UNDERSTANDING AND EXPLOITING HOST-COMMENSAL INTERACTIONS TO COMBAT PATHOGENS

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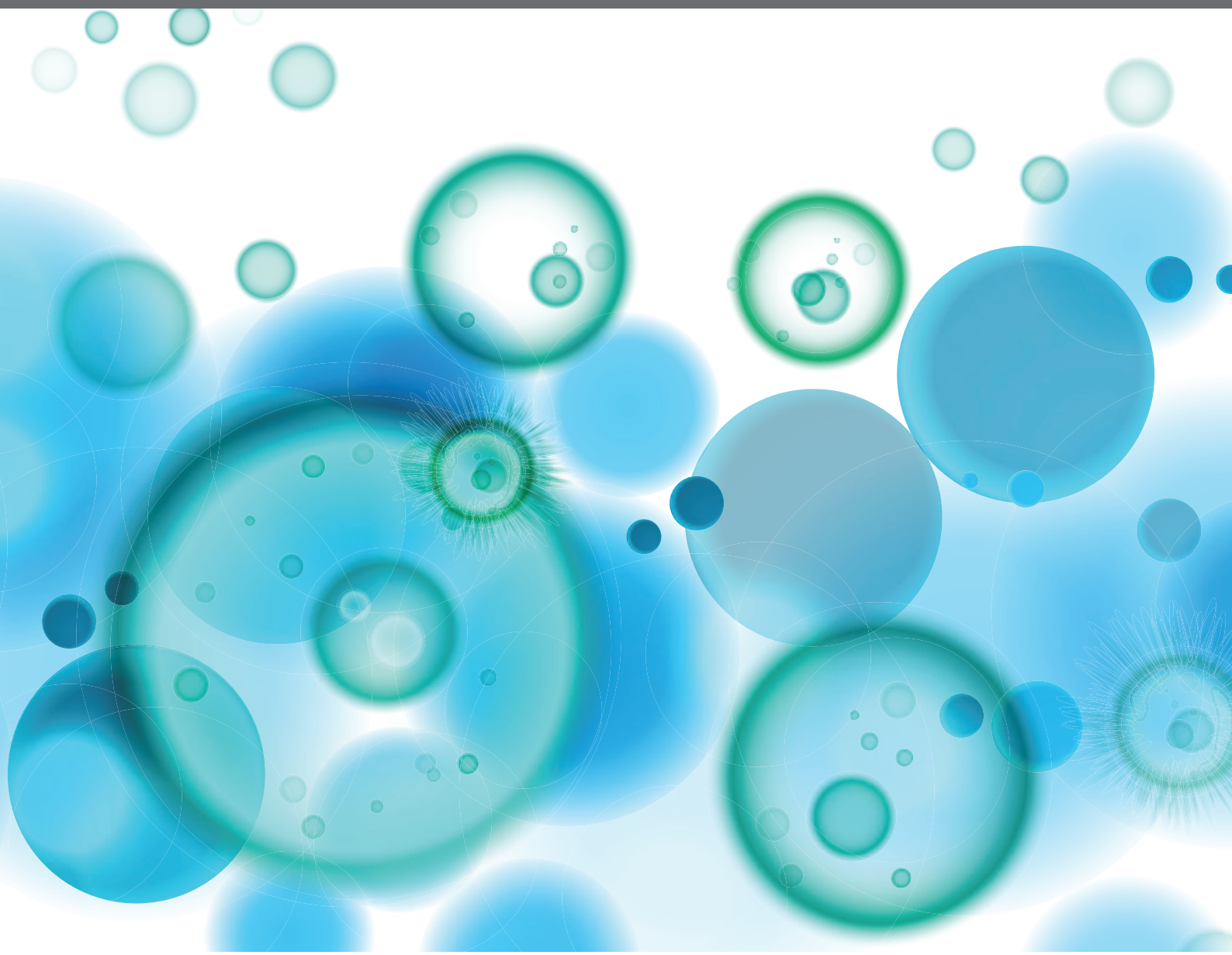

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UNDERSTANDING AND EXPLOITING HOST-COMMENSAL INTERACTIONS TO COMBAT PATHOGENS

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Trillions of commensal microbes reside on and inside the human body, including the intestinal and respiratory tracts, which encompass various microbial taxa, such as bacteria, fungi, archea and viruses. The close proximity of microbes with the host provides an opportunity to continually interact with each other. Advances in research approaches, including high-throughput sequencing, have allowed us to study host and commensal microbes at the genetic and functional levels. Recent studies suggest that commensal microbes play a crucial role in the development of the host's immune system and induce innate and adaptive immune responses against pathogens. It is also becoming apparent that the gut commensals are endowed with a capacity to alter immune responses in organs beyond the intestine, such as the lungs, highlighting the significance of the gut commensals in controlling systemic immunity. On the other hand, the host's immune system possesses the ability to shape the repertoire of commensal microbes and contribute to the establishment of beneficial relationships with them. A better understanding of host-commensal interactions will be important for designing effective vaccines and therapeutics against pathogens.

This Research Topic sheds light on our current understanding of the interplay between the host's immune system and commensal microbes and how this interplay can be exploited for prophylactic and/or therapeutic strategies against pathogens.

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EDITORIAL
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Editorial: Understanding and Exploiting Host-Commensal Interactions to Combat Pathogens

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Editorial on the Research Topic

Understanding and Exploiting Host-Commensal Interactions to Combat Pathogens

The human body harbors an astonishing number of diverse commensal microbes, including bacteria, fungi, and viruses, providing a suitable milieu for microbial growth and multiplication (1, 2). These microbes rarely cause disease and communicate with the host in a mode that is advantageous to both host and microbes (3, 4). In recent years, phylogenetic, metagenomic, and functional studies have been conducted to better understand the complexities of the microbial genome and the effect of the microbiota on the host's immunophysiology. Accumulating evidence has shown that gut commensals regulate the ontogeny and function of the immune system, and contribute to shaping the outcome of immune responses (5). It has also become clear that these commensals have the ability to influence the immune responses at extraintestinal tissues/organs, underscoring their profound impact on local as well as systemic immunity (6). However, alterations in composition, diversity, and metabolic activities of commensal microbes can lead to dysbiosis, which may have detrimental consequences, such as autoimmunity, allergy, asthma, inflammatory bowel disease, cancer, and infection (7). Commensals also hold the potential to cause disease depending on multiple microbial and host factors (8). A much deeper understanding of how commensals communicate with the host is crucial for developing new strategies to prevent and treat diseases.

In this Research Topic, a series of 16 articles, encompassing review, original research, and general commentary articles, provide crucial information on how the interplay between host and commensals takes place and how this could be exploited for designing novel prophylactics/therapeutics against a wide spectrum of disorders, including infectious diseases. In an original research article, Yang et al. report the identification of genetic factors that are involved in the lysis of human neutrophils by *Staphylococcus aureus*, underscoring the mechanism by which this commensal bacterium evades the neutrophilic immune barrier during infection. Likewise, Dai et al. demonstrate that *S. aureus* employs the vancomycin resistance-associated sensor/regulator (VraSR) to increase its survival within macrophages, thereby modulating the process of host-cell autophagy. Overall, these data throw light on important virulence factors used by *S. aureus* to escape innate immunity, highlighting why the innate immune response is incapable of eradicating *S. aureus*. Gao et al. provide evidence that underscores the impact of hydrogen peroxide (H_2O_2) produced by *Streptococcus pneumoniae* on the host's immune responses against pneumococcal

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lung infection. *S. pneumoniae*-secreted H_2O_2 causes damage and leakage of mitochondrial DNA into the cytoplasm, which not only mediates mitochondrial oxidative stress but also promotes IFN-I cascades in lung cells (Gao et al.). Furthermore, Tian et al. show that gut microbiome-derived propionate levels are inversely proportional to the lung inflammation, but not to bacterial immunity, using mouse models of ischemia reperfusion injury and *S. aureus* pneumonia. These studies indicate that microbial products, such as H_2O_2 and propionates, play a significant role in the outcome of host immunity.

Luo et al. describes the inhibitory effect of human cathelicidin antimicrobial peptide LL37 on *Aspergillus fumigatus* infection in mice by directly binding to the fungal mycelia, which follows reduced pulmonary inflammation characterized by decreased histopathological changes and proinflammatory cytokine levels. Woo et al. show a novel mechanism by which the commensal microbiota epigenetically regulate intestinal epithelial cells to downregulate expression of the cell surface glycoprotein C-type lectin 2e (Clec2e), which reduces the efficiency of epithelial cell interaction with the murine enteric pathogen *Citrobacter rodentium*. On the other hand, host immunity can also regulate the composition of the mucosal-associated microbiota. Xiao et al. demonstrate that Toll-like receptor 4 (TLR4), which is a membrane-bound protein expressed on immune cells that identifies microbe-associated molecular patterns, may play a role in the regulation of the distribution and structure of the intestinal mucosal-associated microbiota by vitamin A. Thus, there exists a bidirectional communication between the microbiota and host, which may contribute to maintaining homeostasis in the gut.

The review and commentary articles presented in this Research Topic focus on a variety of interesting areas that include commensal-immune cell interaction and its implications for therapy and prophylaxis of diseases. Humbert et al. review the current literature on the pathophysiology of chronic mucocutaneous candidiasis, which is caused by the fungus *Candida albicans*, in autoimmune polyendocrine syndrome type 1, whereas Iacob and Iacob focus on the importance of the relationship between the intestinal barrier and microbiota, and how dysbiosis can alter this relationship to make the host prone to pathogens. Additionally, it is becoming clearer that the gut microbiota modulates the innate and adaptive immune responses to impact the disease outcome. Cheng et al. shed light on the major mechanisms by which the commensal microbiota boosts the host's innate immunity against infectious agents. Pandiyan et al. review recent evidence on how the gut microbiota influences the function of adaptive immune cells, such as regulatory T cells (Tregs) and Th17 cells, and how the microbiota can be targeted to promote mucosal immunity and ameliorate pathology. Natural killer T (NKT) cells constitute an innate and unconventional population of T cells that perform protective as well as detrimental roles in diverse disease models. A general commentary by Jia focuses on the crucial role played by NKT cells in liver cancer, which is regulated by the gut microbiota-mediated bile acid metabolism.

An interesting review by Forgie et al. discusses the host-microbiota interactions from a dietary point of view. This provides knowledge on how dietary components, such as carbohydrates and proteins, can modulate the host-microbiota interactions to promote resistance against pathogens (Forgie et al.) Studies on the therapeutic applications of the host-microbiota dialogue are crucial for ensuring further translation of the acquired knowledge into health benefits. Li et al. provides a comprehensive review on the beneficial and harmful role of the commensal microbiota in dealing with viral infections and the effect of these infections on the microbiota homeostasis. Khan et al. evaluate emerging data on the contribution of commensal bacteria to host defense against respiratory pathogens and the mechanisms whereby bacteria induce protective immunity. They also discuss how commensal bacteria can be exploited to treat and prevent respiratory infections (Khan et al.). In line with this, Baker and Edlund discuss the therapeutic potential of the oral microbiome in developing strategies to exploit the protective effects of the oral microflora in order to prevent dental caries.

Cumulatively, this Research Topic provides significant knowledge on the mechanisms underlying host and commensal microbe interactions, and the profound impact that these microbes exert on the host's health and disease. This has clinical implications for the prevention and treatment of diseases. The targeting of commensals is gaining momentum as an effective strategy to combat various diseases, including infectious diseases. Successful treatment of severe intestinal infections, caused by antibiotic-resistant bacterial pathogens, using fecal microbiota transplantation, offers an excellent example of how commensal microbes can be used for disease therapy. It is, however, notable that most data on this topic stem from mouse studies. Although mouse experiments remain critical to understand the contribution of the commensal microbiota to health and disease, they pose a concern for the scientific community because of their poor recapitulation of human conditions. For example, the murine and human intestinal microbiota exhibit significant differences in abundance and gene identity, and the murine microbiota composition depends on multiple factors such as rearing facilities and genetic background (9). It is important to consider these facts while translating the knowledge acquired from mouse to human.

Since several commensals/probiotics have been shown to be safe in animal models and humans, it would be worth examining the long-term consequences of their use for the host's well-being *in toto*. With technological advances in this field, approaches that harness the beneficial effects of commensals to prevent diseases and promote health will continue to grow in number. In-depth studies are also needed to focus on the pathogenic potential of commensals/probiotics in individuals under immunosuppression due to malnutrition, chemotherapy, or viral infections. This is crucial because immunocompromised individuals possess an altered microbiota, along with an impaired immune system, which make them highly susceptible to opportunistic infections and cancer.

Finally, we are extremely grateful to all the authors for their significant contribution to this Research Topic as well

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REFERENCES

1. Gevers D, Knight R, Petrosino JF, Huang K, McGuire AL, Birren BW, et al. The Human Microbiome Project: a community resource for the healthy human microbiome. *PLoS Biol*. (2012) 10:e1001377. doi: 10.1371/journal.pbio.1001377
2. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature*. (2007) 449:804–10. doi: 10.1038/nature06244
3. Abt MC, Pamer EG. Commensal bacteria mediated defenses against pathogens. *Curr Opin Immunol*. (2014) 29:16–22. doi: 10.1016/j.coi.2014.03.003
4. Kamada N, Seo SU, Chen GY, Nunez G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol*. (2013) 13:321–35. doi: 10.1038/nri3430
5. Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? *Nat Rev Immunol*. (2010) 10:735–44. doi: 10.1038/nri2850
6. Samuelson DR, Welsh DA, Shellito JE. Regulation of lung immunity and host defense by the intestinal microbiota. *Front Microbiol*. (2015) 6:1085. doi: 10.3389/fmicb.2015.01085
7. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis*. (2015) 26:26191. doi: 10.3402/mehd.v26.26191
8. Henriques-Normark B, Normark S. Commensal pathogens, with a focus on *Streptococcus pneumoniae*, and interactions with the human host. *Exp Cell Res*. (2010) 316:1408–14. doi: 10.1016/j.yexcr.2010.03.003
9. Hugenholtz F, de Vos WM. Mouse models for human intestinal microbiota research: a critical evaluation. *Cell Mol Life Sci*. (2018) 75:149–60. doi: 10.1007/s00018-017-2693-8

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REVIEW
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Chronic Mucocutaneous Candidiasis in Autoimmune Polyendocrine Syndrome Type 1

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Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is an autosomal recessive disease caused by mutations in the autoimmune regulator (AIRE) gene, characterized by the clinical triad of chronic mucocutaneous candidiasis (CMC), hypoparathyroidism, and adrenal insufficiency. CMC can be complicated by systemic candidiasis or oral squamous cell carcinoma (SCC), and may lead to death. The role of chronic *Candida* infection in the etiopathogenesis of oral SCC is unclear. Long-term use of fluconazole has led to the emergence of *Candida albicans* strains with decreased susceptibility to azoles. CMC is associated with an impaired Th17 cell response; however, it remains unclear whether decreased serum IL-17 and IL-22 levels are related to a defect in cytokine production or to neutralizing autoantibodies resulting from mutations in the *AIRE* gene.

Keywords: autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), chronic mucocutaneous candidiasis (CMC), autoimmune regulator (*AIRE*) gene, IL-17, IL-22

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INTRODUCTION

Autoimmune polyendocrine syndrome type 1 (APS-1), also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome, is characterized by the clinical triad of chronic mucocutaneous candidiasis (CMC), hypoparathyroidism, and adrenal insufficiency. This syndrome was formerly known as Whitaker syndrome (1). Accurate diagnosis of this syndrome requires the presence of at least two of these three major components, or only one if a sibling has already been diagnosed with the disease (2). Other autoimmune disorders have also been described, such as hypergonadotrophic hypogonadism, thyroid disease, type 1 diabetes, coeliac disease, liver disease, alopecia, vitiligo, chronic atrophic gastritis, and hypophysitis. These autoimmune disorders are associated with ectodermal dystrophy, asplenia, and the presence of several autoantibodies, even in the absence of corresponding organ dysfunction (3).

APS-1 is a monogenic, autosomal, recessive disease caused by a mutation in the autoimmune regulator (*AIRE*) gene on chromosome 21 (gene map locus 21q22.3) (4). The *AIRE* gene is composed of 14 exons and codes for a 545 amino acid protein (5, 6). The *AIRE* gene is mainly expressed in thymic medullary epithelial cells, which play an important role in the presentation of self-antigens (7, 8), but is also expressed at low levels in the spleen, lymph nodes, pancreas, adrenal cortex, and peripheral blood mononuclear cells. The *AIRE* gene codes for a nuclear

transcriptional regulator protein involved in the ectopic expression of self-antigens in the thymus, leading to the removal of self-reactive thymocytes and generation of peripheral tolerance. The role of peripheral *AIRE* expression, which has been confirmed by mRNA analysis, remains unclear. To date, more than 100 different mutations in this gene, both homogeneous and heterogeneous, have been reported worldwide (9–12).

APECED is a rare syndrome, which has been reported worldwide, but is more prevalent in some historically-isolated homogeneous populations in Finland (1/25000) (4, 13), Sardinia (1/14500) (14), and Iranian Jews (1/9000) (15). APECED is also seen at a lower incidence in Norway, Sweden, Slovenia, Great Britain, Italy, Ireland, and North America (16–21).

Patients with APS-1 suffer from CMC without displaying susceptibility to any other pathogen. CMC is associated with the Finnish mutation c.769C>T (p.Arg257stop) (22). CMC usually affects the oral mucosa, but the nails and skin may also be involved. Esophageal candidiasis results in pain and dysphagia. CMC can be complicated by systemic candidiasis or oral squamous cell carcinomas (SCCs), and may lead to death (23, 24).

CHRONIC MUCOCUTANEOUS CANDIDIASIS

CMC is characterized by recurrent or persistent symptomatic mucocutaneous infections caused by *Candida* species, predominantly *Candida albicans,* affecting the nails, skin, oral cavity, and genital mucosa. The diagnosis of CMC is based on clinical symptoms, associated in most cases with the isolation of *Candida* from body sites (25). The first case of syndromic CMC was described by Thorp and Handley (26).

C. albicans is a ubiquitous, diploid, dimorphic yeast that resides as a commensal organism on the mucosae and in the gastrointestinal tract of healthy individuals. Mucosal candidiasis results from a change in mucosal homeostasis leading to disequilibrium between the yeast and its host. Opportunistic mucosal infection, deep organ, or systemic infection in immunocompromised patients usually arise from *Candida* colonizing the digestive tract (27). Systemic candidiasis can be diagnosed using a number of non-culture based assays (28) but no biological markers are currently available for the diagnosis of "culture-negative" CMC.

Most cases of CMC are sporadic and are secondary to other medical conditions such as HIV infection with T-cell deficiency, diabetes, immunosuppressive therapies like anti-cytokine blockers, antibiotic or steroid therapy (25, 29, 30). CMC is also more rarely favored by genetic disorders (i.e., familial CMC) that can be inherited. These cases of primary CMC are due mainly to innate immunodeficiency disorders. They have been reviewed by Puel et al. (31) and were classified as primary immunodeficiency disease by the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency in 2015 (32).

Three types of immnuodeficiency can be distinguished depending on the genetic abnormalities associated with CMC:

(i) Severe underlying immunodeficiency (ID), including severe combined immunodeficiency (SCID), or CD25 deficiency. SCID is a body of diseases characterized by the inability to produce T-cells leading to Th17 cell deficiency (32, 33). Individuals with SCID are susceptible to a whole range of infections caused by bacteria and viruses. Subjects with autosomal recessive CD25 deficiency have a decrease in T-cell and Th17 cell numbers, and a consequent high incidence of viral and bacterial infections (31, 33).

(ii) HIES, CARD9, IL12Rb1 deficiency, GOF-STAT1, and APECED/APS1 are syndromes where CMC has additional specific clinical features. Autosomal dominant hyper IgE syndrome (AD-HIES) is characterized by impaired production of Th17 cells and Th17-derived cytokines caused by an autosomal dominant mutation of *STAT3* (31, 34). AD-HIES is associated with several infectious diseases, including staphylococcal skin abscesses, bacterial pneumonia, and CMC. Recent literature has also described an autosomal recessive hyper-IgE syndrome (AR-HIES), caused by a deficiency in DOCK8, resulting in a variety of symptoms and diseases including atopy, autoimmunity risk, malignancies, recurrent viral/bacterial infections, and CMC. Patients who are deficient in DOCK8 have decreased Th17 cells (35–38). Low concentrations of critical candidacidal peptides, including histatins and β-defensin 2 (BD2), which are activated by IL-17, are found in the saliva of individuals with HIES. Saliva from healthy individuals usually contains high concentrations of β-defensins and histatins, which have direct candidacidal activity. IL-17A, but not IL-22, acts directly on human salivary glands, which might explain why saliva from HIES patients is deficient in histatins and the increased susceptibility of these individuals to CMC. Antimicrobial peptides are secreted by skin epithelial cells only when they are stimulated with Th17 cytokines and classical proinflammatory cytokines (e.g., TNF-α, IL-1β, IFN-γ) (34). Autosomal recessive tyrosine kinase deficiency is another type of combined immunodeficiency that mimics the symptoms of AR-HIES (31–33).
CMC and invasive *Candida* infection have also been associated with a deficiency in autosomal recessive caspase recruitment domain-containing protein 9 (CARD9) (39–41). CARD9 is an intracellular adaptor involved in Dectin 1 and Dectin 2 signaling, the main pathogen recognition receptors for *C. albicans* glycans. The number of circulating IL-17-producing cells and IL-17 secretion have been reported to be decreased in CARD9-deficient patients (40, 42, 43). However, these findings remain open to debate (33). With regard to *Candida* infections, mutations in Dectin-1 and Dectin-2 have also been studied in murine models. In humans, a mutation in the early-stop codon for Dectin-1 (Y238X) has been reported in a family with recurrent vulvovaginal candidiasis. Experiments *in vitro* demonstrated that monocytes and neutrophils from homozygous patients lacking Dectin-1 expression are defective in cytokine production, including IL-17, when

stimulated with *C. albicans*. However, phagocytosis and yeast cell killing remained normal (44, 45). Another report demonstrated that patients receiving hematopoietic stem cell transplants who were heterozygous for Y238X had an increased incidence of gastrointestinal *Candida* colonization (46). Although Dectin-2-deficient mice had higher mortality and a higher kidney fungal burden after infection with *C. albicans* (47), the impact of Dectin-2 mutations on the human host response to *C. albicans* infection remains unclear.

