering, long-term, the trajectory, development, and function of blood cell precursors in addition to any potential effects on terminally differentiated mature cells.

ROLE OF BUTYRATE IN HUMAN ALLERGIC ASTHMA

The severity of allergic asthma in mouse models and humans appears to correlate with the presence of butyrate producing intestinal commensals and, in some instances the presence of butyrate directly. Despite this association, delineating the mechanisms and testing the causal role for butyrate in the attenuation of atopy and asthma has proven difficult. Part of the difficulty is that butyrate has wide-spread functions in intestinal homeostasis that may have an indirect impact on peripheral immune functions (45). In addition, butyrate has the potential to epigenetically alter cell fate(s) so that the biological effects of butyrate exposure may be observed long after the initial exposure (33, 45, 46). Thus, when considering its effects, one may need to consider that alterations in cell function may reflect an exposure to butyrate that occurred much earlier in the life history of a cell or lineage. Additionally, the presence of butyrate and the commensals that produce it *in vivo*, may also herald exposure to several other potentially immune modulating metabolites produced by the same bacteria that may contribute to the ultimate phenotype of the individual or experimental animal (6, 47). Finally, studies that measure fecal SCFA and butyrate often fail to report blood plasma concentrations, which are difficult to accurately measure. A recent study showed that in adult normobiotic human males receiving twice daily 2 g sodium butyrate for 4 weeks, the plasma levels of butyrate were not significantly altered compared to pre-treatment levels (23). These treatments did, however, have a marginal anti-inflammatory effect on peripheral blood mononuclear cells (PBMCs) stimulated with innate ligands (23). **Figure 3** illustrates various effects of butyrate on immune cells in allergic asthma.

Despite evidence of oral SCFA administration attenuating allergic inflammation in murine studies (41, 43) treatments to successfully prevent the development of allergies in human remain unclear. However, the PASTURE study suggests that infants on a diet composed of yogurt, fish, vegetables, and/or fruits have an increased level of fecal butyrate. Children with the highest level of butyrate were less likely to develop asthma and food allergy (41). Cait et al. (48) found that bacterial butyrate production protects children from developing atopy. Specifically, the CHILD study showed that the genes encoding for butyrate fermentation and carbohydrate-active enzymes (CAZymes) which degrade human milk oligosaccharides (HMOs) were depleted in children at age 3 months. Therefore, butyrate production genes may be used as biomarkers to monitor infants who have the genetic propensity (familial history) or other risk factors for atopy.

EFFECTS OF BUTYRATE ON IMMUNE CELL SUBSETS IN ALLERGIC ASTHMA

Eosinophils

Eosinophil influx into the lung parenchyma is a hallmark feature of the most common form of allergic asthma. During allergic inflammation, interleukin 5 (IL-5), IL-13, and granulocyte macrophage colony-stimulating factor (GM-CSF) secreted by Th2 cells and innate lymphoid cells (ILCs) type 2 promote the survival of eosinophils (49–51). Recent *in vitro* studies using human peripheral blood eosinophils show that butyrate promotes eosinophil apoptosis and limits their adhesion to endothelial cells under flow in response to CCL24 (eotaxin-2). Butyrate, but not propionate, also inhibited eosinophil migration in response to CCL24 (52).

Both GPR41 and GPR43 transcripts are expressed by human eosinophils (52) but only GPR43 appears to be translated and expressed on the cell surface (53, 54). Unlike acetate and propionate, butyrate does not induce Ca^{2+} release or stimulate reactive oxygen species (ROS) production via GPR43 (52). However, butyrate (3–10 mM) and propionate (10 mM) induce apoptosis of human eosinophils from allergic-donors 18 h after exposure (52). Surprisingly, this effect appears to be independent of GPR41/43 receptor stimulation and instead depends on influx of these SCFA into eosinophils via monocarboxylate transporters (52). Exposure of eosinophils to butyrate or propionate induces the intrinsic apoptotic pathway including morphological changes in cell size and nucleus structure, mitochondrial depolarization, caspase-3/7 activation, and reduced expression of pro-survival factors myeloid cell leukemia 1 (MCL-1) and B cell lymphoma extra-large (BCL-XL) (52). Importantly, only eosinophils isolated from allergic donor peripheral blood exhibit sensitivity to butyrate- or propionate-induced caspase-3/7 activation and apoptosis (52). Intriguingly, eosinophils from non-allergic donors require priming with IL-5 before butyrate or propionate