Other studies have demonstrated that patients with IL-12 or IL-23 signaling defects have an increased risk of developing CMC (31, 33, 48). Patients with CMC, autoimmune manifestations, other mild bacterial or viral infections, intracranial aneurysms, or SCC also had heterozygous missense gain-of-function (GOF) mutations of STAT1. The development of IL-17-producing T-cells is impaired in these patients, as a result of hyperactivity of STAT1 that inhibits STAT3 signaling. This phenomenon results in increased STAT1-dependent cellular responses that repress IL-17-producing T-cell responses, such as IFN-γ, and/or enhanced IL-6, IL-21, and IL-23 STAT1 responses, which normally activate STAT3 and induce IL-17 T-cell production (31).

(iii) Isolated CMC has also been described in subjects with IL-17RA, IL-17RC, ACT1, IL-17F, and RORγt deficiency, where CMC is the only presenting feature of the disease. Some families have also been identified with autosomal-dominant mutations in the gene coding for IL-17F, or autosomal-recessive mutations in the gene coding for IL-17 receptor A or IL-17 receptor C as risk factors for CMC (31–33).

These familial cases of CMC demonstrate that IL-17 plays a pivotal role in human epithelial immunity to *C. albicans*. Another piece of evidence supporting the central role of Th17 cytokines in CMC and mucosal immunity to yeasts is the observation of cases of CMC in the Phase 2 trial of secukinumab, a human anti-IL17 receptor antibody for the treatment of Crohn's disease (49, 50). This review aims to focus on the characteristics of familial CMC associated with APECED syndrome.

CHRONIC MUCOCUTANEOUS CANDIDIASIS AND APECED

Clinical Description

CMC is the most common infection occurring in APECED patients (77–100%) (19, 21, 23, 51–53), except in Iranian Jews (17%) (15). CMC is also the most common first clinical manifestation of APECED syndrome (40–93%) (20, 23, 52, 54, 55). Median age at diagnosis is usually <5-years-old (1.0–6.5years) (20, 21, 23, 54, 55). According to the Finnish series, one-sixth of patients had developed CMC by 1.0 year, half by 5 years, 70% by 10 years, 94% by 20 years, and 97% by 30 years of age (56).

The clinical course of CMC varies from periodic to chronic, and its severity varies between individuals. The oral cavity was involved in 100% of patients in the Finnish cohort (23). In the Norwegian cohort, 40% of patients had angular cheilitis (53). In the mild oral form, CMC causes ulceration, redness, and soreness of the corners of the mouth. In more severe cases, the entire mouth is involved making it impossible to consume acidic or spicy foods. In the hyperplastic form, the tongue and buccal mucous membranes are covered by white or gray plaques and hyperkeratosis. In the atrophic form, the mucosa is erythematous and may be speckled with areas of leukoplakic or nodules (56). *Candida* onychomycosis is often associated with mucosal *Candida* lesions in childhood and is very difficult to eliminate (56). CMC affected the nails in 72% of patients in the Irish cohort of Collins et al. (52), and less frequently the skin (10–17%) (21, 23). Esophageal CMC occurred in 5–22% of patients in a European series (23, 52, 53), and in 51% of patients in a recent American study (21). Esophageal candidiasis often occurs without the typical form or symptoms of oral candidiasis, and can be complicated by substernal pain, dysphagia, and stenosis. Esophageal stenosis requires endoscopic dilation (23, 52–54). In the digestive tract, CMC can cause abdominal pain, flatulence, and diarrhea, which may be severe. Symptomatic intestinal candidiasis may also be present in the absence of oral disease (56).

In some patients, CMC may also be complicated by systemic candidiasis, although evidence is lacking that dissemination occurs from the oral cavity. Systemic candidiasis is very rare, even in APECED patients, and is frequently associated with immunosuppressive therapy. In an Italian cohort of 41 patients, one patient died from systemic candidiasis after the onset of immunosuppressive treatment (54), and in a French cohort, one patient died from systemic candidiasis after the onset of immunosuppressive treatment for large granular lymphocytic leukemia (57). In a Finnish cohort, one patient developed an abscess on the pericardium and small intestine (23). One isolated case of chronic *Candida* otitis has been described (53).

Genotype-Phenotype Correlation

The prevalence of CMC is reportedly higher in patients with the major Finnish *AIRE* mutation R257X than with other mutations (22). The prevalence is <20% in Iranian Jews affected by the Y85C mutation (15). Kisand et al. studied 160 APECED patients with the most severe mutations, R257X (Finnish) and R139X (Sardinian) and the Norwegian mutation, 967-979Δ13 In contrast to the study of Puel et al. (58), CMC was less prevalent in patients with the homozygous mutation 967-979Δ13 than with the other two mutations (59).

Squamous Cell Carcinoma and CMC

CMC has been reported to be involved in carcinogenesis as cancer often develops at the site of fungal lesions. Several cases of oral carcinoma have been described in association with CMC of the oral cavity and esophagus, suggesting that oral candidiasis may be carcinogenic. The most common morphological entity of these cancers is SCC (29, 60, 61). In patients with chronic *Candida* infection, oral CMC is often associated with esophageal cancer (62), and good clinical practice should include regular monitoring, every 2–3 years, by endoscopy. CMC is induced by immunosuppressive therapy rather than SCC itself.

The first report of oral SCC associated with APECED syndrome was published in 1975 in a patient who died of

metastatic disease at 27-years of age (63). A case report published in 2010 recorded the presence of three separate oral SCCs in a 40-year-old woman with APECED (61). In the Finnish case series, 6/91 APECED patients >25-years of age (10.5%) developed SCC, four died from the disease, and two developed oral colonization with *Candida* with decreased susceptibility to azole antifungals. One patient developed SCC without symptoms of oral candidiasis (23, 60). In the Norwegian cohort, 3/52 patients (6%) developed SCC at an early age (53). SCC was not reported in a recent American case series (21).

In the general population, mean age at diagnosis for oral and esophageal SCC is >62-years, and the disease is uncommon in young adults (64). The patients diagnosed with oral SCC in the Finnish cohort were between 29- and 44-years of age (23). The high rate of oral SCC in young patients with APECED demonstrates the possible carcinogenic potential of *C. albicans* when associated with the specific immunodeficiencies characteristic of this syndrome. Therefore, appropriate screening and adequate management of the infection and areas of oral dysplasia is necessary to reduce the risk of malignancy. Each erosive, ulcerated lesion should be biopsied, and each dysplastic lesion should be treated surgically (65).

In the general population, there are many risk factors for oral and esophageal SCC. Extrinsic factors include alcohol, tobacco, betel quid, immunosuppression, radiation, oncogenic viruses (human papilloma virus), and *Candida* infection, while intrinsic factors include immune defects, iron or vitamin A deficiency, malnutrition, and defects in tumor suppressor genes (64, 66, 67). Of the six APECED patients who developed SCC in the Finnish cohort, four were smokers and one had received immunosuppressive therapy (23). Therefore, extrinsic factors for SCC should be controlled in APECED patients as well as in CMC.

The role of chronic *Candida* infection in the etiopathogenesis of oral SCC is unclear. Possible mechanisms by which oral *Candida* infection might contribute to cancer development include: (i) metabolism of procarcinogens (such as the conversion of ethanol to acetaldehyde by *Candida*); (ii) production of carcinogens (such as the production of nitrosamine by *Candida* species); and (iii) induction of chronic inflammation, with the production of cytokines that enhance cell proliferation and inhibit apoptosis (24, 64, 65, 68, 69).

C. albicans and Decreased Susceptibility to Antifungal Treatment

Oropharyngeal candidiasis (mainly *C. albicans* and *C. glabrata*) is the most common fungal infection in patients with human immunodeficiency virus (HIV), and long-term use of azoles in this population has been reported to cause loss of susceptibility of *C. albicans* to fluconazole (70, 71). Candidiasis is usually caused by the yeast *C. albicans* in APECED patients, unlike in HIV patients (23, 52, 72–75). In a group of Finnish patients with APECED, non-*C. albicans* spp. were reported in only 7/56 patients (12.5%) (72). Because of the high prevalence of CMC in APECED patients and the risk of SCC, lifelong management of candidiasis with antifungal treatment is necessary (76). Topical intermittent treatment is more frequently prescribed than systemic antifungals, which are restricted to periods of severe symptoms and systemic candidiasis. Unlike in HIV patients, APECED patients with CMC treated with fluconazole have a high risk of becoming colonized with *C. albicans* with decreased susceptibility to fluconazole (76). Emergence of resistance does not appear to be a problem during short-term use, as shown in 43 isolates of *C. albicans* from 23 Finnish APECED patients.

Resistance seems to be correlated to the number of years of antifungal drug use, and is mainly due to the use of triazoles. Rautemaa et al. identified *C. albicans* isolates with decreased susceptibility to fluconazole in 11/56 (20%) Finnish patients (72). In the Irish cohort study of 16 patients with APECED, McManus described 11/16 (69%) patients with clinical signs of oral *Candida* infection and oral *Candida* isolates were recovered from 12/16 (75%) patients. Surprisingly, clinical signs suggestive of candidiasis did not always correlate with microbiological evidence of infection, and yeasts were isolated from only 32% of patients. The susceptibility of sequentially recovered isolates to fluconazole and itraconazole was examined; 11/16 (69%) patients had received antifungal treatment with fluconazole or itraconazole. Four of these 11 patients (36%) had isolates that were resistant or had reduced susceptibility to azoles (74).

Multiple mechanisms could lead to azole resistance in *C. albicans*. Some genes of *C. albicans* involved in ergosterol synthesis can be upregulated and in this way confer resistance to azoles, in particular the *ERG11* gene and CDR1, CDR2, and MDR1 encoded efflux pumps. In the study of McManus, acquisition of such mutations was revealed in *C. albicans* strains isolated during the longitudinal follow-up of patients. *C. albicans* strains recovered sequentially from nine Finnish patients were studied. The major molecular mechanisms leading to azole resistance were GOF mutations in *TAC1*, contributing to overexpression of CDR1 and CDR2, point mutations in *ERG11*, and six new *TAC1* mutations were detected (77). To avoid the emergence of azole resistance, McManus et al. proposed only prescribing azole therapy in patients with mycologically-documented *Candida* infection.

How Should CMC Be Managed in APECED Patients?

Husebye et al. suggested that oral infection should be strictly controlled to prevent the development of cancer. These authors highlighted the importance of having good oral hygiene with abstention from smoking and excessive alcohol consumption, and to avoid eating acidic or spicy food, and toothpastes containing powerful whitening agents. Meticulous oral hygiene is recommended in CMC patients, using both toothpaste and chlorhexidine solution, at bedtime and long-term, with the continual use of two topical polyene drugs. In line with this recommendation, patients should hold 1–2 mL of nystatin suspension in their mouth for several minutes and then allow an amphotericin B lozenge to dissolve without chewing. Both drugs should be spread to every part of the mouth with the tongue and finally swallowed. This regimen should be continued for 4–6 weeks or for at least 1 week after the resolution of symptoms. The use of these two polyene antifungal drugs is important in clinical

practice and both are well-tolerated due to a lack of absorption from the gut and a low rate of yeast resistance to these drugs.

Azole agents should be restricted to 2–3 courses per year to avoid decreased susceptibility. This treatment can be followed by prophylactic treatment consisting of 1 week of a polyene antifungal every 3 weeks and 1 week of chlorhexidine mouth rinse twice a day, if CMC becomes recurrent. Prophylactic treatment should be administered more frequently if symptoms persist, and even become daily treatment. In the case of failure of antifungal therapy, it is necessary to alert microbiologists to the possibility of a mixed infection and to use chromogenic media to detect mixed species, which would not be picked up with classical Sabouraud glucose agar. It is also recommended that all species isolated undergo antifungal susceptibility testing so that treatment can be adapted accordingly. Angular cheilitis should be treated by application of natamycin, amorolphine hydrochloride cream, or chlorhexidine gel several times a day, continuing for 4–5 days after the corners of the mouth have healed. Miconazole gel can also be used, while bearing in mind the risk of selection of azole-resistant strains. In order to avoid CMC relapses, antifungal treatment should be prescribed simultaneously for oral candidiasis and angular cheilitis, and biopsy of any lesion should be considered if mucositis with ulceration fails to respond to treatment within 2 weeks. *Candida* esophagitis and digestive CMC with diarrhea should also be treated with the same drug regimen for 1 or 2 weeks. If esophagitis persists, esophagoscopy and mycological sampling should be performed. The use of high-dose fluconazole (200–300 mg once a day for 1 week) must be restricted to severe cases and failure of topical therapy. Vaginal *Candida* infection should be treated with a short course of vaginal fluconazole, while fingernail candidiasis is very hard to eliminate and systemic medication is often necessary for 6 weeks. In general terms, prolonged intravenous (IV) antifungal therapy may be required and administered after obtaining expert medical advice (56, 75).

Adjunct immunotherapy with normal pooled immunoglobulin or IV immunoglobulin G (IVIG) in combination with antifungal agents is another treatment option. IVIG is a pooled IgG therapeutic preparation obtained from the plasma of several thousand healthy donors. In addition to its safety profile, IVIG could benefit CMC-associated APECED as a result of multiple mechanisms, including a reduction of inflammation by targeting various arms of the immune system, inhibition of autoantibody production by B-cells, and B-cell anergy (78). Further, antibodies in IVIG to fungal antigens may also help in pathogen neutralization (79, 80). As high-dose IVIG (1–2 g/kg) is known to inhibit Th17 responses *in vitro* (81, 82) and *in vivo*, including experimental allergic bronchopulmonary aspergillosis (78, 83), studies are required regarding appropriate dose of IVIG for CMC-associated APECED.

Pathophysiology of CMC Associated With APECED

The most prevalent autoantibodies detected in APECED patients are those neutralizing cytokines, especially type I IFNs and TH17-related cytokines, with a prevalence of 100% for IFN-ω (84, 85) and >90% for IL-22. IFN-ω autoantibodies are also found in thymomas (86), with a high rate of specificity and sensitivity. These autoantibodies are highly disease specific. Antibodies neutralizing IFN-ω are not correlated with CMC, and high titers of anti-IFN-ω antibodies are found in patients without CMC. Antibody titers in patients with APECED are almost always higher at diagnosis and persist for decades, representing a reliable biomarker for APECED syndrome (84, 85). Neutralizing autoantibodies against IL-17A, IL-17F, and IL-22 are also present at diagnosis. Autoantibodies against IL-22 and IL-17F seem to be more prevalent in APECED patients with CMC than in those without CMC, suggesting that type Th17 cytokines are central in human epithelial immunity against *Candida* infection (**Figure 1**). In contrast to the study of Kisand et al. (59) who reported

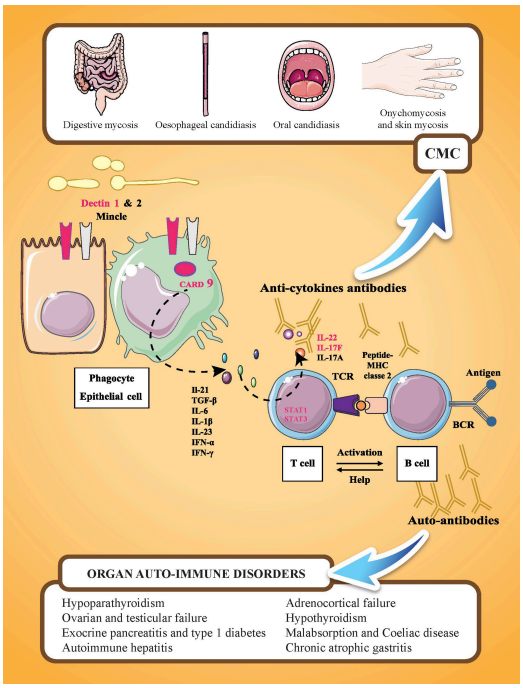

FIGURE 1 | Familial CMC and APECED syndrome**.** APECED syndrome is characterized by the association of endocrine autoimmune disorders (such as hypoparathyroidism, hypothyroidism, adrenocortical insufficiency, and gonadal failure), non-endocrine autoimmune disorders (such as autoimmune hepatitis, celiac disease, and chronic atrophic gastritis), and chronic mucocutaneous candidiasis (CMC). These manifestations are related to the presence of tissue specific antibodies and cytokine antibodies. IL-17 mediated immunity is represented by cooperation between cells recognizing *C. albicans* (phagocytes and epithelial cells) and IL-17 cytokine-producing cells (T-cells). On *C. albicans* recognition by PRRs (pathogen recognition receptors; including Dectin-1, Dectin-2, or Mincle), the adaptor molecule CARD9 mediates the induction of pro-inflammatory cytokines, such as IL 1β, IL-6, and IL-23. On binding to their receptors expressed on T-lymphocytes, pro-inflammatory cytokines, such as IL-6 or IL-23, activate T-lymphocytes via the transcription factor STAT3 resulting in their differentiation into IL-17-producing T-cells. Genes in which mutations are associated with CMC are indicated in pink: dectin 1, CARD 9, STAT 1, STAT3, IL22, and IL17F. IL-17RA and IL-12RB1 are not represented. Y designates cytokine-neutralizing autoantibodies that develop in AIRE-deficient (APECED and rare thymoma cases) patients.

that autoantibodies neutralizing IL-22 and IL-17F (but not those against IL-17A) were correlated with CMC in a study of 162 APECED patients, a study by Sarkadi et al. (87) reported high levels of autoantibodies against IL-17A in APECED patients with severe CMC. However, recent experimental data have shown that autoantibodies against IL-17A that develop in older *AIRE*-deficient mice do not confer susceptibility to oropharyngeal candidiasis, while monoclonal antibodies that cross-react with murine IL-22 derived from patients increase the mucosal fungal burden (88).

Occasional or weakly binding autoantibodies against IL-6, IL-9, IL-12, IL-21, IL-23, IL-26, IL-29, and RANTES have been reported in APECED patients in addition to autoantibodies against Th17 cytokines; however, their role in the development of CMC has not been demonstrated clearly (59).

The production of type I IFNs by dendritic cells is not impaired in APECED patients (89). *In vitro*, IL-22 and IL-17F production by peripheral blood mononuclear cells of APECED patients, stimulated by *Candida* antigens or polyclonal stimuli, was decreased in patients with CMC (59, 90, 91). The production of IL-17A was not impaired and even appeared to be increased (90, 91). The pathogenesis of CMC is believed to be associated with impaired Th17 cell responses, similar to several other primary immunodeficiencies associated with CMC. Th17 cytokines (IL-17A, IL-17F, and IL-22) influence epithelial cells by inducing the production of chemokines and antimicrobial peptides that exert direct antifungal activity. Additionally, IL-22 promotes epithelial barrier integrity, especially in synergy with TNF-α co-secreted by Th22 cells (92). Moreover, the production of IL-22 is severely impaired by skin-populating T-cells from APECED patients (93).

CONCLUSION

In APECED patients, CMC is associated with an impaired Th17 cell response. However, it remains unclear whether decreased serum IL-17 and IL-22 levels are related to a defect in cytokine production or to neutralizing autoantibodies resulting from mutations in the *AIRE* gene. Further investigations to develop new host- or pathogen-derived biomarkers are needed to improve the diagnosis of CMC and for a better understanding of human epithelial immunity against *C. albicans* infection.

AUTHOR CONTRIBUTIONS

BS and LH conceived the framework of the review. BS, LH, MC, JB, and M-CV wrote the manuscript. EP-L and J-LW revised the manuscript. LH and MC created the Figure. All authors have read and approved the final version of the manuscript.