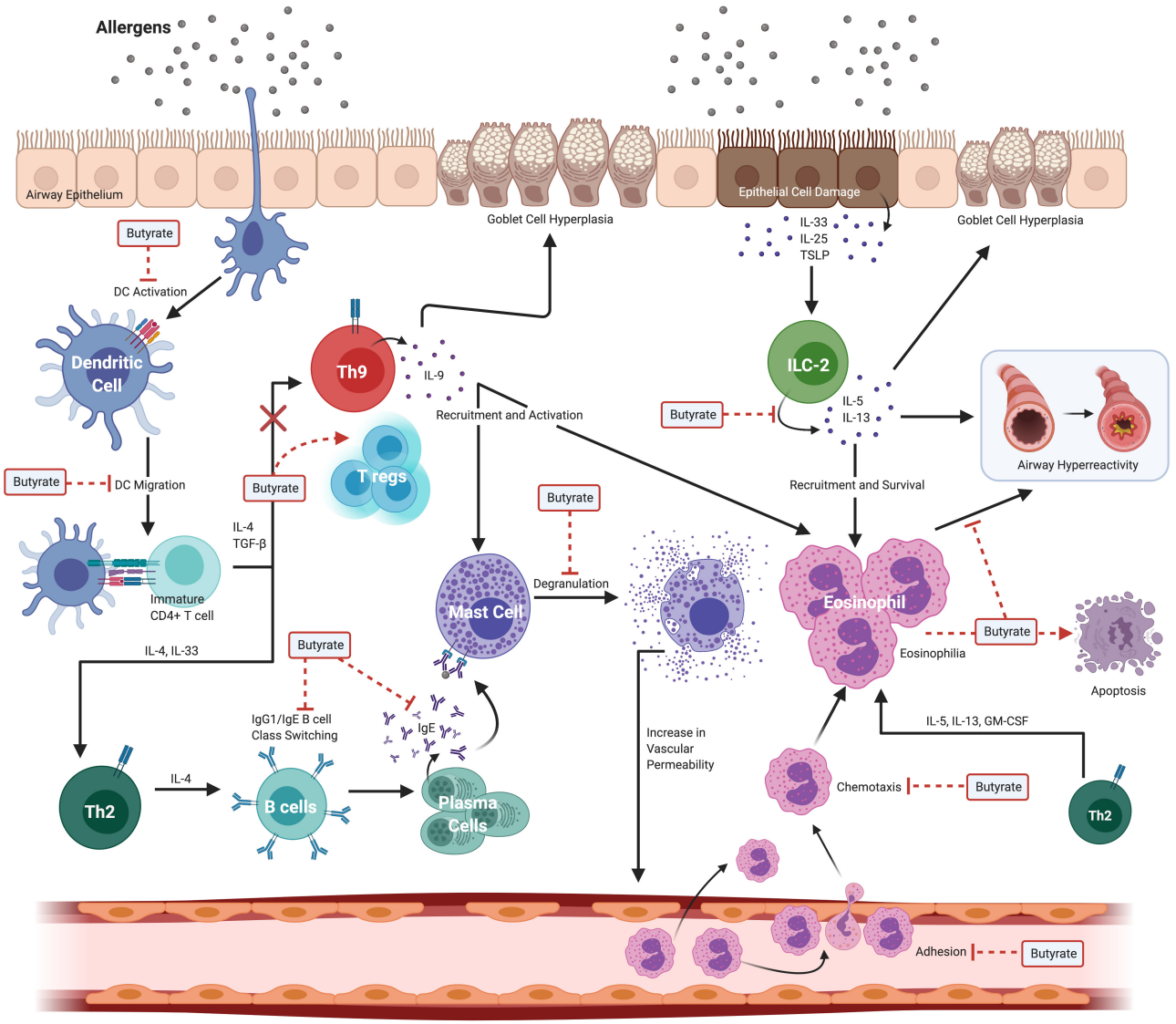


FIGURE 3 | Widespread effects of butyrate on immune cells in allergic asthma. Allergic asthma is a complex inflammatory disease with several immune cells involved in the pathogenesis. Exposure to an allergen induces eosinophilia, airway hyperreactivity, and goblet cell hyperplasia. These effects are collectively driven by dendritic cells (DCs), Th2 cells, Th9 cells, ILC2s, B cells, mast cells, and eosinophils. Butyrate ameliorates allergic asthma by modulating various steps in pathways of different immune cell compartments. Butyrate suppresses both DC activation and migration to local lymph nodes where activated DCs function to stimulate immature/naive CD4+ T cells to polarize to the Th2 lineage. In the B cell compartment, butyrate suppresses both B cell isotype class switching and plasma cell differentiation leading to decreased levels of circulating IgE. Subsequent binding of allergens and cross-linking of surface bound IgE to Fc receptors expressed on mast cells induces degranulation; however, butyrate inhibits IgE-mediated mast cell degranulation. In the Th9 cell lineage, butyrate functions to divert the fate of naïve CD4+ T cells from Th9 to FoxP3+ regulatory-T cells (T regs) effectively promoting a regulatory phenotype. In ILC2s, butyrate suppresses the secretion of IL-5 and IL-13 cytokines that have downstream effects on eosinophils. Butyrate inhibits both the adhesion of eosinophils to the blood vessel endothelium, chemotaxis in response to CCL24, and directly promotes eosinophil apoptosis. Created with https://biorender.com/.

promotes caspase-3/7 activation (52). This suggests that these SCFA exert their effects through inhibition of pro-survival pathways regulated by IL-5 in activated eosinophils.

Butyrate and propionate regulate eosinophil survival and migration via inhibition of class IIa HDACs. Indeed, both a pan-specific HDACi (trichostatin A (TSA)) and a class IIa-specific HDACi (MC1568) also promote dose-dependent apoptosis of eosinophils (52). Both propionate and butyrate induce acetylation of histone 3 (H3) starting at 3 h after exposure and peaking at 18 h (52). Notably, the biological effect of these SCFAs on eosinophils is most robust selectively after 18-h, coinciding with epigenetic regulation of gene expression by HDAC (52). Exposure of allergic donor eosinophils to butyrate is associated with decreased transcript and protein expression of homing, extracellular matrix, and chemotactic receptors including integrin alpha-4 (CD49d), CD44, and CCR3,

respectively (52). Notably, butyrate and propionate also potently reduce IL-5 receptor alpha (*IL5RA*) transcript expression (52).