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REFERENCES

1. Whitaker J, Landing BH, Esselborn VM, Williams RR. The syndrome of familial juvenile hypoadrenocorticism, hypoparathyroidism and superficial moniliasis. *J Clin Endocrinol Metab.* (1956) 16:1374–87.
2. Neufeld M, Maclaren N, Blizzard R. Autoimmune polyglandular syndromes. *Pediatr Ann.* (1980) 9:154–62.
3. Proust-Lemoine E, Saugier-Veber P, Wémeau J-L. Polyglandular autoimmune syndrome type I. *Presse Méd.* (2012) 41:e651–62. doi: 10.1016/j.lpm.2012.10.005
4. Aaltonen J, Björses P, Sandkuijl L, Perheentupa J, Peltonen L. An autosomal locus causing autoimmune disease: autoimmune polyglandular disease type I assigned to chromosome 21. *Nat Genet.* (1994) 8:83–7. doi: 10.1038/ng0994-83
5. Finnish-German APECED Consortium. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet.* (1997) 17:399–403. doi: 10.1038/ng1297-399
6. Su MA, Anderson MS. Aire: an update. *Curr Opin Immunol.* (2004) 16:746–52. doi: 10.1016/j.coi.2004.09.009
7. Ramsey C, Bukrinsky A, Peltonen L. Systematic mutagenesis of the functional domains of AIRE reveals their role in intracellular targeting. *Hum Mol Genet.* (2002) 11:3299–308. doi: 10.1093/hmg/11.26.3299
8. Pereira LE, Bostik P, Ansari AA. The development of mouse APECED models provides new insight into the role of AIRE in immune regulation. *Clin Dev Immunol.* (2005) 12:211–6. doi: 10.1080/17402520500212589
9. Björses P, Aaltonen J, Vikman A, Perheentupa J, Ben-Zion G, Chiumello G, et al. Genetic homogeneity of autoimmune polyglandular disease type I. *Am J Hum Genet.* (1996) 59:879–86.
10. Björses P, Halonen M, Palvimo JJ, Kolmer M, Aaltonen J, Ellonen P, et al. Mutations in the AIRE gene: effects on subcellular location and transactivation function of the autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy protein. *Am J Hum Genet.* (2000) 66:378–92. doi: 10.1086/302765
11. Heino M, Peterson P, Kudoh J, Shimizu N, Antonarakis SE, Scott HS, et al. APECED mutations in the autoimmune regulator (AIRE) gene. *Hum Mutat.* (2001) 18:205–11. doi: 10.1002/humu.1176
12. Zhu W, Hu Z, Liao X, Chen X, Huang W, Zhong Y, et al. A new mutation site in the AIRE gene causes autoimmune polyendocrine syndrome type 1. *Immunogenetics* (2017) 69:643–51 doi: 10.1007/s00251-017-0995-5
13. Ahonen P, Miettinen A, Perheentupa J. Adrenal and steroidal cell antibodies in patients with autoimmune polyglandular disease type I and risk of adrenocortical and ovarian failure. *J Clin Endocrinol Metab.* (1987) 64:494–500. doi: 10.1210/jcem-64-3-494
14. Rosatelli MC, Meloni A, Meloni A, Devoto M, Cao A, Scott HS, et al. A common mutation in Sardinian autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients. *Hum Genet.* (1998) 103:428–34.
15. Zlotogora J, Shapiro MS. Polyglandular autoimmune syndrome type I among Iranian Jews. *J Med Genet.* (1992) 29:824–6.
16. Pearce SH, Cheetham T, Imrie H, Vaidya B, Barnes ND, Bilous RW, et al. A common and recurrent 13-bp deletion in the autoimmune regulator gene in British kindreds with autoimmune polyendocrinopathy type 1. *Am J Hum Genet.* (1998) 63:1675–84. doi: 10.1086/302145
17. Myhre AG, Halonen M, Eskelin P, Ekwall O, Hedstrand H, Rorsman F, et al. Autoimmune polyendocrine syndrome type 1 (APS I) in Norway. *Clin Endocrinol.* (2001) 54:211–7. doi: 10.1046/j.1365-2265.2001.01201.x
18. Dominguez M, Crushell E, Ilmarinen T, McGovern E, Collins S, Chang B, et al. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in the Irish population. *J Pediatr Endocrinol Metab.* (2006) 19:1343–52. doi: 10.1515/JPEM,2006.19.11.1343
19. Wolff ASB, Erichsen MM, Meager A, Magitta NF, Myhre AG, Bollerslev J, et al. Autoimmune polyendocrine syndrome type 1 in Norway: phenotypic variation, autoantibodies, and novel mutations in the autoimmune regulator

gene. *J Clin Endocrinol Metab.* (2007) 92:595–603. doi: 10.1210/jc.2006-1873
20. Valenzise M, Fierabracci A, Cappa M, Porcelli P, Barcellona R, De Luca F, et al. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy: report of seven additional sicilian patients and overview of the overall series from sicily. *Horm Res Paediatr.* (2014) 82:127–32. doi: 10.1159/000363537
21. Ferre EM, Rose SR, Rosenzweig SD, Burbelo PD, Romito KR, Niemela JE, et al. Redefined clinical features and diagnostic criteria in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *JCI Insight* (2016) 1:13. doi: 10.1172/jci.insight.88782
22. Halonen M, Eskelin P, Myhre A-G, Perheentupa J, Husebye ES, Kämpe O, et al. AIRE mutations and human leukocyte antigen genotypes as determinants of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy phenotype. *J Clin Endocrinol Metab.* (2002) 87:2568–74. doi: 10.1210/jcem.87.6.8564
23. Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J Clin Endocrinol Metab.* (2006) 91:2843–50. doi: 10.1210/jc.2005-2611
24. Zhu F, Willette-Brown J, Song N-Y, Lomada D, Song Y, Xue L, et al. Autoreactive T cells and chronic fungal infection drive esophageal carcinogenesis. *Cell Host Microbe* (2017) 21:478–93.e7. doi: 10.1016/j.chom.2017.03.006
25. Eyerich K, Eyerich S, Hiller J, Behrendt H, Traidl-Hoffmann C. Chronic mucocutaneous candidiasis, from bench to bedside. *Eur J Dermatol.* (2010) 20:260–5. doi: 10.1684/ejd.2010.0910
26. Thorpe ES, Handley HE. Chronic tetany and chronic mycelial stomatitis in a child aged four and one-half years. *Am J Dis Child* (1929) 38:328–38. doi:10.1001/archpedi.1929.01930080104011
27. de Repentigny L, Lewandowski D, Jolicoeur P. Immunopathogenesis of oropharyngeal candidiasis in human immunodeficiency virus infection. *Clin Microbiol Rev.* (2004) 17:729–59. doi: 10.1128/CMR.17.4.729-759.2004.
28. Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, et al. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. *Clin Microbiol Infect.* (2012) 18:19–37. doi: 10.1111/1469-0691.12039
29. Rosa DD, Pasqualotto AC, Denning DW. Chronic mucocutaneous candidiasis and oesophageal cancer. *Med Mycol.* (2008) 46:85–91. doi: 10.1080/13693780701616023.
30. Mohammadi F, Javaheri MR, Nekoeian S, Dehghan P. Identification of Candida species in the oral cavity of diabetic patients. *Curr Med Mycol.* (2016) 2:1–7.doi: 10.18869/acadpub.cmm.2.2.4
31. Puel A, Cypowyj S, Maródi L, Abel L, Picard C, Casanova J-L. Inborn errors of human IL-17 immunity underlie chronic mucocutaneous candidiasis. *Curr Opin Allergy Clin Immunol.* (2012) 12:616. doi: 10.1097/ACI.0b013e328358cc0b.
32. Picard C, Al-Herz W, Bousfiha A, Casanova J-L, Chatila T, Conley ME, et al. Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015. *J Clin Immunol.* (2015) 35:696–726. doi: 10.1007/s10875-015-0201-1.
33. Okada S, Puel A, Casanova J-L, Kobayashi M. Chronic mucocutaneous candidiasis disease associated with inborn errors of IL-17 immunity. *Clin Transl Immunol.* (2016) 5:e114. doi: 10.1038/cti.2016.71
34. Sowerwine KJ, Holland SM, Freeman AF. Hyper-IgE syndrome update. *Ann N Y Acad Sci.* (2012) 1250:25–32. doi: 10.1111/j.1749-6632.2011.06387.x
35. Zhang Q, Davis JC, Lamborn IT, Freeman AF, Jing H, Favreau AJ, et al. Combined Immunodeficiency Associated with DOCK8 Mutations. *N Engl J Med.* (2009) 361:2046–55. doi: 10.1056/NEJMoa0905506
36. Su HC. DOCK8 (Dedicator of cytokinesis 8) deficiency. *Curr Opin Allergy Clin Immunol.* (2010) 10:515–20. doi: 10.1097/ACI.0b013e32833fd718
37. Su HC, Jing H, Zhang Q. DOCK8 deficiency. *Ann N Y Acad Sci.* (2011) 1246:26–33. doi: 10.1111/j.1749-6632.2011.06295.x
38. Biggs CM, Keles S, Chatila TA. DOCK8 deficiency: insights into pathophysiology, clinical features and management. *Clin Immunol.* (2017) 181:75–82. doi: 10.1016/j.clim.2017.06.003
39. Grumach AS, de Queiroz-Telles F, Migaud M, Lanternier F, Filho NR, Palma SMU, et al. A homozygous CARD9 mutation in a Brazilian patient with deep dermatophytosis. *J Clin Immunol.* (2015) 35:486–90. doi: 10.1007/s10875-015-0170-4
40. Celmeli F, Oztoprak N, Turkkahraman D, Seyman D, Mutlu E, Frede N, et al. Successful granulocyte colony-stimulating factor treatment of relapsing *Candida albicans* meningoencephalitis caused by CARD9 deficiency. *Pediatr Infect Dis J.* (2016) 35:428–31. doi: 10.1097/INF.0000000000001028
41. Jones N, Garcez T, Newman W, Denning D. Endogenous Candida endophthalmitis and osteomyelitis associated with CARD9 deficiency. *BMJ Case Rep* (2016) 2016:bcr2015214117. doi: 10.1136/bcr-2015-214117. [Epub ahead of print].
42. Glocker E-O, Hennigs A, Nabavi M, Schäffer AA, Woellner C, Salzer U, et al. A homozygous CARD9 mutation in a family with susceptibility to fungal infections. *N Engl J Med.* (2009) 361:1727–35. doi: 10.1056/NEJMoa0810719
43. Drewniak A, Gazendam RP, Tool ATJ, van Houdt M, Jansen MH, van Hamme JL, et al. Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. *Blood* (2013) 121:2385–92. doi: 10.1182/blood-2012-08-450551
44. Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spriel AB, Venselaar H, et al. Human Dectin-1 deficiency and mucocutaneous fungal infections. *N Engl J Med.* (2009) 361:1760–7. doi: 10.1056/NEJMoa0901053.
45. Rosentul DC, Plantinga TS, Oosting M, Scott WK, Velez Edwards DR, Smith PB, et al. Genetic variation in the Dectin-1/CARD9 recognition pathway and susceptibility to candidemia. *J Infect Dis.* (2011) 204:1138–45. doi: 10.1093/infdis/jir458.
46. Plantinga TS, van der Velden WJFM, Ferwerda B, van Spriel AB, Adema G, Feuth T, et al. Early stop polymorphism in human DECTIN-1 is associated with increased Candida colonization in hematopoietic stem cell transplant recipients. *Clin Infect Dis.* (2009) 49:724–32. doi: 10.1086/604714
47. Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* (2010) 32:681–91. doi: 10.1016/j.immuni.2010.05.001
48. Vinh DC. Insights into human antifungal immunity from primary immunodeficiencies. *Lancet Infect Dis.* (2011) 11:780–92. doi: 10.1016/S1473-3099(11)70217-1
49. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PDR, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* (2012) 61:1693–700. doi: 10.1136/gutjnl-2011-301668
50. Colombel JF, Sendid B, Jouault T, Poulain D. Secukinumab failure in Crohn's disease: the yeast connection? *Gut* (2013) 62:800–1. doi: 10.1136/gutjnl-2012-304154
51. Ahonen P, Myllärniemi S, Sipilä I, Perheentupa J. Clinical variation of Autoimmune Polyendocrinopathy–Candidiasis–Ectodermal Dystrophy (APECED) in a series of 68 patients. *N Engl J Med.* (1990) 322:1829–36. doi: 10.1056/NEJM199006283222260
52. Collins SM, Dominguez M, Ilmarinen T, Costigan C, Irvine AD. Dermatological manifestations of autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy syndrome. *Br J Dermatol.* (2006) 154:1088–93. doi: 10.1111/j.1365-2133.2006.07166.x
53. Bruserud Ø, Oftedal BE, Landegren N, Erichsen MM, Bratland E, Lima K, et al. A longitudinal follow-up of autoimmune polyendocrine syndrome type 1. *J Clin Endocrinol Metab.* (2016) 101:2975–83. doi: 10.1210/jc.2016-1821
54. Betterle C, Greggio NA, Volpato M. Autoimmune polyglandular syndrome type 1. *J Clin Endocrinol Metab.* (1998) 83:1049–55. doi: 10.1210/jcem.83.4.4682
55. Meloni A, Willcox N, Meager A, Atzeni M, Wolff ASB, Husebye ES, et al. Autoimmune polyendocrine syndrome type 1: an extensive longitudinal study in sardinian patients. *J Clin Endocrinol Metab.* (2012) 97:1114–24. doi: 10.1210/jc.2011-2461

56. Husebye ES, Perheentupa J, Rautemaa R, Kämpe O. Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. *J Intern Med*. (2009) 265:514–29. doi: 10.1111/j.1365-2796.2009.02090.x
57. Proust-Lemoine E, Saugier-Véber P, Lefranc D, Dubucquoi S, Ryndak A, Buob D, et al. Autoimmune polyendocrine syndrome type 1 in North-Western France: AIRE gene mutation specificities and severe forms needing immunosuppressive therapies. *Horm Res Paediatr*. (2010) 74:275–84. doi: 10.1159/000297714
58. Puel A, Döffinger R, Natividad A, Chrabieh M, Barcenas-Morales G, Picard C, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med*. (2010) 207:291–7. doi: 10.1084/jem.20091983.
59. Kisand K, Wolff ASB, Podkrajšek KT, Tserel L, Link M, Kisand KV, et al. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *J Exp Med*. (2010) 207:299–308. doi: 10.1084/jem.20091669
60. Rautemaa R, Hietanen J, Niissalo S, Pirinen S, Perheentupa J. Oral and oesophageal squamous cell carcinoma – A complication or component of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, APS-I). *Oral Oncol*. (2007) 43:607–13. doi: 10.1016/j.oraloncology.2006.07.005
61. Böckle BC, Wilhelm M, Müller H, Götsch C, Sepp NT. Oral mucous squamous cell carcinoma—an anticipated consequence of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). *J Am Acad Dermatol*. (2010) 62:864–8. doi: 10.1016/j.jaad.2009.06.061
62. Koo S, Kejariwal D, Al-Shehri T, Dhar A, Lilic D. Oesophageal candidiasis and squamous cell cancer in patients with gain-of-function STAT1 gene mutation. *United Eur Gastroenterol J*. (2017) 5:625–31. doi: 10.1177/2050640616684404
63. Richman RA, Rosenthal IM, Solomon LM, Karachorlu KV. Candidiasis and multiple endocrinopathy: with oral squamous cell carcinoma complications. *Arch Dermatol*. (1975) 111:625–7.
64. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma—an update. *CA Cancer J Clin*. (2015) 65:401–21. doi: 10.3322/caac.21293
65. Shephard MK, Schifter M, Palme CE. Multiple oral squamous cell carcinomas associated with autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy. *Oral Surg Oral Med Oral Pathol Oral Radiol*. (2012) 114:e36–42. doi: 10.1016/j.oooo.2012.04.013
66. Binnie WH, Rankin KV, Mackenzie IC. Etiology of oral squamous cell carcinoma. *J Oral Pathol*. (1983) 12:11–29.
67. Mehrotra R, Yadav S. Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations. *Indian J Cancer* (2006) 43:60–6. doi: 10.4103/0019-509X.25886
68. Swidergall M, Solis NV, Lionakis MS, Filler SG. EphA2 is an epithelial cell pattern recognition receptor for fungal β-glucans. *Nat Microbiol*. (2018) 3:53–61. doi: 10.1038/s41564-017-0059-5
69. Zhu W, Phan QT, Boontheung P, Solis NV, Loo JA, Filler SG. EGFR and HER2 receptor kinase signaling mediate epithelial cell invasion by *Candida albicans* during oropharyngeal infection. *Proc Natl Acad Sci USA*. (2012) 109:14194–9. doi: 10.1073/pnas.1117676109
70. Rex JH, Rinaldi MG, Pfaller MA. Resistance of Candida species to fluconazole. *Antimicrob Agents Chemother*. (1995) 39:1–8.
71. Heald AE, Cox GM, Schell WA, Bartlett JA, Perfect JR. Oropharyngeal yeast flora and fluconazole resistance in HIV-infected patients receiving long-term continuous versus intermittent fluconazole therapy. *AIDS* (1996) 10:263–8.
72. Rautemaa R, Richardson M, Pfaller M, Perheentupa J, Saxén H. Reduction of fluconazole susceptibility of *Candida albicans* in APECED patients due to long-term use of ketoconazole and miconazole. *Scand J Infect Dis*. (2008) 40:904–7. doi: 10.1080/00365540802275853
73. Rautemaa R, Richardson M, Pfaller MA, Perheentupa J, Saxén H. Activity of amphotericin B, anidulafungin, caspofungin, micafungin, posaconazole, and voriconazole against *Candida albicans* with decreased susceptibility to fluconazole from APECED patients on long-term azole treatment of chronic mucocutaneous candidiasis. *Diagn Microbiol Infect Dis*. (2008) 62:182–5. doi: 10.1016/j.diagmicrobio.2008.05.007
74. McManus BA, McGovern E, Moran GP, Healy CM, Nunn J, Fleming P, et al. Microbiological screening of irish patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy reveals persistence of *Candida albicans* Strains, gradual reduction in susceptibility to azoles, and incidences of clinical signs of oral candidiasis without culture evidence. *J Clin Microbiol*. (2011) 49:1879–89. doi: 10.1128/JCM.00026-11
75. Proust-Lemoine E, Guyot S. Polyendocrinopathies auto-immunes de type 1 et pathologies buccales. *Presse Méd*. (2017) 46:853–63. doi: 10.1016/j.lpm.2017.05.029
76. Rautemaa R, Richardson M, Pfaller M, Koukila-Kähkölä P, Perheentupa J, Saxén H. Decreased susceptibility of *Candida albicans* to azole antifungals: a complication of long-term treatment in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients. *J Antimicrob Chemother*. (2007) 60:889–92. doi: 10.1093/jac/dkm299
77. Siikala E, Rautemaa R, Richardson M, Saxen H, Bowyer P, Sanglard D. Persistent *Candida albicans* colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) patients. *J Antimicrob Chemother*. (2010) 65:2505–13. doi: 10.1093/jac/dkq354
78. Galeotti C, Kaveri SV, Bayry J. IVIG-mediated effector functions in autoimmune and inflammatory diseases. *Int Immunol*. (2017) 29:491–8. doi: 10.1093/intimm/dxx039
79. Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Intravenous immunoglobulin for infectious diseases: back to the pre-antibiotic and passive prophylaxis era? *Trends Pharmacol Sci*. (2004) 25:306–10. doi: 10.1016/j.tips.2004.04.002
80. Ferrara G, Zumla A, Maeurer M. Intravenous immunoglobulin (IVIg) for refractory and difficult-to-treat infections. *Am J Med*. (2012) 125:1036.e1–8. doi: 10.1016/j.amjmed.2012.01.023
81. Maddur MS, Vani J, Hegde P, Lacroix-Desmazes S, Kaveri SV, Bayry J. Inhibition of differentiation, amplification, and function of human TH17 cells by intravenous immunoglobulin. *J Allergy Clin Immunol*. (2011) 127:823–30. doi: 10.1016/j.jaci.2010.12.1102
82. Saha C, Das M, Patil V, Stephen-Victor E, Sharma M, Wymann S, et al. Monomeric immunoglobulin A from plasma inhibits human Th17 responses *in vitro* independent of FcαRI and DC-SIGN. *Front Immunol*. (2017) 8:275. doi: 10.3389/fimmu.2017.00275
83. Bozza S, Käsermann F, Kaveri SV, Romani L, Bayry J. Intravenous immunoglobulin protects from experimental allergic bronchopulmonary aspergillosis via a sialylation-dependent mechanism. *Eur J Immunol*. (2018). doi: 10.1002/eji.201847774. [Epub ahead of print].
84. Meager A, Visvalingam K, Peterson P, Möll K, Murumägi A, Krohn K, et al. Anti-interferon autoantibodies in autoimmune polyendocrinopathy syndrome type 1. *PLoS Med*. (2006) 3:e289. doi: 10.1371/journal.pmed.0030289
85. Meloni A, Furcas M, Cetani F, Marcocci C, Falorni A, Perniola R, et al. Autoantibodies against type I interferons as an additional diagnostic criterion for autoimmune polyendocrine syndrome type I. *J Clin Endocrinol Metab*. (2008) 93:4389–97. doi: 10.1210/jc.2008-0935
86. Kisand K, Lilic D, Casanova J-L, Peterson P, Meager A, Willcox N. Mucocutaneous candidiasis and autoimmunity against cytokines in APECED and thymoma patients: clinical and pathogenetic implications. *Eur J Immunol*. (2011) 41:1517–27. doi: 10.1002/eji.201041253
87. Sarkadi AK, Taskó S, Csorba G, Tóth B, Erdos M, Maródi L. Autoantibodies to IL-17A may be Correlated with the Severity of Mucocutaneous Candidiasis in APECED Patients. *J Clin Immunol*. (2014) 34:181–93. doi: 10.1007/s10875-014-9987-5
88. Bichele R, Kärner J, Truusalu K, Smidt I, Mändar R, Conti HR, et al. IL-22 neutralizing autoantibodies impair fungal clearance in murine oropharyngeal candidiasis model. *Eur J Immunol*. (2018) 48:464–70. doi: 10.1002/eji.201747209
89. Kisand K, Link M, Wolff ASB, Meager A, Tserel L, Org T, et al. Interferon autoantibodies associated with AIRE deficiency decrease the expression of IFN-stimulated genes. *Blood* (2008) 112:2657–66. doi: 10.1182/blood-2008-03-144634
90. Ng WF, von Delwig A, Carmichael AJ, Arkwright PD, Abinun M, Cant AJ, et al. Impaired TH17 responses in patients with

chronic mucocutaneous candidiasis with and without autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy. *J Allergy Clin Immunol.* (2010) 126:1006–15. doi: 10.1016/j.jaci.2010.08.027

91. Ahlgren KM, Moretti S, Lundgren BA, Karlsson I, Åhlin E, Norling A, et al. Increased IL-17A secretion in response to *Candida albicans* in autoimmune polyendocrine syndrome type 1 and its animal model. *Eur J Immunol.* (2011) 41:235–45. doi: 10.1002/eji.200939883
92. Kisand K, Peterson P. Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. *J Clin Immunol.* (2015) 35:463–78. doi: 10.1007/s10875-015-0176-y
93. Laakso SM, Kekäläinen E, Heikkilä N, Mannerström H, Kisand K, Peterson P, et al. *In vivo* analysis of helper T cell responses in patients with autoimmune polyendocrinopathy – candidiasis – ectodermal dystrophy provides evidence in support of an IL-22 defect. *Autoimmunity* (2014) 47:556–62. doi: 10.3109/08916934.2014.929666.

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Exploiting the Oral Microbiome to Prevent Tooth Decay: Has Evolution Already Provided the Best Tools?

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To compete in the relatively exposed oral cavity, resident microbes must avoid being replaced by newcomers. This selective constraint, coupled with pressure on the host to cultivate a beneficial microbiome, has rendered a commensal oral microbiota that displays colonization resistance, protecting the human host from invasive species, including pathogens. Rapid increases in carbohydrate consumption have disrupted the evolved homeostasis between the oral microbiota and dental health, reflected by the high prevalence of dental caries. Development of novel modalities to prevent caries has been the subject of a breadth of research. This mini review provides highlights of these endeavors and discusses the rationale and pitfalls behind the major avenues of approach. Despite efficacy, fluoride and other broad-spectrum interventions are unlikely to further reduce the incidence of dental caries. The most promising methodologies in development are those that exploit the exclusive nature of the healthy oral microbiome. Probiotics derived from the dental plaque of healthy individuals sharply antagonize cariogenic species, such as *Streptococcus mutans.* Meanwhile, targeted antimicrobials allow for the killing of specific pathogens, allowing reestablishment of a healthy microbiome, presumably with its protective effects. The oral microbiota manufactures a massive array of small molecules, some of which are correlated with health and are likely to antagonize pathogens. The prohibitive cost associated with sufficiently rigorous clinical trials, and the status of dental caries as a non-life-threatening condition will likely continue to impede the advancement of new therapeutics to market. Nevertheless, there is room for optimism, as it appears evolution may have already provided the best tools.

Keywords: oral probiotics, oral treatment, *Streptococcus*, caries, antimicrobial small molecules

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INTRODUCTION

Evolution within a microbiota is driven by the requirement of each taxa to compete and persist within the host. Meanwhile, hosts are under strong selective pressure to modulate their microbiota to ensure that it confers a benefit. Unlike individual microbes, the sophisticated immune system of mammals can easily influence an entire resident microbial community, and benefit from doing so. For this reason, the human microbiota has been described as an "ecosystem on a leash" (Foster et al., 2017). With multiple microenvironments allowing for a large diversity of taxa, as well as consistent exposure to the external environment and food, the oral cavity presents a highly unique circumstance for the interaction of the human microbiota and the host. Constant exposure to foreign microbes selects for oral taxa which are particularly skilled at direct competition—they

must avoid being replaced! As a consequence, the oral microbiome displays colonization resistance, which is beneficial to the host (He et al., 2014). Therefore, it is probable that the immunology of the oral cavity has also evolved to tolerate, and even facilitate, maintenance of a commensal, yet fiercely territorial, oral microbiome which prevents the establishment of foreign invaders, including pathogens.

Humans have a long history of co-evolution with our resident bacteria, and evidence suggests that our ancient hominid microbiota was more diverse and stable than that of modern humans (Adler et al., 2013; Moeller et al., 2014, 2016). Two dietary shifts, brought about by the development of agriculture (∼7,500 years ago) and the Industrial Revolution (∼200 years ago) (Adler et al., 2013), significantly and rapidly increased the consumption of carbohydrates (particularly sucrose, in the case of the latter). These changes have perturbed the homeostasis of the oral microbiome and dental health, causing dental caries to become the most common chronic disease worldwide, affecting 60–90% of children and adults in industrialized countries (reviewed in Pitts et al., 2017). This review highlights therapeutic strategies, both contemporary and developing, that exploit the protective effects of the healthy oral flora in an effort to prevent dental caries.

A BRIEF OVERVIEW OF DENTAL PLAQUE ECOLOGY

Typically, the earliest colonizers of the tooth surface are commensal streptococci, such as *Streptococcus mitis, Streptococcus sanguinis, Streptococcus gordonii,* and other closely related taxa. These species are the most avid binders of the naked, pellicle-coated tooth surface. Once these species have bound, they provide a more complex substrate to which other species can now bind. To help ensure their continued success, the majority of taxa within the mitis and sanguinis groups stanchly antagonize newcomers using the production of alkali, bacteriocins, and H_2O_2. In the absence of a carbohydrate-rich diet, these commensal streptococci tend to remain at high abundances in dental plaque. This dominance is strongly associated with good dental health. With frequent consumption of carbohydrates, particularly when concurrent with a lack of oral hygiene, increased bacterial production of a glucan matrix is favored, emeshing cells and preventing diffusion of metabolites. This allows for development of emergent properties of the dental plaque, such as acidic microenvironments resulting from carbohydrate fermentation. Typically, the saliva in the mouth has sufficient buffering capacity to neutralize the organic acids produced by bacterial metabolism, and repair acid-damaged enamel. However, the increased thickness and density of exopolysaccharide-rich plaque prevents both diffusion of saliva into the biofilm and diffusion of acids out of the biofilm. The commensal early colonizers are comparatively not well-adapted to acidic conditions, allowing for a further enrichment of acid-tolerant (aciduric) taxa such as *Streptococcus mutans, Veillonella* spp., and *Lactobacillus* spp. With progression of this positive feedback loop, the rate of net acid damage (demineralization) of the tooth enamel outpaces repair (remineralization), leading to clinical disease.

With an arsenal of extracellular glucosyltransferases (Gtfs), *S. mutans* is particularly adept at producing a glucan matrix from sucrose, and therefore is considered a keystone species in caries pathogenesis (Bowen, 2016; Bowen et al., 2018). Competition between the early colonizers of the teeth and cariogenic species, particularly *S. mutans,* has been well-documented and acknowledged for decades (Marquis, 1995; Huang et al., 2018). Interested readers are directed to four excellent and recent reviews covering the above topics in more depth (Abranches et al., 2018; Bowen et al., 2018; Lamont et al., 2018; Redanz et al., 2018). This battleground over the ecological niche of the tooth surface represents a significant opportunity for intervention and subsequent prevention of caries. If the balance of power can be tipped in favor of health-associated organisms, it is possible that pathogenesis of caries can be halted. **Figure 1** provides an overview of dental caries pathogenesis and the major preventative strategies discussed in this review.

CURRENT CONTROL MEASURES: DIET AND FLUORIDE

Diet

Caries is not a classic infectious disease, but the consequence of an ecological shift. Indeed, pathogenic species are necessary, but not sufficient, to cause disease—a constant supply of carbohydrates is also required. In addition to dental caries, the carbohydrate-laden, highly processed modern Western diet has led to pandemics of obesity, type II diabetes, cardiovascular disease, as well as related metabolic disorders and cancers. As with caries, a large and growing body of evidence is linking these conditions to diet via microbial mediators (reviewed in Gilbert et al., 2018; Zmora et al., 2018). Education of the general public to the importance of diet, healthy dietary habits, and the significant association of diet, the microbiota, and health issues remains paramount. In addition to the dissemination of current dietary recommendations, improving the accessibility of healthy foods is a goal worthy of considerable attention. A return to a more primitive and unprocessed diet is likely to have significant health benefits by supporting the microbial profiles with which we have the proper evolutionary rapport to underpin a mutualistic relationship. This includes, of course, the microbial profiles on the tooth surface.

Fluoride

Fluoride treatments, including fluoridated toothpaste and drinking water, have been used to combat dental caries for more than 50 years. Fluoride prevents and treats dental caries by promoting favorable remineralization of the tooth enamel while concomitantly impairing bacterial metabolism (Pitts et al., 2017). Although the efficacy of fluoride treatments is well-documented, clearly the current prevalence of the disease illustrates that fluoride alone is insufficient to prevent dental caries in many situations. Other, more stringent, antimicrobial agents are available for dental use (e.g., chlorhexidine and triclosan),

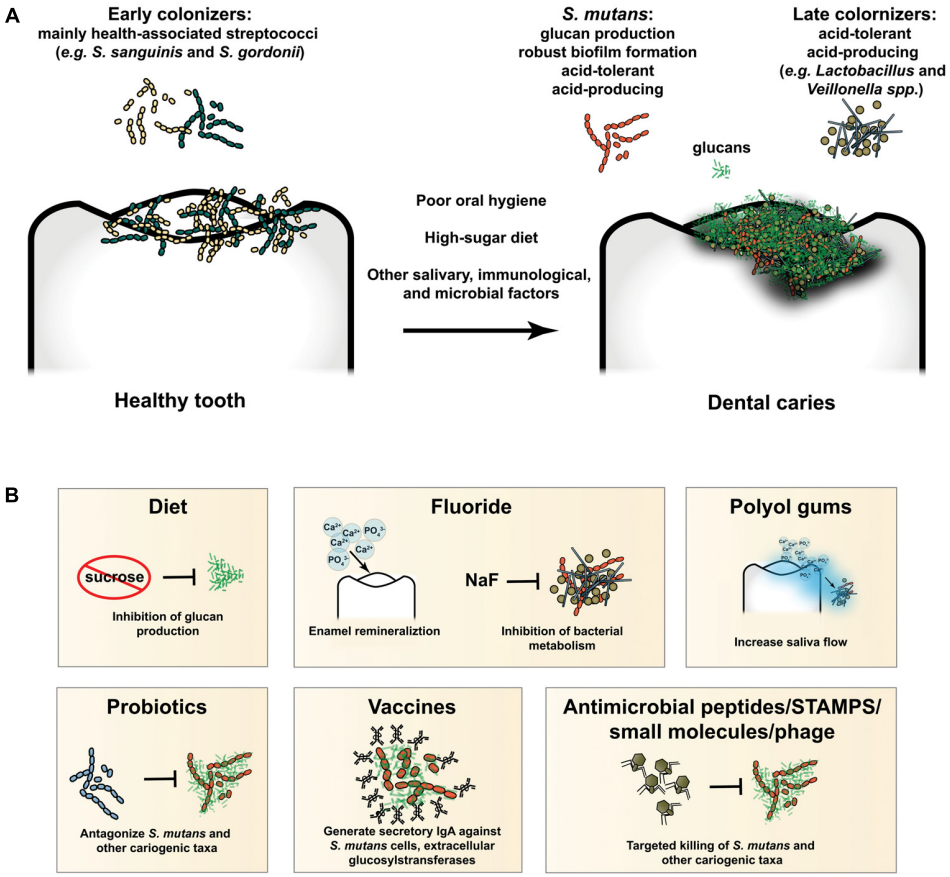

FIGURE 1 | (A) Overview of caries pathogenesis. Early colonizers of the tooth are mainly health-associated streptococcal species, such as *S. sanguinis* and *S. gordonii,* as well as other closely related taxa. Poor oral hygiene, a high-sugar diet, and other salivary, immunological, and microbial factors lead to development of pathogenic biofilms (i.e., dysbiosis). *S. mutans* produces a glucan matrix, which leads to robust biofilm formation and colonization by taxa which could not have bound the tooth surface unassisted (late colonizers). Production of acid within the biofilm selects for increasingly acid-tolerant cariogenic organisms, such as *S. mutans, Lactobacillus spp., Veillonella spp.,* and others. Unchecked, the process will destroy the protective enamel coating of the tooth and lead to clinical disease. **(B)** Preventative and therapeutic modalities in current use or development. A diet low in refined sugars, such as sucrose, will inhibit production of glucans and formation of cariogenic biofilms. Fluoride both promotes remineralization of tooth enamel and inhibits potentially cariogenic bacterial metabolism. Polyol gums increase saliva flow, delivering ions for tooth remineralization and promoting clearance of bacteria from the tooth surface. Probiotics antagonize and prevent establishment and outgrowth of pathogenic species, such as *S. mutans.* Immune priming via vaccination leads to elevated levels of secretory IgA, which binds target epitopes on *S. mutans* and other cariogenic targets, preventing binding and biofilm formation, and promoting clearance from the oral cavity. Antimicrobial peptides, STAMPS, small molecules, and phage promote targeted killing of specific cariogenic taxa, such as *S. mutans.*

but all are similarly broad-spectrum. As such, reengineering of a dysbiotic oral microbiome is likely to generate a more positive outcome than its total destruction. The development of approaches to specifically alter plaque composition and prevent outgrowth of cariogenic species, such as *S. mutans,* remains a highly attractive objective. These approaches fall into several broad categories, discussed below.

PREVENTATIVE APPROACHES IN DEVELOPMENT

Prebiotics

Prebiotics are food or supplements which are administered to modulate the microbiome for the benefit of the host. Arginine has demonstrated success as a prebiotic to prevent dental caries (reviewed in Nascimento, 2018). Arginine can be broken down by commensal arginolytic species (e.g., *S. sanguinis* and *S. gordonii*) to generate ammonia, an alkaline molecule that buffers the organic acids in dental plaque. These reactions are performed by the arginine deiminase system (ADS). In addition to contributing to a more alkaline pH, the breakdown of arginine by the ADS also generates ATP, providing a bioenergetic advantage to the commensal streptococci (Bowen et al., 2018). Furthermore, arginine has been shown to inhibit the growth, pathogenic potential, and stress response mechanisms of *S. mutans*, thereby preventing caries pathogenesis through multiple mechanisms (Chakraborty and Burne, 2017). Higher cost, as well as controversy regarding the protective effects of arginine and the integrity of several clinical trials

(Astvaldsdottir et al., 2016; Richards, 2017), have impeded dentifrices containing both fluoride and arginine from becoming widely available. As laboratory evidence for the protective effects of arginine continues to accumulate (Agnello et al., 2017; Huang et al., 2017; Zheng et al., 2017), more rigorous clinical trials could perhaps lead to widespread availability of arginine-containing commercial therapeutics. Recent studies have identified several other compounds; Met-Pro, succinic acid, beta-methyl-D-galactoside and *N*-acetyl-D-mannosamine as prebiotic candidates for caries prevention. These molecules were able to promote the dominance of health-associated organisms in a multispecies *in vitro* culture (Slomka et al., 2017, 2018). Whether these effects can be translated into *in vivo* studies remains to be investigated.

Exploiting Oral Immunology

Caries is not immediately life-threatening, thus selective pressure on the host to thwart the condition is not terribly strong when compared to a disease like smallpox. On the other hand, teeth are a highly valued organ involved in obtaining and digesting food, self-defense, speech/communication, and even sexual attraction (Koussoulakou et al., 2009). It is likely no accident that moieties in the saliva provide binding sites and nourishment for specific species (i.e., the early colonizers), which are largely benign. Saliva flow, and the components of saliva have great influence over which taxa are able to persist in the mouth, and which are cleared (Marsh et al., 2016). Individuals with reduced salivary flow have a greatly increased prevalence of caries. Approaches to increase salivary flow are likely to assist in buffering acids, supplying antimicrobial peptides and antibodies, and preventing dysbiosis and caries from occurring. Chewing gums containing polyols, such as xylitol, provide salivary stimulus without fermentable carbohydrates. Furthermore, certain polyols, particularly erythritol (de Cock, 2018), also have antimicrobial activities, furthering their utility as a preventative modality (Makinen, 2010). Although auspicious, more rigorous research into the effects of these polyol molecules on overall systemic health is warranted; several other sugar substitutes have been recently shown to wreak havoc on the gut microbiota and promote disease (Suez et al., 2014; Rother et al., 2018).

Considering the adaptive arm of the immune system, levels of salivary IgA against immunogenic *S. mutans* epitopes, such as GTFs and glucan-binding proteins (GBPs), inversely correlate with colonization of *S. mutans* and caries prevalence (Taubman and Smith, 1993; Nogueira et al., 2005, 2012). Research investigating the feasibility of active or passive immunization against dental caries has been sporadic. Early investigations on the topic were excellently reviewed (Taubman and Nash, 2006). More recent studies have explored vaccination using a recombinant P1 adhesin antigen (Batista et al., 2017), a DNA-based vaccine against glucosyltransferases and surface proteins (Jiang et al., 2017), and a glycoconjugate vaccine based on rhamnan surface polysaccharides (St Michael et al., 2018). Unfortunately, past, present, and likely future, translational endeavors to move anti-caries vaccine research into clinical trials face significant regulatory and investment headwinds due to the fact that it is a non-life-threatening disease. There are currently no licensed vaccines to prevent dental caries.

Probiotics

Aside from providing "food" for the oral microbiome to modulate ecology, ecology can also be directly altered by either selectively adding or removing particular species from the oral community. Attempted probiotic strategies to prevent caries either have sought to add health-associated taxa to bolster the capacity of the microbiome to resist dysbiosis, or to replace cariogenic strains with genetically modified mutants which are competitive, yet less pathogenic. A number of studies have explored the use of *Lactobacillus* and *Bifidobacterium,* traditionally the genera used in probiotic formulations for digestive health, in the prevention of dental caries. Despite some encouraging results (Lin et al., 2018), there is widespread skepticism concerning the use of these genera as anti-caries probiotics. Both *Lactobacillus* and *Bifidobacterium*, are acidogenic and aciduric, meaning that they may actually contribute directly to caries formation under the proper conditions, a fear supported by several studies (reviewed in Philip et al., 2018). In addition, most lactobacilli and bifidobacteria are residents of the gut, meaning they are not well-adapted for long-term persistence in the human mouth. They lack capabilities to bind to the salivary pellicle or even nascent dental plaques.

Species with a higher likelihood of outcompeting *S. mutans* are found already residing in the healthy oral cavity. Studies in other environments have illustrated that the best probiotics for preventing the growth of pathogens both occupy the same ecological niche as the pathogen, and produce compounds that directly antagonize the pathogen (Schlatter et al., 2017). *Streptococcus dentisani* and *Streptococcus* A12 are two recently described species which show particular promise as potential probiotics (Huang et al., 2016; Lopez-Lopez et al., 2017). Both species are active colonizers of the tooth surface, increase the pH of dental plaque through the arginolytic pathway, and inhibit the growth of mutans streptococci. In addition, *Streptococcus* A12 produces a challisin-like protease that disrupts pheromone signaling by *S. mutans,* inhibiting production of the bacteriocins which *S. mutans* utilizes to poison its competitors (Huang et al., 2016). Meanwhile, *S. dentisani* utilizes its own arsenal of bacteriocins to kill multiple cariogenic species (Lopez-Lopez et al., 2017). *Streptococcus salivarius* has also been examined in a probiotic context (Kurasz et al., 1986; Di Pierro et al., 2015). However, similar to lactobacilli and bifidobacteria, broad skepticism remains over the use of *S. salivarius* strains as dental plaque probiotics. *S. salivarius* is typically an inhabitant of the soft surfaces of the mouth and is thought to have limited ability to colonize the tooth surface and directly compete with *S. mutans in situ* (Philip et al., 2018).

The other major strategy used in probiotic approaches to prevent caries is displacement of native *S. mutans* strains with *S. mutans* strains engineered to have low pathogenicity. Two examples of this technique have been reported, utilizing *S. mutans* mutants deficient in intracellular polysaccharide metabolism (Tanzer et al., 1982) or lactic acid production

(Hillman et al., 2007). Despite preliminary results supporting the potential of these strains as anti-caries probiotics, no further research or studies in humans have been reported. Overall, although newer candidates, such as *S. dentisani* and *Streptococcus* A12, provide encouragement, no formulations of probiotics have been tested in rigorous clinical trials and successfully received endorsement for the prevention of dental caries from a regulatory agency or professional organization (Gruner et al., 2016; Burne, 2018).

Recent studies have explored the capacity of the biosynthetic gene clusters (BGCs) encoded by the oral microbiome to produce compounds which modulate oral ecology (Donia et al., 2014; Aleti et al., 2018). Specific BGCs appear to be associated with health or disease states. Comparative statistical modeling illustrated that certain BGCs were inversely correlated with the abundance of cariogenic species, such as *S. mutans* and *Lactobacillus* spp. This indicates that the molecular products of these BGCs may be priority therapeutic leads and that the strains harboring these BGCs are prime probiotic candidates, inviting further investigation.

Antimicrobial Peptides/STAMPS

As opposed to adding species to the community to prevent or alleviate dysbiosis, various approaches to remove problematic species, such as *S. mutans*, have been explored. Such targeted methods would presumably restore a healthy oral microbiome. Recent reports showed that the antimicrobial peptides ZXR-2 and CLP-4 efficiently killed *S. mutans* biofilms, however, specificity for *S. mutans* was not shown (Chen et al., 2017; Min et al., 2017). Specifically targeted antimicrobial peptides (STAMPS) are synthetic peptides consisting of a targeting domain to invoke specificity and a killing domain to exert antimicrobial action against the targeted species (Eckert et al., 2006b). C16G2 is a STAMP designed to specifically kill *S. mutans*, and several studies have shown that C16G2 is capable of targeted killing of *S. mutans* while leaving commensal streptococci unharmed. Furthermore, C16G2 was able to remodel the composition of a complex bacterial community, eliminating *S. mutans* and allowing for enrichment of organisms associated with dental health (Eckert et al., 2006a; Guo et al., 2015). C16G2 has proceeded to clinical trials in a number of formulations, the results of which will be of significant interest.

Small Molecules

Several small molecules have been proposed as agents to prevent caries through disruption of *S. mutans* biofilms. The small molecule 3F1 selectively dispersed *S. mutans* biofilms and served to modestly reduce caries in rodent model, however, no changes in the oral microbiomes were reported aside from a moderate reduction in *S. mutans* as measured by CFUs (Garcia et al., 2017). A 2016 study identified a quinoxaline derivative capable of inhibiting the GtfC enzyme of *S. mutans*. The compound indeed successfully reduced the ability of *S. mutans* to form biofilms and reduced caries in a rat model (Ren et al., 2016). Although both of these approaches dispersed *S. mutans* biofilms, there was minimal effect on overall dental plaque ecology. It is likely that this will allow rapid reformation of the problematic community and require perpetual application of these therapeutics. A recent study utilized a drug repositioning approach to identify 126 compounds with activity against *S. mutans* (Saputo et al., 2018). How many of these leads are specific to *S. mutans* remains yet to be determined.

Phage

Conceptually, bacteriophage is a very attractive approach to combat cariogenic pathogens, and one that has received relatively little attention. Although the few phages known to infect *S. mutans* were lytic, and completely eliminated viable counts from single-species biofilms, the phage demonstrated a highly stringent host specificity, which was considered a significant disadvantage, particularly in light of the high intra-species diversity exhibited by *S. mutans* (reviewed in Szafranski et al., 2017). No testing in multi-species communities or further studies have been reported to date.

CONCLUSION AND PERSPECTIVES

Because of its status as a key pathogenic species, much of the development of novel caries therapeutics has focused on *S. mutans,* specifically. *S. mutans* is certainly a justifiable target, associated with caries in the majority of cases and unparalleled in its ability to form insoluble glucans from sucrose. However, it is not the singular cause of the disease, and caries does occasionally occur without detectable *S. mutans* levels. Future research efforts would benefit from embracing this perspective and tempering expectations for approaches that fail to do so. It is clear that a dramatic reduction in the prevalence of dental caries through current modalities (such as fluoride and dietary modification) alone is unlikely to be realized. Fortunately, evolution has shaped territorial commensal taxa which antagonize cariogenic species. Exploitation of this relationship, whether by directly supporting the dominance of commensal taxa, or via targeted killing of their pathogenic competitors, is a promising course of therapeutic development. Although several of these approaches have produced encouraging results, properly controlled rigorous human studies are needed, the cost of which is likely to be a significant deterrent. Nevertheless, there is room for optimism, as it appears evolution may have already provided the best tools in the form of our commensal defenders and their natural products.