The *in vitro* assays outlined above required low mM concentrations of butyrate to affect cell activity. Systemic administration (intravenous) of sodium butyrate to mice at a dose of 1 g/kg achieves transient (30–60 min) plasma butyrate concentrations in the 1–10 mM range (24). In an OVA-induced mouse model of asthma, daily administration of 1 g/kg systemic butyrate (i.p.) during the challenge phase resulted in decreased eosinophilia and lower concentrations of type 2 cytokines (IL-4,−5,−13) in the bronchoalveolar lavage fluid (BALF) and attenuated airway hyperresponsiveness (52). Thus, mM plasma concentrations of butyrate, even transiently, can alter allergic eosinophilia and type 2 allergic response *in vivo*. It is important to note that these experimental butyrate concentrations are not normally achievable through microbially-produced butyrate.

Mast Cells

Mast cells are key effector cells in allergic inflammation and can initiate and propagate inflammation in atopic disease. Circulating immunoglobulin isotype E (IgE) binds to high-affinity Fc receptors expressed on the surface of mast cells. The subsequent binding of allergens and cross-linking of surface-bound IgE triggers the release of preformed granules containing proteases, lysozymes, an array of cytokines, histamine, and eicosanoids. The release of histamine, a vasoactive amine, increases vascular permeability that leads to inflammation. Mast cells are found near both vascular and lymphatic vessels and are in mucosal and barrier tissue sites such as the gastrointestinal tract, lung airways, and skin where they play a pivotal role in driving allergic inflammation [reviewed in (55, 56)].

Butyrate treatment (5 mM, 24 h exposure) of precision cut lung slices harvested from OVA-sensitized guinea pig demonstrated greatly decreased allergen-induced histamine release and attenuated airway contraction (57). In addition, butyrate and propionate (but not acetate) potently inhibit IgE- and non-IgE mediated human and mouse mast cell degranulation and IL-6 secretion. These effects are independent of GPR41, GPR43, and PPARγ but depend on butyrate's function as an HDACi. Butyrate exposure reduces the expression of transcripts for Bruton's tyrosine kinase (*BTK)*, spleen tyrosine kinase (*SYK)*, and linker of activated T cells (*LAT*) in human mast cells. These are tyrosine kinases that are well-known to propagate IgE receptor signaling pathways (57) upstream of degranulation and cytokine secretion. In mice, butyrate exposure reduces the expression of levels of BTK and SYK but not LAT proteins. ChIP-Seq analysis of histone 3 lysine 27 (H3K27) acetylation in butyrate-treated primary human mast cell cultures revealed increased global H3K27 acetylation levels as would be expected in the presence of a pan-specific HDACi (57). Curiously, H3K27 acetylation levels near the transcriptional start sites of *BTK*, *SYK*, and *LAT* are markedly ***reduced*** after butyrate treatment (57). This suggests that, although butyrate acts on mast cells by evoking broad histone acetylation (as an HDACi), it also selectively promotes deacetylation near promoter regions of genes associated with FcεRI-mediated mast cell activation with the consequence of attenuated gene expression (57). The mechanism behind how butyrate evokes selective deacetylation remains unexplored.