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REFERENCES

Abranches, J., Zeng, L., Kajfasz, J. K., Palmer, S. R., Chakraborty, B., Wen, Z. T., et al. (2018). Biology of oral Streptococci. *Microbiol. Spectr.* 6. doi: 10.1128/microbiolspec.GPP3-0042-2018

Adler, C. J., Dobney, K., Weyrich, L. S., Kaidonis, J., Walker, A. W., Haak, W., et al. (2013). Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions. *Nat. Genet.* 45, 450–455. doi: 10.1038/ng.2536

Agnello, M., Cen, L., Tran, N. C., Shi, W., Mclean, J. S., and He, X. (2017). Arginine improves pH homeostasis via metabolism and microbiome modulation. *J. Dent. Res.* 96, 924–930. doi: 10.1177/0022034517707512

Aleti, G., Baker, J. L., Tang, X., Alvarez, R., Dinis, M., Tran, N. C., et al. (2018). Identification of the bacterial biosynthetic gene clusters of the oral microbiome illuminates the unexplored social language of bacteria during health and disease. *bioRxiv* [Preprint]. doi: 10.1101/431510

Astvaldsdottir, A., Naimi-Akbar, A., Davidson, T., Brolund, A., Lintamo, L., Attergren Granath, A., et al. (2016). Arginine and caries prevention: a systematic review. *Caries Res.* 50, 383–393. doi: 10.1159/000446249

Batista, M. T., Ferreira, E. L., Pereira, G. S., Stafford, P., Maeda, D., Rodrigues, J. F., et al. (2017). LT adjuvant modulates epitope specificity and improves the efficacy of murine antibodies elicited by sublingual vaccination with the N-terminal domain of Streptococcus mutans P1. *Vaccine* 35, 7273–7282. doi: 10.1016/j.vaccine.2017.11.007

Bowen, W. H. (2016). Dental caries - not just holes in teeth! A perspective. *Mol. Oral Microbiol.* 31, 228–233. doi: 10.1111/omi.12132

Bowen, W. H., Burne, R. A., Wu, H., and Koo, H. (2018). Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol.* 26, 229–242. doi: 10.1016/j.tim.2017.09.008

Burne, R. A. (2018). Getting to know "The Known Unknowns": heterogeneity in the oral microbiome. *Adv. Dent. Res.* 29, 66–70. doi: 10.1177/0022034517735293

Chakraborty, B., and Burne, R. A. (2017). Effects of arginine on growth, virulence gene expression, and stress tolerance by *Streptococcus mutans*. *Appl. Environ. Microbiol.* doi: 10.1128/AEM.00496-17 [Epub ahead of print].

Chen, L., Jia, L., Zhang, Q., Zhou, X., Liu, Z., Li, B., et al. (2017). A novel antimicrobial peptide against dental-caries-associated bacteria. *Anaerobe* 47, 165–172. doi: 10.1016/j.anaerobe.2017.05.016

de Cock, P. (2018). Erythritol functional roles in oral-systemic health. *Adv. Dent. Res.* 29, 104–109. doi: 10.1177/0022034517736499

Di Pierro, F., Zanvit, A., Nobili, P., Risso, P., and Fornaini, C. (2015). Cariogram outcome after 90 days of oral treatment with *Streptococcus salivarius* M18 in children at high risk for dental caries: results of a randomized, controlled study. *Clin. Cosmet. Investig. Dent.* 7, 107–113. doi: 10.2147/CCIDE.S93066

Donia, M. S., Cimermancic, P., Schulze, C. J., Wieland Brown, L. C., Martin, J., Mitreva, M., et al. (2014). A systematic analysis of biosynthetic gene clusters in the human microbiome reveals a common family of antibiotics. *Cell* 158, 1402–1414. doi: 10.1016/j.cell.2014.08.032

Eckert, R., He, J., Yarbrough, D. K., Qi, F., Anderson, M. H., and Shi, W. (2006a). Targeted killing of Streptococcus mutans by a pheromone-guided "smart" antimicrobial peptide. *Antimicrob. Agents Chemother.* 50, 3651–3657.

Eckert, R., Qi, F., Yarbrough, D. K., He, J., Anderson, M. H., and Shi, W. (2006b). Adding selectivity to antimicrobial peptides: rational design of a multidomain peptide against *Pseudomonas* spp. *Antimicrob. Agents Chemother.* 50, 1480–1488.

Foster, K. R., Schluter, J., Coyte, K. Z., and Rakoff-Nahoum, S. (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nature* 548, 43–51. doi: 10.1038/nature23292

Garcia, S. S., Blackledge, M. S., Michalek, S., Su, L., Ptacek, T., Eipers, P., et al. (2017). Targeting of *Streptococcus mutans* biofilms by a novel small molecule prevents dental caries and preserves the oral microbiome. *J. Dent. Res.* 96, 807–814. doi: 10.1177/0022034517698096

Gilbert, J. A., Blaser, M. J., Caporaso, J. G., Jansson, J. K., Lynch, S. V., and Knight, R. (2018). Current understanding of the human microbiome. *Nat. Med.* 24, 392–400. doi: 10.1038/nm.4517

Gruner, D., Paris, S., and Schwendicke, F. (2016). Probiotics for managing caries and periodontitis: systematic review and meta-analysis. *J. Dent.* 48, 16–25. doi: 10.1016/j.jdent.2016.03.002

Guo, L., Mclean, J. S., Yang, Y., Eckert, R., Kaplan, C. W., Kyme, P., et al. (2015). Precision-guided antimicrobial peptide as a targeted modulator of human microbial ecology. *Proc. Natl. Acad. Sci. U.S.A.* 112, 7569–7574. doi: 10.1073/pnas.1506207112

He, X., Mclean, J. S., Guo, L., Lux, R., and Shi, W. (2014). The social structure of microbial community involved in colonization resistance. *ISME J.* 8, 564–574. doi: 10.1038/ismej.2013.172

Hillman, J. D., Mo, J., Mcdonell, E., Cvitkovitch, D., and Hillman, C. H. (2007). Modification of an effector strain for replacement therapy of dental caries to enable clinical safety trials. *J. Appl. Microbiol.* 102, 1209–1219. doi: 10.1111/j.1365-2672.2007.03316.x

Huang, X., Browngardt, C. M., Jiang, M., Ahn, S. J., Burne, R. A., and Nascimento, M. M. (2018). Diversity in antagonistic interactions between commensal oral Streptococci and *Streptococcus mutans*. *Caries Res.* 52, 88–101. doi: 10.1159/000479091

Huang, X., Palmer, S. R., Ahn, S. J., Richards, V. P., Williams, M. L., Nascimento, M. M., et al. (2016). A highly arginolytic *Streptococcus* species that potently antagonizes *Streptococcus mutans*. *Appl. Environ. Microbiol.* 82, 2187–2201. doi: 10.1128/AEM.03887-15

Huang, X., Zhang, K., Deng, M., Exterkate, R. A. M., Liu, C., Zhou, X., et al. (2017). Effect of arginine on the growth and biofilm formation of oral bacteria. *Arch. Oral Biol.* 82, 256–262. doi: 10.1016/j.archoralbio.2017.06.026

Jiang, H., Hu, Y., Yang, M., Liu, H., and Jiang, G. (2017). Enhanced immune response to a dual-promoter anti-caries DNA vaccine orally delivered by attenuated *Salmonella* typhimurium. *Immunobiology* 222, 730–737. doi: 10.1016/j.imbio.2017.01.007

Koussoulakou, D. S., Margaritis, L. H., and Koussoulakos, S. L. (2009). A curriculum vitae of teeth: evolution, generation, regeneration. *Int. J. Biol. Sci.* 5, 226–243. doi: 10.7150/ijbs.5.226

Kurasz, A. B., Tanzer, J. M., Bazer, L., and Savoldi, E. (1986). In vitro studies of growth and competition between *S. salivarius* TOVE-R and mutans streptococci. *J. Dent. Res.* 65, 1149–1153. doi: 10.1177/00220345860650090701

Lamont, R. J., Koo, H., and Hajishengallis, G. (2018). The oral microbiota: dynamic communities and host interactions. *Nat. Rev. Microbiol.* 16, 745–759. doi: 10.1038/s41579-018-0089-x

Lin, T. H., Lin, C. H., and Pan, T. M. (2018). The implication of probiotics in the prevention of dental caries. *Appl. Microbiol. Biotechnol.* 102, 577–586. doi: 10.1007/s00253-017-8664-z

Lopez-Lopez, A., Camelo-Castillo, A., Ferrer, M. D., Simon-Soro, A., and Mira, A. (2017). Health-associated niche inhabitants as oral probiotics: the case of *Streptococcus dentisani*. *Front. Microbiol.* 8:379. doi: 10.3389/fmicb.2017.00379

Makinen, K. K. (2010). Sugar alcohols, caries incidence, and remineralization of caries lesions: a literature review. *Int. J. Dent.* 2010:981072. doi: 10.1155/2010/981072

Marquis, R. E. (1995). Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *J. Ind. Microbiol.* 15, 198–207. doi: 10.1007/BF01569826

Marsh, P. D., Do, T., Beighton, D., and Devine, D. A. (2016). Influence of saliva on the oral microbiota. *Periodontology* 2000, 80–92. doi: 10.1111/prd.12098

Min, K. R., Galvis, A., Williams, B., Rayala, R., Cudic, P., and Ajdic, D. (2017). Antibacterial and antibiofilm activities of a novel synthetic cyclic lipopeptide against cariogenic *Streptococcus mutans* UA159. *Antimicrob. Agents Chemother.* 61:e00776. doi: 10.1128/AAC.00776-17

Moeller, A. H., Caro-Quintero, A., Mjungu, D., Georgiev, A. V., Lonsdorf, E. V., Muller, M. N., et al. (2016). Cospeciation of gut microbiota with hominids. *Science* 353, 380–382. doi: 10.1126/science.aaf3951

Moeller, A. H., Li, Y., Mpoudi Ngole, E., Ahuka-Mundeke, S., Lonsdorf, E. V., Pusey, A. E., et al. (2014). Rapid changes in the gut microbiome during human evolution. *Proc. Natl. Acad. Sci. U.S.A.* 111, 16431–16435. doi: 10.1073/pnas.1419136111

Nascimento, M. M. (2018). Potential uses of arginine in dentistry. *Adv. Dent. Res.* 29, 98–103. doi: 10.1177/0022034517735294

Nogueira, R. D., Alves, A. C., Napimoga, M. H., Smith, D. J., and Mattos-Graner, R. O. (2005). Characterization of salivary immunoglobulin A responses in children heavily exposed to the oral bacterium *Streptococcus mutans*: influence of specific antigen recognition in infection. *Infect. Immun.* 73, 5675–5684. doi: 10.1128/IAI.73.9.5675-5684.2005

Nogueira, R. D., Sesso, M. L., Borges, M. C., Mattos-Graner, R. O., Smith, D. J., and Ferriani, V. P. (2012). Salivary IgA antibody responses to *Streptococcus mitis* and *Streptococcus mutans* in preterm and fullterm newborn children. *Arch. Oral Biol.* 57, 647–653. doi: 10.1016/j.archoralbio.2011.11.011

Philip, N., Suneja, B., and Walsh, L. J. (2018). Ecological approaches to dental caries prevention: paradigm shift or shibboleth? *Caries Res.* 52, 153–165. doi: 10.1159/000484985

Pitts, N. B., Zero, D. T., Marsh, P. D., Ekstrand, K., Weintraub, J. A., Ramos-Gomez, F., et al. (2017). Dental caries. *Nat. Rev. Dis. Primers* 3:17030. doi: 10.1038/nrdp.2017.30

Redanz, S., Cheng, X., Giacaman, R. A., Pfeifer, C. S., Merritt, J., and Kreth, J. (2018). Live and let die: hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome. *Mol. Oral Microbiol.* 33, 337–352. doi: 10.1111/omi.12231

Ren, Z., Cui, T., Zeng, J., Chen, L., Zhang, W., Xu, X., et al. (2016). Molecule targeting glucosyltransferase inhibits *Streptococcus mutans* biofilm formation and virulence. *Antimicrob. Agents Chemother.* 60, 126–135. doi: 10.1128/AAC.00919-15

Richards, D. (2017). Little evidence available for arginine and caries prevention. *Evid. Based Dent.* 18:71. doi: 10.1038/sj.ebd.6401251

Rother, K. I., Conway, E. M., and Sylvetsky, A. C. (2018). How non-nutritive sweeteners influence hormones and health. *Trends Endocrinol. Metab.* 29, 455–467. doi: 10.1016/j.tem.2018.04.010

Saputo, S., Faustoferri, R. C., and Quivey, R. G. Jr. (2018). A drug repositioning approach reveals that *Streptococcus mutans* is susceptible to a diverse range of established antimicrobials and nonantibiotics. *Antimicrob. Agents Chemother.* 62:e1674-17. doi: 10.1128/AAC.01674-17

Schlatter, D., Kinkel, L., Thomashow, L., Weller, D., and Paulitz, T. (2017). Disease suppressive soils: new insights from the soil microbiome. *Phytopathology* 107, 1284–1297. doi: 10.1094/PHYTO-03-17-0111-RVW

Slomka, V., Hernandez-Sanabria, E., Herrero, E. R., Zaidel, L., Bernaerts, K., Boon, N., et al. (2017). Nutritional stimulation of commensal oral bacteria suppresses pathogens: the prebiotic concept. *J. Clin. Periodontol.* 44, 344–352. doi: 10.1111/jcpe.12700

Slomka, V., Herrero, E. R., Boon, N., Bernaerts, K., Trivedi, H. M., Daep, C., et al. (2018). Oral prebiotics and the influence of environmental conditions in vitro. *J. Periodontol.* 89, 708–717. doi: 10.1002/JPER.17-0437

St Michael, F., Yang, Q., Cairns, C., Vinogradov, E., Fleming, P., Hayes, A. C., et al. (2018). Investigating the candidacy of the serotype specific rhamnan polysaccharide based glycoconjugates to prevent disease caused by the dental pathogen Streptococcus mutans. *Glycoconj. J.* 35, 53–64. doi: 10.1007/s10719-017-9798-z

Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C. A., Maza, O., et al. (2014). Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature* 514, 181–186. doi: 10.1038/nature13793

Szafranski, S. P., Winkel, A., and Stiesch, M. (2017). The use of bacteriophages to biocontrol oral biofilms. *J. Biotechnol.* 250, 29–44. doi: 10.1016/j.jbiotec.2017.01.002

Tanzer, J. M., Fisher, J., and Freedman, M. L. (1982). Preemption of Streptococcus mutans 10449S colonization by its mutant 805. *Infect. Immun.* 35, 138–142.

Taubman, M. A., and Nash, D. A. (2006). The scientific and public-health imperative for a vaccine against dental caries. *Nat. Rev. Immunol.* 6, 555–563. doi: 10.1038/nri1857

Taubman, M. A., and Smith, D. J. (1993). Significance of salivary antibody in dental disease. *Ann. N. Y. Acad. Sci.* 694, 202–215. doi: 10.1111/j.1749-6632.1993.tb18354.x

Zheng, X., He, J., Wang, L., Zhou, S., Peng, X., Huang, S., et al. (2017). Ecological effect of arginine on oral microbiota. *Sci. Rep.* 7:7206. doi: 10.1038/s41598-017-07042-w

Zmora, N., Suez, J., and Elinav, E. (2018). You are what you eat: diet, health and the gut microbiota. *Nat. Rev. Gastroenterol. Hepatol.* 16, 35–56. doi: 10.1038/s41575-018-0061-2

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A Genome-Wide Screen Identifies Factors Involved in *S. aureus*-Induced Human Neutrophil Cell Death and Pathogenesis

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Staphylococcus aureus is a commensal organism in approximately 30% of the human population and colonization is a significant risk factor for invasive infection. As a result of this, there is a great need to better understand how *S. aureus* overcomes human immunity. Neutrophils are essential during the innate immune response to *S. aureus*, yet this microorganism uses multiple evasion strategies to avoid killing by these immune cells, perhaps the most catastrophic of which is the rapid induction of neutrophil cell death. The aim of this study was to better understand the mechanisms underpinning *S. aureus*-induced neutrophil lysis, and how this contributes to pathogenesis in a whole organism model of infection. To do this we screened the genome-wide Nebraska Transposon Mutant Library (NTML) in the community acquired methicillin resistant *S. aureus* strain, USA300, for decreased ability to induce neutrophil cell lysis. Out of 1,920 *S. aureus* mutants, a number of known regulators of cell lysis (including the master regulators accessory gene regulator A, *agrA* and Staphylococcus exoprotein expression protein S, *saeS*) were identified in this blinded screen, providing validity to the experimental system. Three gene mutations not previously associated with cell death: *purB, lspA,* and *clpP* were found to be significantly attenuated in their ability to induce neutrophil lysis. These phenotypes were verified by genetic transductants and complemented strains. *purB* and *clpP* were subsequently found to be necessary for bacterial replication and pathogenesis in a zebrafish embryo infection model. The virulence of the *clpP* mutant was restored in a neutrophil-depleted zebrafish model, suggesting the importance of ClpP in mechanisms underpinning neutrophil immunity to *S. aureus*. In conclusion, our work identifies genetic components underpinning *S. aureus* pathogenesis, and may provide insight into how this commensal organism breaches innate immune barriers during infection.

Keywords: *Staphylococcus aureus*, neutrophils, cell death, methicillin resistant *S. aureus* (MRSA), zebrafish

INTRODUCTION

Staphylococcus aureus has long been recognized as a highly adaptive and dangerous human pathogen, yet this microorganism colonizes the nose of ∼30% of the human population without any ill effects (1). Highly virulent methicillin resistant *S. aureus* (MRSA) strains can also be carried asymptomatically by healthy individuals (2). Interventions such as hospitalization or episodes of immunosuppression can result in invasive *S. aureus* infection, which can manifest in multiple forms from superficial skin abscesses to necrotising pneumonia or life-threatening bacteraemia. Colonization is a significant risk factor for pathogenic infection (3, 4). Considering the speed and efficiency with which *S. aureus* acquires resistance to antibiotics, the shift from silent passenger to life-threatening pathogen is all the more concerning.

Neutrophils are a critical defense in controlling colonization and active infection with *S. aureus* (5, 6). Yet this microorganism uses multiple evasion strategies to avoid killing by these innate immune cells [reviewed in (7)]. Perhaps the most catastrophic of these strategies is the induction of neutrophil cell death. This not only eradicates a critical element of the early immune response, but also results in inflammation and tissue damage which intensifies disease. *S. aureus* has been shown to upregulate cell death pathway genes and promote neutrophil apoptosis as well as programmed necrosis (8–10). *S. aureus* also produces a number of cytolytic toxins including Panton-Valentine leukocidin (PVL), phenol-soluble modulins (PSMs), α-hemolysin, and the leukotoxin LukAB (11). Cytolytic toxin production is intimately linked with pathogenesis, including in community-acquired MRSA (CA-MRSA) infection (12, 13). Understanding host-pathogen interactions in the context of CA-MRSA is imperative since these strains spread rapidly between individuals and are able to cause disease in healthy people. The increased virulence of CA-MRSA has been attributed in part to its resistance to neutrophil-mediated killing, including via the induction of neutrophil lysis (14).

Previous studies have taken candidate approaches to the study of components involved in neutrophil lysis, which may have resulted in an incomplete picture of the genetics underpinning cell death. Here we have performed an unbiased genome-wide study to identify additional routes to neutrophil cell death. Using the NTML we have screened all non-essential *S. aureus* genes to identify genetic components that are involved in inducing neutrophil cell lysis. The NTML was created in the CA-MRSA strain USA300, and has been successfully applied in diverse phenotypic screens to identify genes involved in polymicrobial interactions, antimicrobial resistance, and pathogenicity (15–18). A high-throughput, flow cytometric human neutrophil cell death assay has revealed three genes (*purB, lspA,* and *clpP*) that are required for cell lysis. The role of these factors in disease has been determined using a zebrafish embryo model of infection. Our study has provided further evidence for the complex interaction between pathogen and host that determines disease outcome.

MATERIALS AND METHODS

Bacterial Information and Culture

All *S. aureus* strains were grown in brain heart infusion (BHI) broth at 37°C with aeration at 250 rpm unless otherwise stated. USA300 *S. aureus* strain JE2 was used as a positive control. NTML strains were cultured in liquid BHI in 96-well plates. Transductant and complement strains were grown on BHI agar plates followed by overnight inoculation in 250 ml BHI broth. Where required, selection for antibiotic resistance markers was carried out using the following concentrations: ampicillin (Amp, 100 μg/ml); chloramphenicol (Cm, 30 μg/ml); erythromycin (Ery, 2.5 μg/ml); lincomycin (Lin, 12.5 μg/ml); kanamycin (Kan, 50 μg/ml); tetracycline (Tet, 5 μg/ml). The NTML was acquired from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) strain repository. The NTML was constructed based on USA300 FPR3757 chromosomal genome sequence mapped transposition of *bursa aurealis* from the delivery plasmid pBursa into the non-essential protein coding sequences of the wild-type JE2 strain (15). Colony forming unit (CFU) count results were obtained using the Miles and Misra method (19). Bacterial density was quantified by spectrophotometric reading at 600 nm (OD600) using a Jenway 6100 spectrophotometer. NTML strain OD600 was measured using a Perkin VICTOR x3 2030 plate reader, with orbital shaking for 0.2 s before reading.

Genetic Transduction and Complementation

Staphylococcus aureus transduction was performed with *Φ*11 as described previously (20). Transformation of *S. aureus* RN4220 and *E. coli* was carried out by electroporation based on previous methods (21, 22). For genetic complementation, the *lspA* operon was amplified from JE2 genomic DNA with Phusion polymerase (NEB), using primers containing appropriate restriction sites (forward, GAATTCGTACGG GCCCGGGCTTACTTAACCTCCTTCTCC; reverse, CCA TGTAGGCCAAGTCAAATGAATAATTAAGTTCATATT TAATGTCAAAA). The pKASBAR-Kan^R, a plasmid carrying an *attP* encoding site, was inserted with the PCR product to integrate the insert into the *S. aureus* genome via the *attB* site, in the presence of an integrase (23). The plasmid providing integrase, pYL112Δ19, was propagated into *S. aureus* RN4220 strain. From RN4220, the insert was transduced into *lspA* and control strains. The *clpP* mutant and *Φ*85 complemented *clpP*$^+$ strain in 8325 background were kindly provided by Knut Ohlsen (24).

Chemical Complementation

Chemical complementation of *purB* was conducted with adenine (20 mg/ml in 0.5 M HCl) and inosine (50 mg/ml in dH_2O). A final concentration of 20 μg/ml was achieved in BHI agar or RPMI (+10% FCS) media accordingly.

Neutrophil Isolation and Culture

Human neutrophils were isolated by dextran sedimentation followed by plasma-Percoll gradient centrifugation from whole

blood of healthy volunteers as previously described (25, 26). Written informed consent and ethical approval from the South Sheffield Research Ethics Committee (study number STH13927) were obtained. The purity of isolated neutrophils was determined from Diff-Quik (Sigma-Aldrich, St. Louis, MO) stained cytocentrifuge preparations by light microscopy. Neutrophils were suspended at 5×10^6/ml in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, United States) + 10% fetal calf serum (FCS, PromoCell, Heidelberg, Germany) and cultured in 96-well plates at 37°C, 5% CO_2. Phagocytosis assays were performed by incubating neutrophils (in RPMI + 10% FCS) with *S. aureus* strains at a multiplicity of infection (MOI) of 10 for 1 h after which they were cytocentrifuged (Cytospin, Shandon) onto microscope slides (27). Cells were stained with Quik-Diff dyes and *S. aureus* visualized within neutrophils by oil immersion light microscopy. The phagocytic index was calculated using the following formula: (total number of engulfed bacteria/total number of neutrophils) × (number of neutrophils containing bacteria/total number of neutrophils) × 100.