Regulatory T Cells

Butyrate (100–125 μM) and, less potently, propionate (> 1 mM) promote transforming growth factor beta (TGF- β-dependent FoxP3+ regulatory T cell polarization. In this scenario, naïve CD4+ T cells mature into Tregs in response to TCR activation in the presence of Flt3 ligand (Flt3L)-elicited dendritic cells (DCs) (58). This enhanced Treg polarization is partly explained by butyrate-induced enhanced acetylation, in CD4+ T cells, of the *FoxP3* promoter and conserved non-coding sequence 1 (CNS1), an intronic enhancer essential for extrathymic Treg differentiation (58). In addition to its direct effects on T cells, butyrate also enhanced chromatin acetylation (specifically histone 3 (H3)) in the co-stimulatory Flt3L-DCs that provide exogenous help to drive T cell polarization. Furthermore, expression of *Relb,* a transcription factor known to promote DC activation is suppressed by butyrate (58) and this has previously been shown to promote Treg polarization (59, 60). Several non-histone proteins have been identified as direct targets of HDAC deacetylase activity, many of which are transcription factors (36, 61). Hyperacetylation of FoxP3 prevents ubiquitin-dependent degradation and thus stabilizes this protein and, in addition, enhances its DNA binding or association with other HDACs and TFs (34, 35, 61–63). Arpaia et al. (58), showed enhanced levels of acetylated FoxP3 protein in the presence of butyrate. Importantly, direct acetylation of FoxP3 by butyrate-sensitive HDACs has been demonstrated, and HDAC9, a known effector of FoxP3 acetylation is ***not*** butyrate-sensitive. Thus, butyrate is most likely supporting FoxP3 expression rather than directly altering its acetylation state.

In aggregate, the net effect of butyrate on naïve T cells and co-stimulatory DCs is enhanced generation of extrathymic Tregs and a dampened pro-inflammatory milieu. Notably, expression of GPR109A in DCs was not involved in their Treg polarizing activity in response to butyrate (58). In mice rendered dysbiotic by an antibiotic cocktail consisting of ampicillin, vancomycin, neomycin and metronidazole (AVNM), and thus lacking SCFA-fermenting commensal bacteria, FoxP3+ Treg levels could be restored in the spleen and intestinal lymph nodes by supplementing with SCFA in drinking water (36 mM of each) (58). This SCFA supplement returned serum butyrate concentrations of AVNM dysbiotic-mice to levels comparable to normobiotic mice (∼5–6 μM) (58). It is notable that Treg levels in both the intestinal lymph nodes and the spleen were restored with SCFA supplementation, suggesting that relatively low concentration of butyrate in the blood may be sufficient to be immunomodulatory in peripheral tissues and not just the intestinal lumen (58).

$CD4^+$ T_H2 Cells/Dendritic Cells

Dendritic cells (DCs) are key players in the initial recognition of antigen and serve to bridge the innate and adaptive arms of the immune system in allergic inflammation. DCs are professional antigen presenting cells and are responsible for the uptake of the antigen at barrier sites and the subsequent migration to

the local lymph nodes where they present antigen and provide the necessary signals required for T cell activation in the adaptive immune response. For T cells to be activated, DCs must present processed antigen in the form of peptides on MHC class II molecules and provide the costimulatory molecules CD80 and CD86.

Butyrate (2 mM) suppresses lipopolysaccharide (LPS)-induced activation of human monocyte-derived DCs (moDCs) *in vitro* by limiting upregulation of co-stimulatory markers CD40, CD80, and CD83 and reducing DC metabolic activity (64). In addition, exposure of moDCs to butyrate alters the ability of moDCs to promote the polarization of naïve CD4+ T cells toward the IL10-producing type 1 regulatory T cell lineage (FoxP3^{lo} Tr1) (64). Butyrate induces expression of retinaldehyde dehydrogenase (*ALDH1A1*) in moDCs with a consequent increase in retinoic acid (RA) production. The resultant RA functions in an autocrine and paracrine fashion on naïve CD4+ T cells to promote FoxP3^{lo} Tr1 polarization and IL-10 production (64). IL-10 from these cells leads to a subsequent suppression of effector T cell proliferation in the inflamed tissue (64). Interestingly, this butyrate induced effect on moDCs-dependent Tr1 polarization requires both HDACi and GPR109A signaling activity in moDCs (64). Only the combination of niacin (a natural ligand of GPR109A) and the HDACi TSA can promote moDCs Tr1-priming at levels comparable to butyrate alone (64).

More recently, independent studies have shown that butyrate can have a more selective role on DC function in models of allergic diseases. Previously, we reported that the exposure of DCs to butyrate attenuates both DC activation and chemotaxis (43). Transcriptomic analysis of isolated splenic DCs incubated with butyrate (40 mM) and activated by LPS *ex vivo* reveals altered gene expression profiles in two key pathways involved in allergic disease susceptibility: activation of lymphocytes and DC trafficking (43). As stated above, butyrate has been shown to attenuate DC activation by reducing the expression of costimulatory molecules CD80 and CD86. *Ex vivo* studies using isolated DCs from naïve mice shows that butyrate also reduces the expression of CD80 and CD86 after LPS stimulation while, DCs isolated from vancomycin-treated mice in the absence of butyrate show increased expression levels (43). Thus, butyrate-induced reduction in the costimulatory molecules on DCs limits the ability of DCs to activate T cells generally and, more specifically, dampens their ability to polarize T cells to the Th2 lineage.

Butyrate also reduces the chemotactic potential of DCs by attenuating the responsiveness of DCs to CCL19 chemokine (43). *In vitro* studies using a transwell chemotaxis assay show that DCs isolated from naïve mice and incubated with butyrate exhibit decreased chemotaxis in response to CCL19 as compared to DCs incubated without butyrate (43). DCs isolated from vancomycin-treated mice however exhibit increased migration. In a papain-induced mouse model of allergic asthma, DQ-OVA was used to track the migration of DCs *in vivo* from the airways upon the intranasal administration of papain (43). Results from this experiment indicate that vancomycin-treated mice challenged with papain had significantly higher numbers of DCs in the mediastinal lymph nodes compared with naïve challenged mice. Furthermore, the administration of a mixture of SCFAs (butyrate, acetate, and propionate) reduces the trafficking of DCs in vancomycin-treated mice to levels comparable to control mice. In aggregate these studies would argue that butyrate has a profound effect on the adaptive immune response to allergens by dampening the ability of DCs to migrate to the draining lymph nodes and subsequently prime Th2 polarization.

CD4$^+$ T$_H$9 Cells

T helper 9 (Th9) cells are a subset of IL-9 producing CD4+ T cells developmentally related to Treg and Th2 subsets (65). Th9 cell development from naïve CD4+ T cells is highly dependent on the presence of both IL-4 and TGF-β (65, 66). In general, Th9 cells facilitate immune processes involved in parasitic clearance and anti-tumor response (65–67). More recently, the role of Th9 cells in the immunopathology of allergic lung diseases and autoimmunity has been investigated in animal models (68). Th9 cells promote allergic lung inflammation by orchestrating the recruitment and activation of eosinophils and mast cells (in direct response to IL-9 secretion) and by stimulating mucus production by lung epithelial cells (69, 70).

In *in vitro* polarization assays, butyrate (0.25 mM) and less potently, propionate (0.25 mM), divert the fate of naive CD4+ T cells from Th9 cells to FoxP3+ Tregs under conditions that favor Th9 polarization and reinforce FoxP3 expression under Treg polarizing conditions (68). In other words, butyrate negatively modulates Th9 cell differentiation by inducing robust FoxP3 expression during naïve CD4+ T cell polarization. FoxP3 expression directly suppresses IL-9 production in CD4+ T cells (68). As such, the anti-Th9 effect of butyrate is in part due to an effect on cell fate rather than a direct regulatory interaction between butyrate and Th9 cells. In fact, the dampening effect of butyrate on IL-9 production in naïve CD4+ T cells is not observed in mature Th9 cells, which would be expected to promote allergic inflammation (68).