Intracellular Killing Assay

Neutrophils (4.5×10^5 cells/well) were infected with *S. aureus* at an MOI of 5 at 37°C, 5% CO_2 in RMPI + 10% FCS. The number of internalized viable *S. aureus* was determined after 30 min. Cells were centrifuged at 2000 RPM for 2 min, resuspended in 200 μl 1% saponin (in PBS), and incubated at RT for 10 min with constant vortexing to lyse neutrophils. The number of viable *S. aureus* in cell lysates was determined by the Miles and Misra method (19). In parallel wells, lysostaphin (20 μg/ml) was added for 30 min to kill extracellular *S. aureus* (28). Viable intracellular *S. aureus* was determined after a further 60 min (120 min in total) as above.

NTML Neutrophil Cell Death Assay Screen

NTML strains were grown overnight in 96-well plates containing BHI broth following which 10 μl was sub-cultured into 190 μl BHI for 3 h at 37°C. OD_{600} measurements varied from 0.3 to 0.7. Cultures were centrifuged at 5,000 g for 10 min (RT) and pellets were resuspended in 200 μl of RPMI (+10% FCS) for use in cell death assays. Five microliters of each strain were added to 2.5×10^5 neutrophils in individual wells of a 96-well plate to achieve an MOI of 10. The following conditions were also included for each experiment: media control (neutrophils in RPMI + 10% FCS) and JE2 (WT) challenge. Plates were incubated at 37°C, 5% CO_2 for 3 h. Following this, 100 μl cold PBS containing ToPro-3 (100 nM) was added to each well and samples were immediately subjected to flow cytometry using an Attune Autosampler (ThermoFisher, Waltham, MA). To avoid lengthy plate reading times a maximum of two 96-well plates plus controls were assessed in any one experiment. Plates were acquired at a speed of 500 μl/min and stopped once 70 μl of each sample has been aspirated. Cell counts were automatically generated. Flow cytometric data was analyzed by FlowJo software (TreeStar, Ashland OR). FSC/SSC dot plot profiles of media control conditions were used to set a gate around viable neutrophils and absolute cell numbers were automatically enumerated in this gated region for all plots. Events in the viable gate were exclusively ToPro-3 negative (data not shown), verifying cell viability in this population. The genetic identity of mutant strains was not identified until after analysis was completed to avoid bias.

Lactate Dehydrogenase Assay (LDH) Cytotoxicity Assay

Neutrophils (4.5×10^5 cells/well) were incubated in media (RPMI + 10% FCS) alone or challenged with *S. aureus* strains at MOI of 5 for 3 h after which the cultures were centrifuged at 300 g for 5 min. LDH activity was quantified in 50 μl supernatant using Pierce™ LDH Cytotoxicity Assay Kit, according to the manufacturer's instructions.

Zebrafish Models of Infection

Zebrafish embryos <5 days postfertilization (dpf) are not protected under the Animals (Scientific Procedures) Act 1986, but all zebrafish work was carried out according to the details set out in Project License PPL 40/3574. London Wild Type (LWT) strains were maintained in E3 medium at 28°C by following standard protocols and used for all experiments (29). Embryos were dechorionated 1 day prior to bacterial injection. Zebrafish embryos at 24 hpf were anesthetized in 0.02% (w/v) tricaine for 8 min prior to bacterial injection. The stock solution of 0.4% (w/v) 3-amino benzoic acid ester tricaine (Sigma-Aldrich) was made in 20 mM tris-HCl (pH 7). *S. aureus* was microinjected into the circulation valley located ventral to the yolk sac as described previously (29). The inoculum was determined retrospectively by Miles and Misra method. Zebrafish viability was determined by visual assessment based on cessation of heartbeat and circulatory flow, and assessed at time points of 20, 26, 44, 50, 68, 74, and 92 h post-injection. Bacterial growth was assessed from homogenized embryos plated onto BHI agar.

Morpholino Oligonucleotide Depletion of Neutrophils

Morpholino oligonucleotides to *pu.1* (sequence 5′-3′: GATATACTGATACTCCATTGGTGGT) were microinjected into the yolk sac of embryos within 30 min of fertilization (1–4 cell stage) (29, 30). Infection studies were carried out as described above. All survival studies and morpholino data were collected over 3 independent experiments, each comprising of 30 (morpholino and survival studies) or 120 (bacterial growth studies) embryos per group.

Statistical Analysis

The Kaplan-Meier method was used to generate survival curves.

The log-rank (Mantel Cox) test was performed to compare survival curves of different strains. All statistical analysis was completed by GraphPad Prism Version 6.0 by one-way ANOVA or as otherwise stated. Significant differences were indicated as: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, not-significant.

RESULTS

Establishing a High-Throughput *S. aureus*-Induced Neutrophil Lysis Screen

We took an unbiased approach to identifying novel genes related to the induction of neutrophil cell death by high-throughput and blinded screening of a genome-wide *S. aureus* mutant library. Neutrophils were infected with individual NTML strains in 96-well plates at MOI 10 for 3 h following which they were subjected to flow cytometry (**Figure 1**). A total of 1,920 strains were assayed across 20 plates (IDs: 1A-5D) over 11 non-consecutive days and 5 independent neutrophil donors (not pooled). Media treated and JE2 (WT) infected controls were included for each experiment. The absolute number of neutrophils was automatically calculated by an Attune flow cytometer for each strain. **Figure 2** shows the gating strategy [based on known neutrophil FSC/SSC profiles (31)] used to determine the viable neutrophil population. Events outside the rectangular gate include cell debris and contaminating mononuclear and red blood cells. Viable neutrophils in media control treated conditions typically comprised between 80 and 90% of the total events (**Figure 2A**), which equated to absolute numbers of 28,944 ± 2,212 (mean ± SEM) across all experiments. A profound loss of viable neutrophils was seen in JE2 infected samples where absolute numbers equated to 14,936 ± 2,171 (mean ± SEM) across all experiments. **Figure 2B** shows dot plots of 4 representative NTML strains, showing 2 strains (L-lactate permease; *lctP* and ABC transporter ATP-binding/permease protein; SAUSA300_2375) that induced neutrophil cell death at comparable levels to JE2, and 2 "hit" strains (maltose ABC transporter, permease protein, *ganP,* and ABC transporter, permease protein, *vraG*) that were attenuated in their ability to induce neutrophil cell death. The attenuated strains have a greater number of events in the viable gated region compared to JE2. For each 96-well plate, the mean and 2 standard deviations from the mean (+2 STDEV) viable neutrophil number for all NTML strains were calculated (**Figure 2C** presents representative data from a single plate, 1A) and a box is drawn around strains identified as hits. A number of strains resulted in increased neutrophil lysis, where viable neutrophils were almost undetectable. While this is of great interest, and may reflect a loss of a negative regulatory mechanism of cell death, we did not further pursue these mutants, since our objective was to identify genes that played a positive role in the induction of neutrophil lysis. As a measure of screen robustness and "hit" sensitivity we were able to identify genes required for neutrophil lysis including the master regulators *saeS* and *agrA* (32, 33), both of which resulted in increased numbers of viable neutrophils (**Figure 2D**).

The *S. aureus* Genes *purB, lspA,* and *clpP* Regulate Neutrophil Lysis

The objective of this screen was to identify genetic mutations that resulted in a defect in neutrophil cell death. Attenuated strains were identified as those with a viable neutrophil count > +2 STDEV of the plate mean and/or as those with a visibly different FSC/SSC profile (see **Figure 2D**). This latter strategy was adopted in order to maximize the number of hits identified in this initial screen round. Any false positives among these would be weeded out in the subsequent focused screen. 118 NTML strains were taken forward into a second round of focused screening, where each strain (incubated with neutrophils at MOI 10 for 3 h as per the primary screen) was tested in 3 independent experiments and presented as a box and whiskers plot (**Supplemental Figure 1**). As in the primary screen, attenuated hits were identified based

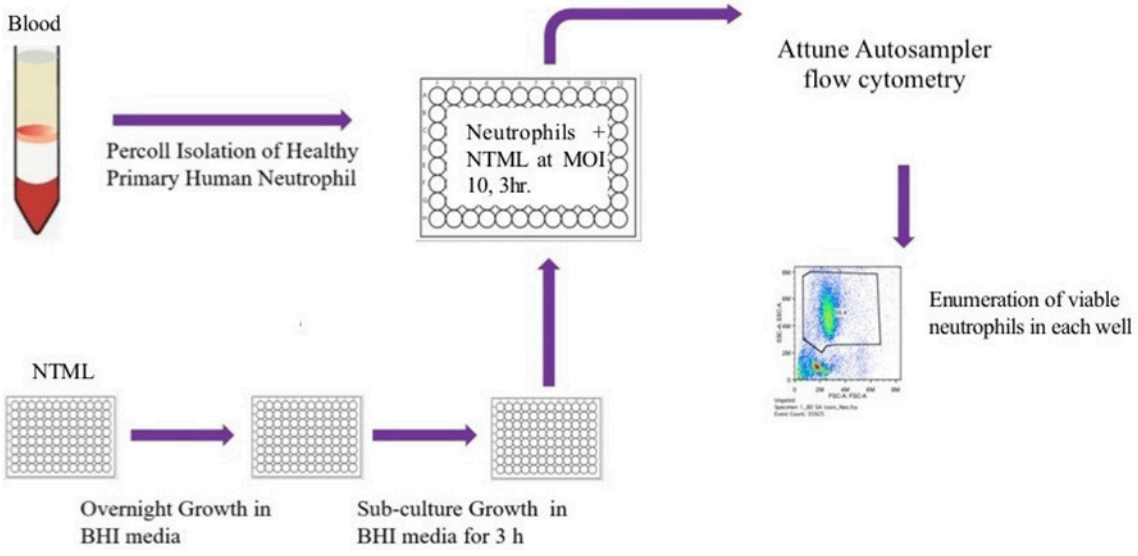

FIGURE 1 | Flow diagram illustrating the NTML neutrophil cell death screen. The NTML was grown overnight in 96-well plates and sub-cultured for a further 3 h on the day of the screen. Neutrophils were isolated from human blood and infected with library strains at MOI 10 for 3 h 96-well plates were subjected to an Attune Autosampler flow cytometer for absolute numbers of viable neutrophils.

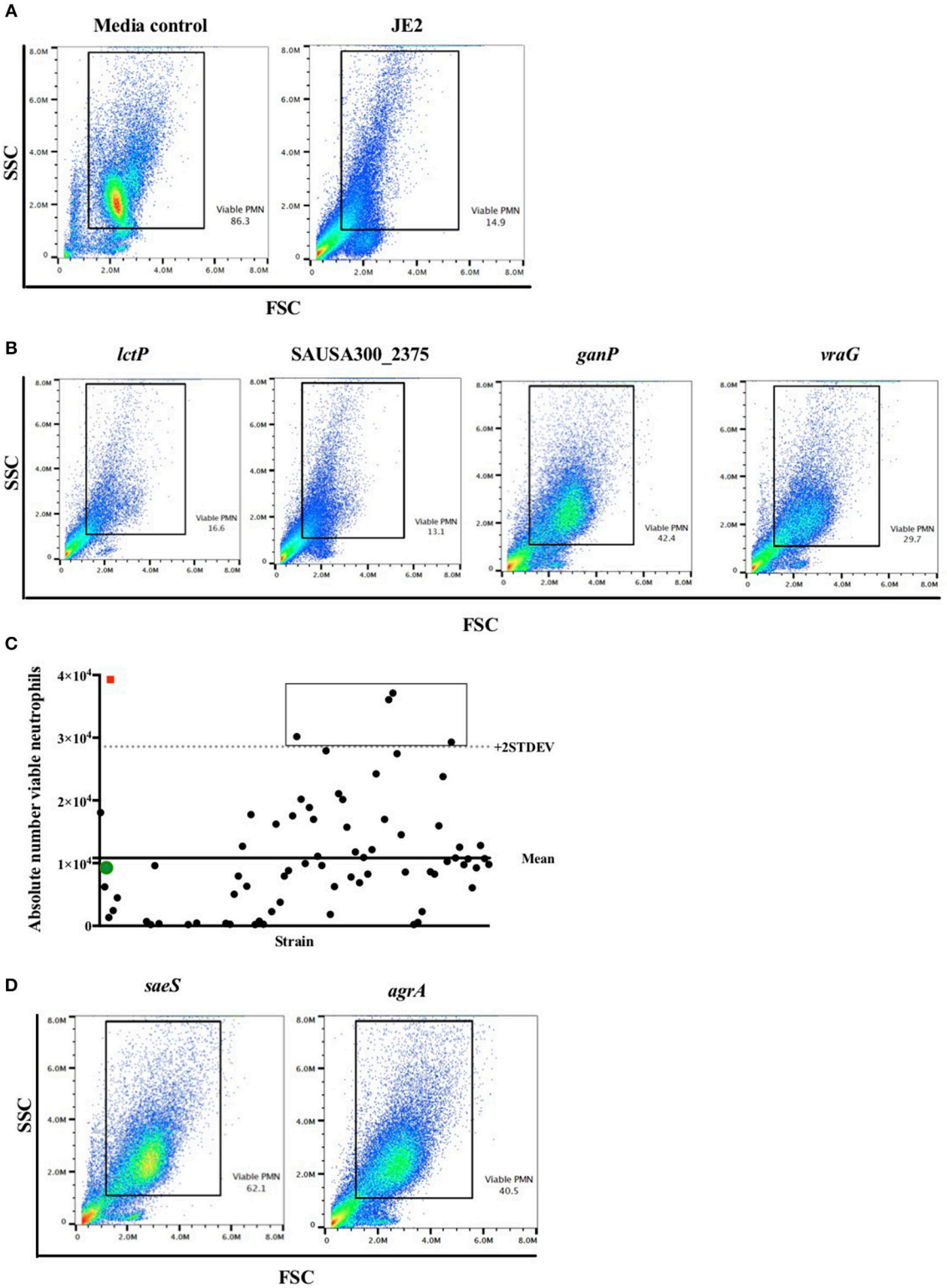

FIGURE 2 | Flow cytometry viable neutrophil gating strategy identifies known lytic factors as proof of concept. Neutrophils were infected with individual NTML strains in 96-well plates at MOI 10 for 3 h following which they were subjected to flow cytometry. **(A)** Illustrative flow cytometry FSC/SSC dot plots for media control and JE2 infected samples. The rectangular gated region defines viable cells based on typical FSC and SSC profiles. Viable PMN (neutrophils) values are expressed as a percentage of events within the gated region. **(B)** Representative FSC/SSC dot plots of four randomly selected NTML strains for illustration. **(C)** Absolute number of

(Continued)

FIGURE 2 | viable neutrophils is calculated from viable gated region for each strain and plotted by strain (solid circles represent individual strains). Panel shows data from a single plate for illustration. Mean of all NTML strains (solid line) and mean +2 STDEV (dotted line) is shown. Open icons denote media treated (red square) and JE2-infected (green circle) controls for comparison. The rectangular box is drawn around strains identified as "hits." **(D)** FSC/SSC dot plots of "hits" *saeS* and *agrA* mutant strains showing increased viable neutrophils compared to JE2 infected samples. Data is generated from a single representative experiment.

on viable cell number > +2 STDEV of the plate mean. Thirty four strains were found to be attenuated (**Supplemental Table 1**). Strains were ranked based on the level of attenuation, that is to say the more viable neutrophils remaining, the greater the attenuation, and listed in descending order. As expected, some of the most attenuated strains included mutations in genes known to be profound modulators of neutrophil death, including leukocidins, *saeS,* and *agrA*. Note inclusion of the genes: *ganP* and *vraG* as highlighted in **Figure 2B**. To determine whether the attenuated neutrophil lysis was due to a bacterial growth defect and therefore reduced MOI, the number of viable neutrophils was correlated with OD_{600} following 3 h growth in BHI. There was no correlation between number of viable neutrophils and OD_{600} (**Supplemental Figure 2**), suggesting the extent of the attenuation was not because of differences in MOI.

Of the attenuated strains identified in the second screen, 17 of the most attenuated mutants were further validated in transduction studies. To do this the transposon insert for each strain identified in the screen was transduced back into the parent strain (*S. aureus* JE2) and transductants were rescreened to establish that the mutant phenotype was specifically associated with each Tn insertion. Neutrophils were infected with transductants at an MOI 10 for 3 h and viable neutrophils enumerated by flow cytometry as above. Transductants (3 clones of each mutation) of 5 original mutants were attenuated (> +2STDEV of the WT strain, JE2) including the known cytolytics *lukAB* and *saeS*, as well as adenylosuccinate lyase (*purB*) and the ATP-dependent Clp protease proteolytic subunit (*ClpP*, **Figure 3A**). *ClpP* in addition to the lipopeptidase *lsp*A, which was moderately attenuated, were taken further into genetic complementation studies. Genetic complements of *lspA* and *clpP* (indicated by $^{+}$) were able to induce neutrophil cell lysis to levels comparable to JE2 (**Figure 3B**, $^{*}p < 0.05$ mutant vs. complement). The integration of empty pKB had no effect on neutrophil lysis. Representative flow cytometry plots showing FSC/SSC profiles and viable neutrophils are shown in **Supplemental Figures 3A–D**. To determine neutrophil lysis by an alternative method, we performed an LDH assay and show that *clpP* and *purB*, but not *lspA* resulted in significantly attenuated cytotoxicity (**Supplemental Figure 3E**). The lack of attenuation by *lspA* in this assay may reflect membrane damage leading to leakage of LDH, but without complete cell lysis (as indicated by flow cytometry plots that are consistent with intact cells, **Supplemental Figure 3B**). Since PurB is required for purine synthesis, the *purB* mutant was chemically complemented by the addition of adenine and inosine to BHI agar during overnight growth of *S. aureus* and/or during neutrophil infection. The presence of adenine and inosine to BHI had no effect on neutrophil viability. Addition of adenine and inosine to RPMI during the infection partially restored the ability of the *purB* mutant to induce neutrophil cell lysis, although this was not statistically significant (**Figure 3C**). This indicates that *S. aureus*-induced neutrophil cell death is dependent on purines and highlights the specific requirement of purines during the infection period. Phagocytosis assays revealed the *clpP* mutant was phagocytosed significantly less avidly than the *purB* and *lspA* mutants (**Figure 4A**), but all strains were killed equally well by human neutrophils (**Figure 4B**). This suggests that the attenuation in neutrophil cell death is not a result of altered killing of *S. aureus*.

PurB and ClpP Are Necessary for Bacterial Replication and Pathogenesis in a Zebrafish Embryo Infection Model

Since *clpP, lspA,* and *purB* mutants were defective in causing neutrophil lysis and therefore may not overcome neutrophil defenses during infection, we hypothesized these strains would have altered pathogenicity *in vivo*. To test this, mutants were studied in a zebrafish embryo infection model (29). Survival rate in PBS injected embryos was >90% (data not shown). As expected, infection with JE2 resulted in profound embryo death (**Figures 5A,B**, solid line). The *lspA* mutant (dotted line) caused significant mortality at rates comparable to JE2 (**Figure 5A**). In contrast, *purB* and *clpP* mutants (dashed lines) failed to kill embryos, with almost maximum survival at 92 h (**Figures 5A,B**). To determine whether a phagocyte response was critical for host immunity to strains, myeloid cells were depleted in zebrafish embryos with a morpholino to pu.1 (30, 34). Consistent with previous studies (34), depletion of neutrophils increased the speed at which embryos died following infection with JE2 with >95% embryos dead by 24 h (**Figure 5C**). Compared to JE2, all mutants delayed embryo death by 24 h but with the exception of *purB*, all went on to completely overcome the zebrafish by 48 h. The *clpP* mutant was able to mount an overwhelming infection in the absence of neutrophils but not in the presence of neutrophils which suggests neutrophils are key in the control of this strain.