In an OVA-model of allergic lung inflammation, administration of butyrate (250 μL of 1 M butyrate, i.p.) to C57Bl/6 mice during both the systemic OVA sensitization and challenge phase attenuated disease severity by limiting eosinophilia (68). Systemic butyrate treatment weakly, but significantly, reduced the frequency (2.8 2%) of IL-9 producing CD4+ T cells in the lung. Adoptive transfer of Th9 cells, but not naïve CD4+ cells, or administration of IL-9 reversed the disease-dampening effect of butyrate (68). Together, these results suggest that the presence of butyrate *in vivo*, skews Th polarization away from pro-inflammatory T cells fates in favor of suppressing fates (Tregs) and thus ameliorates allergic lung disease. Notably, the butyrate dose used to observe these phenotypes (equivalent to about 0.8–1 g/kg) likely produces a transient (up to 60 min) spike in plasma butyrate to the 1–10 mM range.

Type 2 Innate Lymphoid Cells

ILCs are a distinct population of hematopoietic cells that arise from common lymphoid progenitors (CLP). ILCs are part of the innate immune system and they function to orchestrate immunity, inflammation, and tissue repair (71–73). Their role

in mucosal immunity has been studied extensively. Subsets of ILCs (natural killer (NK) cells, ILC1s, ILC2s, and ILC3s) are defined by differential expression of cell surface proteins, transcription factors and effector cytokines and closely mimic T cells in form and function (cytotoxic T cells, Th1, Th2, and Th17 cells, respectively). In fact, the most prominent distinguishing characteristic between T cells and ILCs is the distinct lack of a TCR in the latter. Thus, they can, for all intents and purposes, be considered innate counterparts of these highly specialized adaptive immune subsets (74, 75). ILC2s, as promoters of Th2 immunity, are of most interest in the context of asthma and allergic diseases. Recent studies have revealed the role ILC2s in IL-33 driven Th2 and Treg cell expansion in the lungs. Expression of the costimulatory molecule, OX40 ligand (OX4L), by ILC2 is critical for orchestrating an effective Th2 immune response and ILC2-derived IL-13 has been shown to potentiate memory Th2 cell responses by inducing dendritic cell release of Th2 attracting chemokine CCL17 (76, 77).

Ex vivo exposure of IL-33 expanded lung ILC2s to butyrate (0.5–1 mM) reveals a dose-dependent reduction *Il13* and *Il5* gene expression and IL-5 and IL-13 cytokine secretion (78). Notably, acetate and propionate treatment have no effect on ILC2 cytokine secretion. Butyrate suppression of IL33-induced IL-5 and IL-13 secretion has also been observed in both allergen-induced and naïve ILC2s (78). Butyrate also potently inhibits IL-5 and IL-13 secretion by IL-33-stimulated human ILC2s sorted from healthy donor PBMCs (78). Recall that butyrate only potently promoted apoptosis in IL-5-primed eosinophils (52), so this represents a distinct mechanism. In addition, butyrate does not alter IL-17A and IFN-γ production by *Rag2*$^{-/-}$ ILC3s (78).

In *Alternaria alternata* models of allergic lung inflammation, both chronic systemic (6 weeks and 150 mM butyrate in drinking water) and local intranasal (50 μL of 10 mM butyrate daily) (78) administration of butyrate dampens disease severity. Attenuation of ILC2-mediated lung inflammation by butyrate is marked by decreased ILC2 secretion of IL-5 and IL-13 (78). Normally these cytokines act synergistically to induce lung eosinophilia, goblet cell hyperplasia, and airway hyperresponsiveness (79). Concurrent with a reduction in IL-5 and IL-13, total eosinophil counts in the BALF of butyrate-treated mice were significantly reduced (78). Prior work has also shown that butyrate induces Treg cell expansion. While Tregs can suppress ILC2-driven airway hyperreactivity and inflammation, it is intriguing that the attenuation of these features in the *A alternata* asthma model was not attributed to Treg expansion (80). Adoptive transfer of human PBMCs into NOD-SCID IL2rγ$^{-/-}$ mice under conditions that promote human ILC2 (hILC2) development demonstrates that butyrate administered to the airways (i.n.) also attenuates the appearance of IL-5 and IL-13 producing hILC2s in response to intranasal IL-33 (78).