To define whether increased zebrafish survival was associated with lack of bacterial replication, CFU counts from viable and dead embryos were determined. CFU counts of up to 10^7 were recovered from dead embryos infected with JE2, even at early timepoints, indicating rapid replication *in vivo* (**Figure 6A**). For mutants that were less efficient at killing zebrafish (*clpP* and *purB*), CFU counts in both dead and viable zebrafish were markedly lower at between 10^2 and 3×10^5 (**Figures 6B,C**). The results suggest that *clpP* and *purB* mutants had limited capacity to replicate within embryos, and were therefore unable

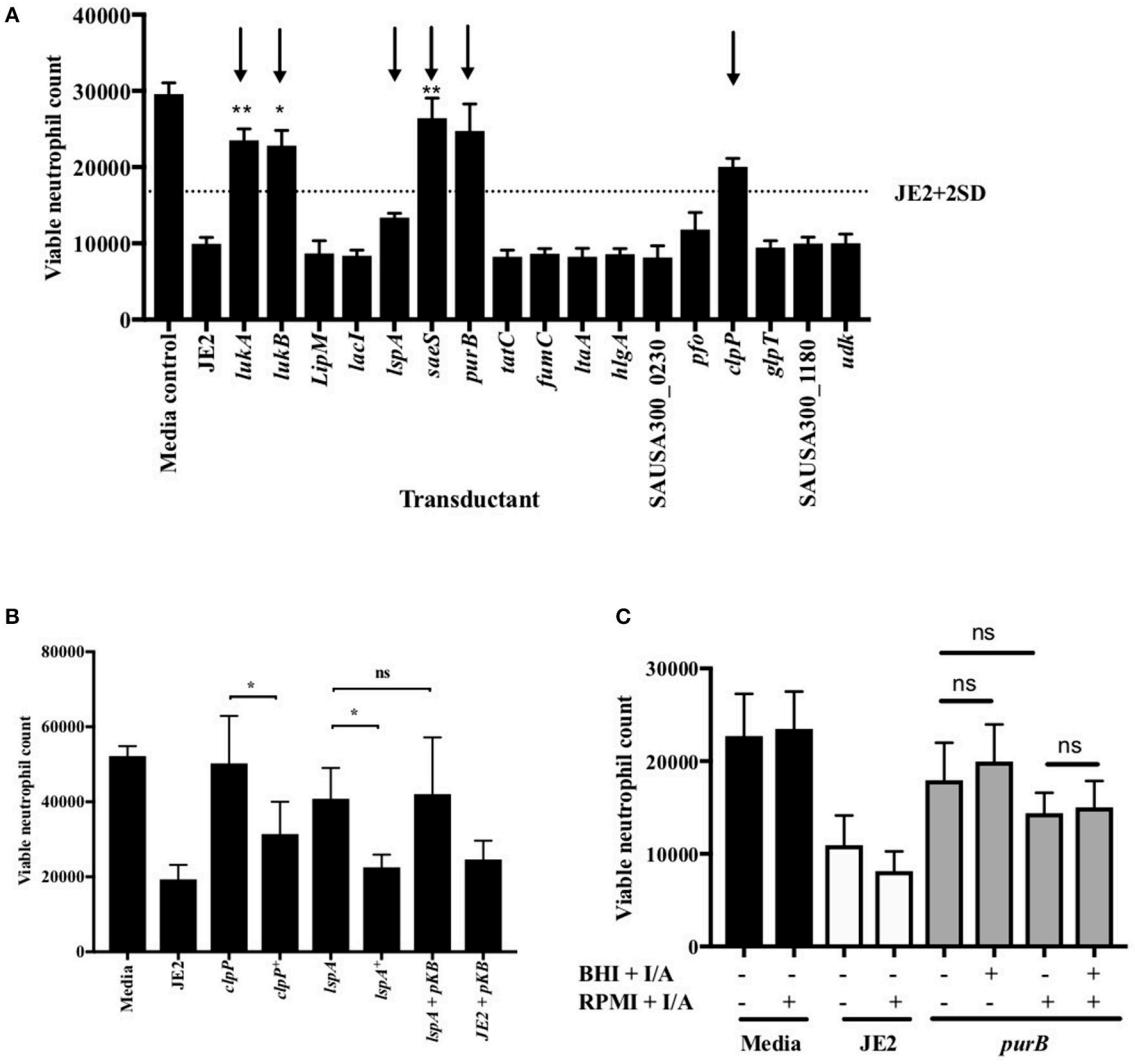

FIGURE 3 | Validation of attenuation by genetic transduction and complementation. **(A)** Neutrophils were co-incubated with media, JE2 or *S. aureus* transductants at MOI 10 for 3 h, followed by flow cytometry. Viable neutrophil counts were generated as previously described. JE2 + 2D is illustrated by dotted line and attenuated strains to be taken forward indicated by arrows ($n = 3$). **(B)** Neutrophils were co-incubated with media, JE2, transductants, or complements (indicated by $^{+}$) at MOI 10 for 3 h. JE2 and *lspA* transductant was also incubated in the presence empty pKasbar plasmid (pKB) ($n = 6$). **(C)** Chemical complementation of *purB* mutant was performed by addition of both inosine and adenine (I/A) to solid BHI agar during bacterial growth or RPMI media during neutrophil infection (indicated by single $^{+}$) or both (indicated by double $^{++}$) at a final concentration of 0.02 mg/ml. The absence of I/A in RPMI or BHI is indicated by ($^{-}$). Neutrophils were incubated with *S. aureus* for 3 h at MOI 10 and viable neutrophils enumerated ($n = 3$). Data expressed at mean ± SEM and analyzed by ANOVA with Bonferoni post-test $^{*}p < 0.05$, $^{**}p < 0.01$. Comparisons were between JE2 and transductant **(A)** or as indicated **(B,C)**.

to overcome the zebrafish. As seen in other zebrafish bloodstream infection models, none of the *S. aureus* strains were completely cleared from any viable embryo over the timecourse studied (29).

DISCUSSION

The importance of neutrophils both in the control of colonization and during active *S. aureus* infection makes them an ideal target for bacterial immune evasion strategies (5, 6). Preserving neutrophil function during infection by preventing *S. aureus*-induced cell death is therefore an attractive therapeutic strategy and here we describe three genes (*purB, clpP,* and *lspA*) with previously unidentified roles in neutrophil cell death. Mutations in two of these genes, *purB* and *clpP,* led to significantly reduced pathogenesis in a zebrafish model of infection. Virulence for *clpP* but not *purB* was restored by depleting neutrophils in zebrafish. Our work suggests that ClpP is a key element of *S. aureus* pathogenicity and therapeutically targeting ClpP may improve outcome during *S. aureus* infections (35–37).

Staphylococcus aureus is well-known to cause a lytic or necrotic like neutrophil cell death via factors that directly damage the cell (38–41). It is unlikely however that any of the gene

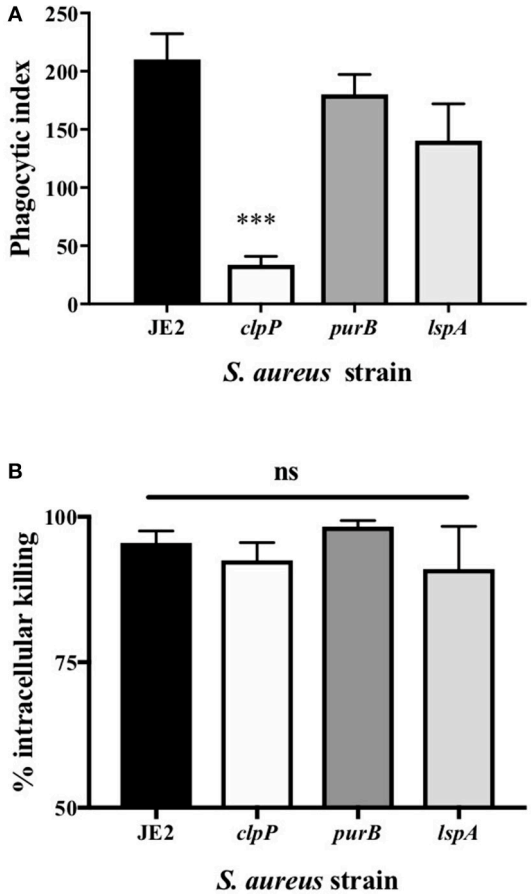

FIGURE 4 | Loss of *clpP* leads to a reduction in phagocytosis but there is no impact on killing by neutrophils **(A)** Neutrophils were incubated with JE2 and *clpP, purB,* or *lspA* strains at MOI 10 for 1 h. Cytocentrifuge slides were prepared and the phagocytic index assessed by light microscopy. Data are expressed as mean ± SEM ($n = 6$). **(B)** Neutrophils were incubated with JE2, *ClpP, purB,* or *lspA* strains at MOI 5 and an intracellular killing assay performed. Graph shows % killing of *S. aureus* by calculating the reduction in the number of viable bacteria at 120 min compared to 30 min for each strain. Data expressed as mean ± SEM ($n = 3$). Data were analyzed by ANOVA with Bonferoni post-test ***$p < 0.001$.

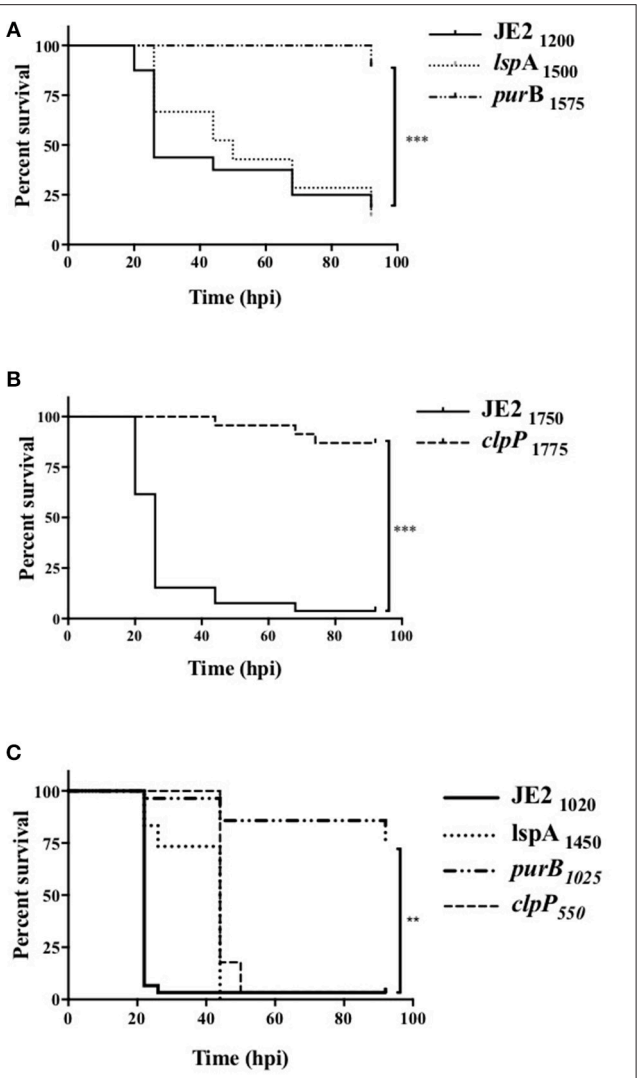

FIGURE 5 | Altered pathogenicity of mutants in zebrafish models of *in vivo* infection. Zebrafish embryos at 24 hpf were injected into the circulation valley with JE2, *lspA, purB,* or *clpP* mutants **(A,B)** transductants. Exact inoculum was determined retrospectively by Miles Misra assays and indicated as subscript values. **(C)** At 30 min post-fertilization zebrafish embryos were injected with a *pu.1* morpholino to delete phagocytes. Embryos were injected at 32 hpf into the circulation valley with the indicated inoculum of JE2, *lspA, purB,* or *clpP* strains. Survival curves over 92 h post-infection (hpi) were calculated by Kaplan-Meier analysis, statistical significance is indicated by **$p < 0.01$, ***$p < 0.001$.

products identified in our study are directly lytic to neutrophils. In the case of PurB, a deficiency in purine biosynthesis and therefore a failure to replicate and express virulence factors in a nutrient poor environment is likely to explain the reduction in neutrophil lysis. This was in part confirmed by the addition of adenine and inosine during infection of neutrophils, which partially increased neutrophil lysis. *S. aureus purB* mutants have been recently shown by our group to have reduced pathogenesis, and *purA* mutants were found to be attenuated in a *S. aureus* murine abscess study (16, 42). In support of this we observed an attenuated phenotype of the *purA* mutant in the primary screen and this was taken forward into the secondary screen. The level attenuation however, was not as robust as *purB* and therefore was not carried forward into further studies.<underline >We found the attenuated virulence of the *purB* mutant in zebrafish was unaffected by the absence of neutrophils. This further suggests that a fundamental deficiency to replicate, rather than a failure to overcome innate immunity, was the cause of the attenuation.

The lipoprotein signal peptidase product of *lspA* is localized in the bacterial membrane and required for biogenesis of bacterial lipoproteins (43). Lipoproteins themselves can play essential roles in host-pathogen interactions, for example as pathogen associated molecule patterns (PAMPs) acting via TLRs (44–46). Bacterial lipoproteins can induce apoptosis in other

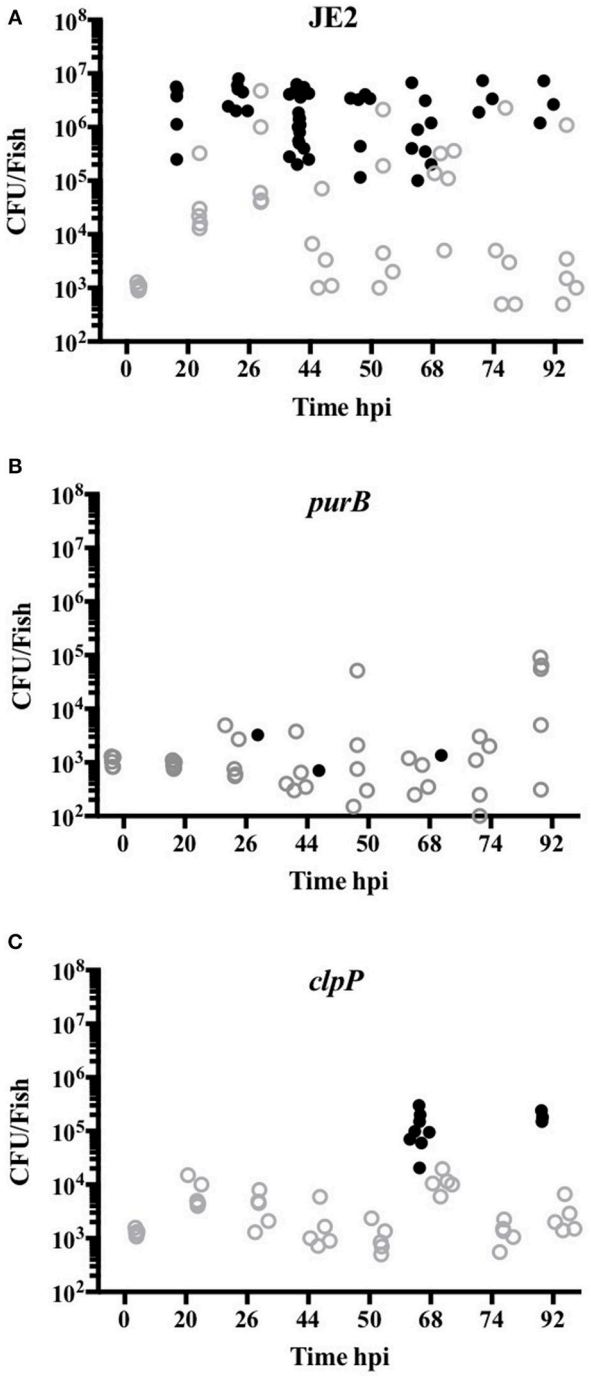

FIGURE 6 | *purB* and *clpP* mutants fail to replicate in zebrafish models of *in vivo* infection. Zebrafish embryos at 24 hpf were injected into the circulation valley with JE2 **(A)**, *purB* **(B)** or *clpP* **(C)** at 950, 1025 and 1275 CFU, respectively. CFU counts from up to five viable (open gray circles) and non-viable (closed black circles) homogenized embryos at selected time points were determined by Miles Misra.

cell types (47). Although we measure a non-apoptotic cell death in this study, it is conceivable that activation of an apoptosis programme by lipoproteins in concert with other death inducing toxins may lead to an overall net effect of lysis and therefore by removing lipoproteins a lytic pathway is prevented. Failure to mature lipoproteins due to loss of LspA or other peptidases has previously been shown to impair growth and pathogenicity, but our study is the first to describe a specific immune modulating role for LspA in *S. aureus* (44, 48). In murine models of *S. aureus* infection an *lsp* mutant failed to induce disease (44) but we show no attenuation in zebrafish embryos. Since *S. aureus* has evolved as a human associated organism it is possible that this tropism may extend to the targets of LspA, explaining the species specificity of the observed effect.

The principal function of Clp proteases is protein degradation, although they are associated with a number of physiological processes (36). *ClpP* mutants are highly susceptible to stress and the attenuated phenotype caused by the *clpP* mutant in our study may be due to lack of stress adaptation in the neutrophil phagosomal environment. *S. aureus* is adept at escape from the richly anti-microbicidal phagosome (49, 50). ClpP deficient *Legionella pneumophila* fails to escape from the endosome-lysosomal pathway in a macrophage cell line and *S. aureus* mutants are unable to replicate intracellularly (35, 51). Interestingly this has also been shown for *lspA* in *Listeria monocytogenes* (52). It is possible that containment within the neutrophil phagosome may prevent post-phagosomal cell lysis for these mutants, but further studies are required. The *clpP* mutant was not phagocytosed as readily, which may be as a result of an impairment in lipoprotein-dependent recognition by neutrophils, leading to a reduction in post-phagocytic cell lysis.

ClpP regulates the expression of a number of bacterial virulence factors such as hemolysin, which may in part account for the attenuation in neutrophil cell death seen in our study (24, 53). As a result, *clpP* mutants are found to be less virulent in animal models (53, 54). We also show attenuation *in vivo,* where the *clpP* mutant fails to replicate in zebrafish embryos, and which is supported by others that demonstrate a failure to replicate in the host (35). A growing number of studies highlight the therapeutic potential of targeting ClpP. A recent study describes a selective, small-molecule inhibitor of ClpP, identified via high-throughput screening and which attenuates virulence in mouse models of *S. aureus* USA300 infection (55). Our work and others suggest therapeutically targeting ClpP may have great promise in treating invasive *S. aureus* infections. In conclusion, our work identifies genetic components underpinning *S. aureus* pathogenesis and provides further evidence for the complex interaction between pathogen and host. These findings provide a greater insight into how this commensal organism breaches innate immune barriers during infection.

AUTHOR CONTRIBUTIONS

DY, SF, and LP wrote the manuscript. DY, YH, IJ, and LC performed the experiments. All authors contributed to experimental design and data analysis.

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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.2019.00045/full#supplementary-material

REFERENCES

1. Graham PL III, Lin SX, Larson EL. A U.S. population-based survey of *Staphylococcus aureus* colonization. *Ann Intern Med.* (2006) 144:318–25. doi: 10.7326/0003-4819-144-5-200603070-00006
2. Bradley SF. MRSA colonisation (eradicating colonisation in people without active invasive infection). *BMJ Clin Evid.* (2015) 2015:0923.
3. Safdar N, Bradley EA. The risk of infection after nasal colonization with *Staphylococcus aureus*. *Am J Med*. (2008) 121:310–5. doi: 10.1016/j.amjmed.2007.07.034
4. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev*. (1997) 10:505–20. doi: 10.1128/CMR.10.3.505
5. Rigby KM, DeLeo FR. Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol*. (2012) 34:237–59. doi: 10.1007/s00281-011-0295-3
6. Archer NK, Harro JM, Shirtliff ME. Clearance of *Staphylococcus aureus* nasal carriage is T cell dependent and mediated through interleukin-17A expression and neutrophil influx. *Infect Immun*. (2013) 81:2070–5. doi: 10.1128/IAI.00084-13
7. Guerra FE, Borgogna TR, Patel DM, Sward EW, Voyich JM. Epic immune battles of history: neutrophils vs. *Staphylococcus aureus*. *Front Cell Infect Microbiol*. (2017) 7:286. doi: 10.3389/fcimb.2017.00286
8. Kobayashi SD, Braughton KR, Whitney AR, Voyich JM, Schwan TG, Musser JM, et al. Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proc Natl Acad Sci USA*. (2003) 100:10948–53. doi: 10.1073/pnas.1833375100
9. Yamamoto A, Taniuchi S, Tsuji S, Hasui M, Kobayashi Y. Role of reactive oxygen species in neutrophil apoptosis following ingestion of heat-killed *Staphylococcus aureus*. *Clin Exp Immunol*. (2002) 129:479–84. doi: 10.1046/j.1365-2249.2002.01930.x
10. Greenlee-Wacker MC, Rigby KM, Kobayashi SD, Porter AR, DeLeo FR, Nauseef WM. Phagocytosis of *Staphylococcus aureus* by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *J Immunol*. (2014) 192:4709–17. doi: 10.4049/jimmunol.1302692
11. Vandenesch F, Lina G, Henry T. *Staphylococcus aureus* hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? *Front Cell Infect Microbiol*. (2012) 2:12. doi: 10.3389/fcimb.2012.00012
12. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med*. (2007) 13:1510–4. doi: 10.1038/nm1656
13. Chua KY, Monk IR, Lin YH, Seemann T, Tuck KL, Porter JL, et al. Hyperexpression of alpha-hemolysin explains enhanced virulence of sequence type 93 community-associated methicillin-resistant *Staphylococcus aureus*. *BMC Microbiol*. (2014) 14:31. doi: 10.1186/1471-2180-14-31
14. Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol*. (2005) 175:3907–19. doi: 10.4049/jimmunol.175.6.3907
15. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, et al. A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* (2013) 4:e00537-12. doi: 10.1128/mBio.00537-12
16. Connolly J, Boldock E, Prince LR, Renshaw SA, Whyte MK, Foster SJ. Identification of *Staphylococcus aureus* factors required for pathogenicity and growth in human blood. *Infect Immun.* (2017) 85:e00337-17. doi: 10.1128/IAI.00337-17
17. Vestergaard M, Leng B, Haaber J, Bojer MS, Vegge CS, Ingmer H. Genome-Wide identification of antimicrobial intrinsic resistance determinants in *Staphylococcus aureus*. *Front Microbiol.* (2016) 7:2018. doi: 10.3389/fmicb.2016.02018
18. Frydenlund Michelsen C, Hossein Khademi SM, Krogh Johansen H, Ingmer H, Dorrestein PC, Jelsbak L. Evolution of metabolic divergence in *Pseudomonas aeruginosa* during long-term infection facilitates a proto-cooperative interspecies interaction. *ISME J*. (2016) 10:1323–36. doi: 10.1038/ismej.2015.220
19. Miles A, Misra S. The estimation of the bactericidal power of the blood. *J Hyg*. (1938) 38:732–49. doi: 10.1017/S002217240001158X
20. Novick RP. Genetic systems in staphylococci. *Methods Enzymol.* (1991) 204:587–636. doi: 10.1016/0076-6879(91)04029-N
21. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* New York, NY: Cold Spring Harbour Laboratory Press. (1989).
22. Schenk S, Laddaga RA. Improved method for electroporation of *Staphylococcus aureus*. *FEMS Microbiol Lett*. (1992) 73:133–8. doi: 10.1111/j.1574-6968.1992.tb05302.x
23. Lee CY, Iandolo JJ. Integration of staphylococcal phage L54a occurs by site-specific recombination: structural analysis of the attachment sites. *Proc Natl Acad Sci USA*. (1986) 83:5474–8. doi: 10.1073/pnas.83.15.5474
24. Michel A, Agerer F, Hauck CR, Herrmann M, Ullrich J, Hacker J, et al. Global regulatory impact of ClpP protease of *Staphylococcus aureus* on regulons involved in virulence, oxidative stress response, autolysis, and DNA repair. *J Bacteriol.* (2006) 188:5783–96. doi: 10.1128/JB.00074-06
25. Haslett C, Guthrie LA, Kopaniak MM, Johnston RB Jr, Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol.* (1985) 119:101–10.
26. Prince LR, Allen L, Jones EC, Hellewell PG, Dower SK, Whyte MK, et al. The role of interleukin-1beta in direct and toll-like receptor 4-mediated neutrophil activation and survival. *Am J Pathol.* (2004) 165:1819–26. doi: 10.1016/S0002-9440(10)63437-2
27. Dick EP, Prince LR, Sabroe I. *Ex vivo*-expanded bone marrow CD34+ derived neutrophils have limited bactericidal ability. *Stem Cells* (2008) 26:2552–63. doi: 10.1634/stemcells.2008-0328
28. Easmon CS, Lanyon H, Cole PJ. Use of lysostaphin to remove cell-adherent staphylococci during in vitro assays of phagocyte function. *Br J Exp Pathol.* (1978) 59:381–5.
29. Prajsnar TK, Cunliffe VT, Foster SJ, Renshaw SA. A novel vertebrate model of *Staphylococcus aureus* infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. *Cell Microbiol.* (2008) 10:2312–25. doi: 10.1111/j.1462-5822.2008.01213.x