At the molecular level, butyrate epigenetically regulates ILC2 *Gata3* expression and limits ILC2 proliferation, but not survival (78). Again, these effects are independent of GPR41/43 (78). Mechanistically, it is likely that butyrate inhibits ILC2 function through histone deacetylase inhibition as both, the effects of dampened IL-5/-13 secretion and suppressed proliferation on IL-33-activated ILC2s, were recapitulated by exposure of ILC2s to the HDACi, trichostatin A (78). *In vitro*, butyrate and TSA significantly induce acetylation of histone H3 at concentrations at or above 0.5 mM and 1 nM, respectively (78). *Gata3* gene expression is downregulated in both mouse and human ILC2s treated with butyrate (78, 81). In addition, butyrate has been shown to modulate the overall metabolic activity of pulmonary ILC2s by decreasing the inherent ability of ILC2s to utilize both oxidative phosphorylation and glycolysis (81). Although epigenetic regulation is likely, the precise molecular mechanism underlying the inhibition of ILC2 proliferation and *Gata3* expression remains to be conclusively resolved. The chromatin landscape surrounding genes associated with ILC2 proliferation has yet to be explored and is a clear prerequisite for resolving the mechanism behind butyrate-induced amelioration of ILC2-driven AHR and inflammation.

B Cells

The role of B cells in allergic lung inflammation and asthma has been explored extensively and it is well-known that Th2 driven disease has a profound effect on B cell maturation and effector functions (82). Specifically, IL-4 elaborated by innate cells and T cells potently stimulates B cell maturation and directed isotype switching to IgG1 and IgE. The Fc domains of these antibodies play key and profound roles in appropriate Th2 effector functions from myeloid cells including mast cells, basophils, and monocyte/macrophages. In addition, as class II MHC expressing cells, B cells are also well-positioned to function as antigen presenting cells during the early phase of allergic priming and their effects on initiating and amplifying Th2 cell responses have been demonstrated through *ex vivo* experiments and *in vivo* models of asthma (83). Conversely, the depletion of B cells with alpha-CD20 antibodies prior to HDM challenge results in reduced allergic inflammation with a characteristic decrease in CD4+CD44+ T cells, effector memory Th cells, eosinophils, and neutrophils. Together, this evidence suggests that B cells and humoral immunity play a critical role in the initiation and perpetuation of allergic asthma (83).

Recent findings have revealed new insights into the epigenetic effects of butyrate on B cell function. Mice fed a high fiber diet show a dose-dependent decrease in local and systemic antibody response with reductions in B cell activation-induced cytidine deaminase (AID/*Aicda*) and B lymphocyte-induced maturation protein 1 (Blimp-1/*Prdm1*) expression, and a decrease in both class-switched B cells and circulating antibodies specifically IgG1, IgA, and IgE (84). AID plays a critical functional role in both isotype switching, a critical step in the production of IgG1 and IgE, and somatic hypermutation that leads to antibody affinity maturation. Blimp-1, in contrast, orchestrates the maturation of activated B cells into terminally differentiated plasma cells and the corresponding switch from membrane bound to secretory immunoglobulin production. Thus, their inhibition effectively squelches the critical maturation of B cells required for an effective humoral immune response. It is intriguing that at low concentrations butyrate seems to enhance class-switch DNA recombination and increase AID and Blimp-1 expression *in vivo* (84). This could be due to concentration specific effects of butyrate on HDAC isoforms within B cells themselves or,

alternatively, an effect of butyrate on other target cells required to produce cytokines that drive isotype switching and plasma cell maturation like IL-4 and IL-6, respectively.

In summary, the epigenetic mechanisms underlying butyrate-mediated modulation of intrinsic B cell function include the inhibition/silencing of genes involved in somatic hypermutation, class-switching, plasma cell differentiation, and development (84). Butyrate negatively regulates expression of *Aicda*/AID and *Prdm1*/Blimp-1 in mouse and human B cells resulting in a dose-dependent reduction in plasma cell differentiation and restraining class-switching to IgG, IgA, and IgE. Remarkably, the epigenetic-induced gene silencing effects of butyrate are achieved through selective upregulation of miRNAs that target *Aicda* and *Prdm1* 3'UTR (84).

CONCLUSIONS

Allergic asthma is a complex inflammatory disease imitated by allergens and tissue damage and propagated by the coordinated activation and recruitment of several immune cell subsets across the innate and adaptive immune spectrum. While the full

EFFECTS OF BUTYRATE ON IMMUNE CELLS IN ALLERGIC ASTHMA

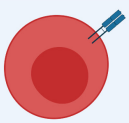
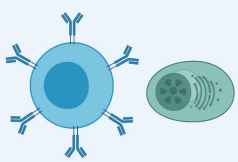
IMMUNE CELL LINEAGE	CELLULAR AND MOLECULAR MECHANISMS OF BUTYRATE	REFERENCES
 Eosinophils	**1.** Activates intrinsic apoptotic pathways: Mitochondrial depolarization, caspase-3/7 activation and reduces expression of MCL-1 and BCL-XL. **2.** Inhibits adhesion and chemotaxis: Reduces transcript and protein expression of Integrin-α-4 (CD49d). CD44, and CCR3.	(52)
 Mast cells	**1.** Reduces transcript expression of Bruton's tyrosine Kinase (*BTK*), spleen tyrosine kinase (*SYK*), and linker of activated T cells (*LAT*). **2.** Increases global H3K27 acetylation levels. **3.** Reduces H3K27 acetylation levels near transcriptional start sites of *BTK, SYK,* and *LAT.*	(57)
 Dendritic Cells (DC)	**1.** Reduces protein expression of costimulatory molecules CD80 and CD86. **2.** Reduces DC chemotactic potential by decreasing the responsiveness of DCs to CCL19.	(43,64)
 Th9 cells	**1.** Diverts the fate of naive $CD4^+$ T cells from Th9 to $FoxP3^+$ T-regulatory cells. **2.** $FoxP3^+$ suppresses IL-9 production in $CD4^+$T cells.	(68)
 ILC-2	**1.** Epigenetically Inhibits IL-5 and IL-13 cytokine secretion. **2.** Epigenetically suppresses GATA3 expression and ILC2 proliferation.	(78,81)
 B cells/Plasma cells	**1.** Reduces expression of activation-induced cytidine deaminase (AID) and B lymphocyte-induced maturation protein 1 (Blimp-1). **2.** Reduces both B cell isotype/class switching and circulating IgG1, IgA, and IgE.	(84)

FIGURE 4 | Cellular and molecular mechanisms of butyrate on immune cells in allergic asthma. Created with https://biorender.com/.

understanding of how SCFAs, in particular, butyrate, influence allergic airway disease pathology remains obscure, a recurring theme has emerged: Butyrate regulates immune cell behavior predominantly through epigenetic modification of cell fate and function (**Figure 4**). Such epigenetic control mechanisms may have a long-term impact on immune cell fate during embryonic development and therefore offers an attractive explanation for the observed narrow "window of opportunity" in early life where commensal bacteria and their metabolites can impact life-long allergic susceptibility and severity. In fact, this window of opportunity appears to end after the nursing stage in humans and mice. Most intriguing, peak influence may extend earlier, into the pre-natal development stage and depend on maternal commensals and their metabolites. Defining these mechanisms more precisely and establishing a causal link to butyrate (and/or other metabolites) will enable a clearer understanding of butyrate's influence on immune cell ontogeny. With evidence that butyrate impacts the epigenetic regulation of mature immune cell subsets it is natural to ask what role butyrate may have on shaping cell fate at much earlier stages in hematopoiesis. Despite the presumably low concentration of butyrate most hematopoietic stem and progenitor cells (HSPCs) may "see" in the bone marrow niche (and perhaps brief forays into circulation), butyrate has the potential to influence cell fates throughout the immune system.

Finally, while it is clear that butyrate can potently influence immune cell functions at mM concentrations present in the intestinal lumen, it remains difficult to explain the more widespread *in vivo* effects of butyrate on inflammatory disease in the lung and other peripheral tissues where the concentration of butyrate is 500-1,000-fold less. Since PBMCs circulate through the intestinal and hepatic supply, it may be that this short term but repeated exposure to high μM concentrations were sufficient to influence cell functions. Alternatively, sustained exposure to low μM levels of butyrate may provide enough HDAC inhibition to alter cell fate in peripheral tissues.

While dietary supplementation of butyrate attenuates lung inflammation in dysbiotic mouse models there are serious limitations to the potential therapeutic utility of butyrate in human asthma. First, and foremost, butyrate supplementation may be most effective perinatally, long before symptoms of allergic lung inflammation or atopic disease are detected. It is likely to be more practical to assess gut microbiota perinatally and identify safe and effective methods to optimize microbial communities for future health. Alternatively, more potent HDAC inhibitors, especially those with HDAC isoform-specificity could have therapeutic potential in established allergic lung disease. Regardless, it is essential to more fully understand how microbial-derived butyrate (and other metabolites) work to tune immune responses.

AUTHOR CONTRIBUTIONS

WY and MRH wrote the manuscript and designed the figures. YL, AC, MH, WM, and KM edited and assisted with the development of the manuscript throughout the writing process. All authors contributed to the article and approved the submitted version.

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