30. Rhodes J, Hagen A, Hsu K, Deng M, Liu TX, Look AT, et al. Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell* (2005) 8:97–108. doi: 10.1016/j.devcel.2004.11.014
31. Prince LR, Graham KJ, Connolly J, Anwar S, Ridley R, Sabroe I, et al. *Staphylococcus aureus* induces eosinophil cell death mediated by alpha-hemolysin. *PLoS ONE* (2012) 7:e31506. doi: 10.1371/journal.pone.0031506
32. Voyich JM, Vuong C, DeWald M, Nygaard TK, Kocianova S, Griffith S, et al. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J Infect Dis*. (2009) 199:1698–706. doi: 10.1086/598967
33. Pang YY, Schwartz J, Thoendel M, Ackermann LW, Horswill AR, Nauseef WM. agr-Dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. *J Innate Immun*. (2010) 2:546–59. doi: 10.1159/000319855
34. Prajsnar TK, Hamilton R, Garcia-Lara J, McVicker G, Williams A, Boots M, et al. A privileged intraphagocyte niche is responsible for disseminated infection of *Staphylococcus aureus* in a zebrafish model. *Cell Microbiol*. (2012) 14:1600–19. doi: 10.1111/j.1462-5822.2012.01826.x
35. Frees D, Chastanet A, Qazi S, Sorensen K, Hill P, Msadek T, et al. Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in *Staphylococcus aureus*. *Mol Microbiol*. (2004) 54:1445–62. doi: 10.1111/j.1365-2958.2004.04368.x
36. Frees D, Gerth U, Ingmer H. Clp chaperones and proteases are central in stress survival, virulence and antibiotic resistance of *Staphylococcus aureus*. *Int J Med Microbiol*. (2014) 304:142–9. doi: 10.1016/j.ijmm.2013.11.009
37. Vahidi S, Ripstein ZA, Bonomi M, Yuwen T, Mabanglo MF, Juravsky JB, et al. Reversible inhibition of the ClpP protease via an N-terminal conformational switch. *Proc Natl Acad Sci USA*. (2018) 115:E6447–56. doi: 10.1073/pnas.1805125115
38. Surewaard BG, de Haas CJ, Vervoort F, Rigby KM, DeLeo FR, Otto M, et al. Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis. *Cell Microbiol*. (2013) 15:1427–37. doi: 10.1111/cmi.12130
39. Greenlee-Wacker MC, Kremserova S, Nauseef WM. Lysis of human neutrophils by community-associated methicillin-resistant *Staphylococcus aureus*. *Blood* (2017) 129:3237–44. doi: 10.1182/blood-2017-02-766253
40. Kobayashi SD, Braughton KR, Palazzolo-Ballance AM, Kennedy AD, Sampaio E, Kristosturyan E, et al. Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun*. (2010) 2:560–75. doi: 10.1159/000317134
41. Anwar S, Prince LR, Foster SJ, Whyte MK, Sabroe I. The rise and rise of *Staphylococcus aureus*: laughing in the face of granulocytes. *Clin Exp Immunol*. (2009) 157:216–24. doi: 10.1111/j.1365-2249.2009.03950.x
42. Lan L, Cheng A, Dunman PM, Missiakas D, He C. Golden pigment production and virulence gene expression are affected by metabolisms in *Staphylococcus aureus*. *J Bacteriol*. (2010) 192:3068–77. doi: 10.1128/JB.00928-09
43. Shahmirzadi SV, Nguyen MT, Gotz F. Evaluation of *Staphylococcus aureus* lipoproteins: role in nutritional acquisition and pathogenicity. *Front Microbiol*. (2016) 7:1404. doi: 10.3389/fmicb.2016.01404
44. Bubeck Wardenburg J, Williams WA, Missiakas D. Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. *Proc Natl Acad Sci USA*. (2006) 103:13831–6. doi: 10.1073/pnas.0603072103
45. Schmaler M, Jann NJ, Gotz F, Landmann R. Staphylococcal lipoproteins and their role in bacterial survival in mice. *Int J Med Microbiol*. (2010) 300:155–60. doi: 10.1016/j.ijmm.2009.08.018
46. Kim NJ, Ahn KB, Jeon JH, Yun CH, Finlay BB, Han SH. Lipoprotein in the cell wall of *Staphylococcus aureus* is a major inducer of nitric oxide production in murine macrophages. *Mol Immunol*. (2015) 65:17–24. doi: 10.1016/j.molimm.2014.12.016
47. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* (1999) 285:736–9. doi: 10.1126/science.285.5428.736
48. Sander P, Rezwan M, Walker B, Rampini SK, Kroppenstedt RM, Ehlers S, et al. Lipoprotein processing is required for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol*. (2004) 52:1543–52. doi: 10.1111/j.1365-2958.2004.04041.x
49. Grosz M, Kolter J, Paprotka K, Winkler AC, Schafer D, Chatterjee SS, et al. Cytoplasmic replication of *Staphylococcus aureus* upon phagosomal escape triggered by phenol-soluble modulin alpha. *Cell Microbiol*. (2014) 16:451–65. doi: 10.1111/cmi.12233
50. Jarry TM, Memmi G, Cheung AL. The expression of alpha-haemolysin is required for *Staphylococcus aureus* phagosomal escape after internalization in CFT-1 cells. *Cell Microbiol*. (2008) 10:1801–14. doi: 10.1111/j.1462-5822.2008.01166.x
51. Zhao BB, Li XH, Zeng YL, Lu YJ. ClpP-deletion impairs the virulence of *Legionella pneumophila* and the optimal translocation of effector proteins. *BMC Microbiol*. (2016) 16:174. doi: 10.1186/s12866-016-0790-8
52. Reglier-Poupet H, Frehel C, Dubail I, Beretti JL, Berche P, Charbit A, et al. Maturation of lipoproteins by type II signal peptidase is required for phagosomal escape of *Listeria monocytogenes*. *J Biol Chem*. (2003) 278:49469–77. doi: 10.1074/jbc.M307953200
53. Frees D, Qazi SN, Hill PJ, Ingmer H. Alternative roles of ClpX and ClpP in *Staphylococcus aureus* stress tolerance and virulence. *Mol Microbiol*. (2003) 48:1565–78. doi: 10.1046/j.1365-2958.2003.03524.x
54. Liu Q, Wang X, Qin J, Cheng S, Yeo WS, He L, et al. The ATP-dependent protease ClpP inhibits biofilm formation by regulating Agr and cell wall hydrolase Sle1 in *Staphylococcus aureus*. *Front Cell Infect Microbiol*. (2017) 7:181. doi: 10.3389/fcimb.2017.00181
55. Gao P, Ho PL, Yan B, Sze KH, Davies J, Kao RYT. Suppression of *Staphylococcus aureus* virulence by a small-molecule compound. *Proc Natl Acad Sci USA*. (2018) 115:8003–8. doi: 10.1073/pnas.1720520115

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Elevated Gut Microbiome-Derived Propionate Levels Are Associated With Reduced Sterile Lung Inflammation and Bacterial Immunity in Mice

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Short-chain fatty acids (SCFA) are important dietary and microbiome metabolites that can have roles in gut immunity as well as further afield. We previously observed that gut microbiome alteration via antibiotics led to attenuated lung inflammatory responses. The rationale for this study was to identify gut microbiome factors that regulate lung immune homeostasis. We first investigated key factors within mouse colonic lumen filtrates (CLF) which could elicit direct inflammatory effects *in vitro*. We identified lipopolysaccharide (LPS) and SCFAs as key CLF ingredients whose levels and inflammatory capacity changed after antibiotic exposure in mice. Specifically, the SCFA propionate appeared to be a key regulator of LPS responses *in vitro*. Elevated propionate: acetate ratios, as seen in CLF after antibiotic exposure, strongly blunted inflammatory responses *in vitro*. *In vivo*, exposure of lungs to high dose propionate, to mimic how prior antibiotic exposure changed SCFA levels, resulted in diminished immune containment of *Staphylococcus aureus* pneumonia. Finally, we discovered an enrichment of propionate-producing gut bacteria in mice with reduced lung inflammation following lung ischemia reperfusion injury *in vivo*. Overall, our data show that propionate levels can distinctly modulate lung immune responses *in vitro* and *in vivo* and that gut microbiome increased production of propionate is associated with reduced lung inflammation.

Keywords: lung injury, short-chain fatty acids, SCFA, acetate, propionate, IR, inflammation

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INTRODUCTION

The human body coexists with a vast commensal microbiome that is increasingly recognized to play important roles in human health, physiology, and disease (Cho and Blaser, 2012; Shreiner et al., 2015; Sender et al., 2016; Alverdy and Krezalek, 2017; Young, 2017). Interactions between the microbiome and host are best understood at specific interfaces, such as the gut, oral cavity and the skin. Additionally, there appears to be a prominent role for gastrointestinal dysbiosis and pathobionts in the etiology of inflammatory bowel disease, other intestinal disorders, and critical illness in general (Alverdy and Krezalek, 2017). From an evolutionary viewpoint, human physiology has evolved closely with specific gut microbiota which, prior to the advent of antibiotics, were likely not exposed to sudden perturbations in composition

over the life cycle of the human host (after early post-natal colonization). These symbiotic organisms may serve purposes besides food digestion and vitamin production, such as during immune education and host defense. While it may be conceptually challenging at first to explain how gut commensal microbial communities affect physiological processes in distant organ systems, a few studies have supported this paradigm (Trompette et al., 2014; Vieira et al., 2015, 2016).

We and others have reported that the gut microbiome influences lung inflammatory responses and these data support the current hypothesis for the existence for a gut-lung axis of communication (Trompette et al., 2014; Marsland et al., 2015; Prakash et al., 2015; Samuelson et al., 2015; Budden et al., 2017). However, the key questions of how communication along this axis occurs still remain unanswered. We speculate that commensal-associated molecular patterns (CAMPs) and metabolites in concert modulate lung inflammatory responses by either directly acting on resident lung immune cells or indirectly through immune reprogramming of circulating immune cells or both. Released CAMPs and metabolites may transit through the intestinal mucosa and portal circulation and travel to the lungs via the liver and heart. Since the pulmonary and gastrointestinal systems are both exposed to environmental and infectious threats, it is plausible that the commensal microbial community influences these two systems. This concept is supported by our earlier published data that lung alveolar macrophages from mice with antibiotic-altered gut microbiota were less responsive to inflammatory ligands than their unaltered counterparts (Prakash et al., 2015). The gut-lung axis may contribute to establishing lung immune homeostasis that could be akin to a baseline state of immune tonicity or injury readiness (reviewed in Lloyd and Marsland, 2017). By understanding the impact of commensal-derived factors on lung immunity, it may be possible to selectively or temporarily regulate lung immunity: for example, by bolstering host defenses during pneumonia or mitigating sterile pneumonitis after gastric acid aspiration.

In this study we focused on identifying factors produced by the gut microbiome that could regulate lung responses to sterile and infectious injuries. We discovered that antibiotic-mediated dysbiosis led to large alterations in SCFA levels, specifically, propionate and acetate levels. Furthermore, propionate appeared to have a concentration-dependent ability to modify LPS inflammatory responses *in vitro*, with low and high concentrations augmenting and blocking inflammatory responses, respectively. High propionate:acetate ratios appeared to skew LPS responses toward less inflammation while low ratios appeared to skew LPS responses toward more inflammation *in vitro*. This was confirmed by the *in vivo* observation that direct administration of high propionate concentrations to mouse lungs resulted in worsening of an experimental pneumonia, likely through the inhibition of beneficial inflammation needed to fight the infection. Finally, examining the microbiomes of mice with varying degrees of sterile lung injury revealed that high propionate-producing bacteria (*Lachnospiraceae*) were enriched significantly in mice that exhibited low lung inflammation phenotypes.

Overall, our study strongly suggests that gut commensal bacteria continuously prime resident cells in distant organ systems, such as the lungs, through shed toxins and specific metabolite compositions. Furthermore, alterations in gut microbiome composition can profoundly change inflammatory responses to sterile injury and pathogenic infectious responses. We had previously shown that reducing lung inflammation results in a deficient host immune response to infection (Tian et al., 2017). Being able to control and modulate lung inflammatory responses through gut microbiome or metabolite manipulation could aid in preventing pulmonary complications from prolonged hospitalization and antibiotic exposure in critically ill patients.

MATERIALS AND METHODS

Animals

Male mice (12–15 weeks old) were either purchased (The Jackson Laboratory, Bar Harbor, ME, United States) or bred at the animal facility at University of California, San Francisco. Purchased mice were allowed to acclimatize to their new housing for at least 1 week before any experiments on them were conducted. Wild-type C57BL/6 and C3H/HeOuJ mice were used in this study.

Only male mice were used in our experiments primarily to reflect the fact that trauma disproportionately affects human males. Based on our previous studies, we used group sizes of 6–10 for all experiments (Prakash et al., 2012, 2015). All mice for a given experiment were either littermates or purchased/bred such that they were age-matched. Mice used in these experiments were randomly chosen either to undergo the various surgeries (sham vs. IR) or treatments (+/− specific treatments); therefore there was no attempt made to blind the individuals conducting the experiments. However, in situations where mice received a treatment or control before IR surgery or infection after surgery, the individual collecting the organs/plasma and generating the ELISA data was unaware of which mice received which specific treatment.

Reagents and Cell Lines

Short-chain fatty acids (SCFA) (acetate, butyrate, propionate, formate), Trichostatin A, polymyxin B, lipopolysaccharide (LPS), and lipoteichoic acid (LTA) were purchased from Sigma-Aldrich, St. Louis, MO, United States. Pam3CysSKKK and FSL-1 were both obtained from EMC Microcollections, Tubingen, Germany. The following cell lines were used in this study: HUVEC (primary human umbilical vein endothelial cells, used at passage 6 or less, Promocell, Heidelberg, Germany), EOMA (129 background endothelial cell line, ATCC, Manassas, VA, United States), SVEC-40 (C3H/HeJ TLR4 mutant background endothelial cell line, ATCC), and MH-S (wild-type BALB/c alveolar macrophage cell line, ATCC).

Antibiotic Treatment

A group of wild-type C3H/HeOuJ background mice were treated with antibiotic containing water *ad libitum* or control

group was given standard drinking water. Antibiotic treatment consisted of neomycin and polymyxin B (final concentrations: 0.6 mg/mL for neomycin and 120 units/mL for polymyxin B, both from Sigma-Aldrich, St. Louis, MO, United States) given in drinking water for 8 weeks as described earlier (Prakash et al., 2015). This combination of antibiotics was chosen specifically for the ability of both to remain within the gut and not be absorbed into circulation; by using this combination to target the gut microbiome, we could focus primarily on its effects without disruption of other microbiomal niches. **Supplementary Figure S6** demonstrates the effects of this combination on the richness, evenness, and diversity of the microbiome after 7 weeks of treatment.

Colonic Lumen Filtrate (CLF)

Stool from mice was obtained in two ways: by scruffing mice and collecting stool as it was produced by the mice; or by collecting small and large intestines of mice and expressing it out of the intestinal cavity. Homogenized stool was then sterile filtered to create CLF and protein concentration measured by bicinchoninic acid (BCA) method. Protein concentrations of CLF were measured using standard assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, United States). CLF sterility was confirmed by plating on LB agar plates. Stool from cages of mice (3 cages, 15 mice total per group) that received either control or antibiotic water were collected, pooled, and CLF was prepared. CLF was added to tissue culture supernatant for various experiments described in the concentrations noted.

SCFA and Amino Acid Measurement

Stool or colonic lumen filtrate (prepared as described above) was sent to PennCHOP metabolomics core facility[1] for SCFA and amino acid analysis. CLF was further filtered using 1.2, 0.65, and 0.22 μm filter plates (Millipore, Billerica, MA, United States). The filtrate was loaded into total recovery vials (Waters, Milford, MA, United States) for analysis. Short chain fatty acids were quantified using a Water Acquity uPLC System with a Photodiode Array Detector and an autosampler (192 sample capacity). Samples were analyzed on a HSS T3 1.8 μm 2.1 × 150 mm column. The flow rate was 0.25 mL/min, the injection volume was 5 μL, the column temperature was 4°C, the sample temperature was 4°C, and the run-time was 25 min per sample. Eluent A was 100 mM sodium phosphate monobasic, pH 2.5, eluent B was methanol, the weak needle wash was 0.1% formic acid in water, the strong needle wash was 0.1% formic acid in acetonitrile, and the seal wash was 10% acetonitrile in water. The gradient was 100% eluent A for 5 min, gradient to 70% eluent B from 5 to 22 min, and then 100% eluent A for 3 min. The photodiode array was set to read absorbance at 215 nm with 4.8 nm resolution. Samples were quantified against standard curves of at least five points run in triplicate. Standard curves were run at the beginning and end of each metabolomics run. Quality control checks (blanks and standards) were run every eight samples. Results were rejected if the standards deviate by greater than ± 5%. Concentrations in the samples were calculated as the measured concentration minus the concentration of the solvent; the range of detection was at least 1 – 100 μmol/g stool.

[1]https://metabolomic.research.chop.edu/

Ventilated Lung Ischemia Reperfusion (Unilateral Left Pulmonary Artery Occlusion) Surgery

A mouse model of unilateral left pulmonary artery (PA) occlusion was used, as we have described previously (Prakash et al., 2012). Briefly, anesthetized mice (using IP tribromoethanol (Avertin®); Sigma-Aldrich) were orally intubated, given buprenorphine (IP; Harry Schein, Melville, NY, United States), and placed on a mini-vent mouse ventilator (Harvard Apparatus, Holliston, MA, United States), using tidal volumes of 0.225 mL (7.5 mL/kg), and a respiratory rate of 180 breaths/min (assuming an average mouse weight of 30 g). A left thoracotomy via the interspace between the 2nd and 3rd ribs was performed and the left PA was identified and ligated using a slip knot suture with 7-0 or 8-0 prolene monofilament suture. The end of the suture was externalized through a narrow bore (27 g) needle to the anterior chest wall. Prior to closure of the thorax, the left lung was reinflated with positive end expiratory pressure (PEEP). Local anesthetic (3-4 drops of 0.25% bupivacaine) was applied topically prior to skin closure. The total period of mechanical ventilation and surgery was approximately 20–25 min. After skin closure, mice were extubated and allowed to recover from anesthesia. After 60 min of ischemia, the ligature on the PA was released and left lung reperfusion started. At the experimental end-point times, mice were euthanized and the blood and lungs were collected.

Blood was collected from anesthetized mice via cardiac puncture using a heparinized syringe, centrifuged (14,000 *g*, 5 min) and the plasma separated, flash frozen in liquid nitrogen and stored at −80°C. Lower portions of the left lungs were excised and placed in Trizol® (Thermo Fisher Scientific, Waltham, MA, United States) at −80°C for RNA isolation. Levels of cytokines and chemokines (described later) were quantified in plasma.

Mice received equivalent durations of mechanical ventilation (20–25 min), and were left spontaneously breathing during their recovery from anesthesia and the remainder of the ischemia period and subsequent reperfusion or equivalent periods in the sham mice.

While this lung IR procedure has high initial survival rates of 80–90% on average, some mice die from irreparable damage to the PA or left bronchus during the slip-knot placement. Mice that did not survive the surgery or the reperfusion period due to technical complications in the surgical procedure (predominantly, left bronchus or left PA injury) were excluded from the study. The overall attrition rate was 10–20%.

Lung Injury Scoring

Lung injury was scored in histology images by one of two methods: semiquantitative visual scoring and by ImageJ analysis of the images for counts of inflammatory cells and %area occupied. The former (semi-quantitative) scoring method (1 = no lung injury and 5 = severe lung injury) was performed

as previously described (Prakash et al., 2015). The latter (quantitative ImageJ) scoring method was performed follows: in brief, the ImageJ freehand selection tool was used to trace the perimeter of each region of interest. The area extending beyond the perimeter of the vessel was cleared, and the color threshold of the image was adjusted using the default method with the following parameters: hue = 0–255, saturation = 0–255, brightness = 130–255, threshold color = white, background = dark, color space = HSB. The image was converted to an 8-bit gray scale, and the threshold was adjusted using the B&W defaults and a range of 0–150. Counts were outlined and summarized using the analyze particles window (size = 0-infinity, circularity = 0.0–1.00). Average percent Area was also calculated. Lung Injury Scores and cutoffs for high vs. low lung injury designation for this study are included in **Supplementary Figure S7**.

S. aureus Experimental Pneumonia

C57BL/6 wild-type mice were pretreated with high (1 mM) or low (0.1 mM) propionate intratracheally (IT) 2 h prior to IT administration (10^8 CFU) of luminescent strain of *S. aureus* (Newman-lux strain generously provided by Alex Horswill, University of Colorado, Denver). IT administration was done under isoflurane anesthesia and with direct visualization. Six hours after infection, mice were live imaged using IVIS® *in vivo* imaging system (see below for more details). Lungs were then collected and imaged *ex vivo* using IVIS® and luminescence was also measured using Cytation5 cell imaging multi-mode reader (BioTek, Winooski, VT, United States).

Staphylococcus aureus (Newman-lux strain) was grown as follows: after an overnight inoculation in LB broth, serial dilutions of the overnight stock was grown at 37C in a 24-well tissue culture dish with orbital shaking in the Cytation5 cell imaging multi-mode reader (BioTek, Winooski, VT, United States). Every 5 min, an OD reading and a luminescence reading (to verify healthy growth of the luminescence producing strain) were obtained. When the mice were ready for infection, the wells that were at OD 0.3 (mid-log phase) were removed and used for IT infection. Later CFU measurements of these innocula provided the actual CFU count administered to the mice as described above.

In vivo Imaging (IVIS®)

C57BL/6 mice after IT propionate and *S. aureus* administration were imaged at the time points noted. Imaging was conducted on the IVIS® Spectrum Instrument (PerkinElmer, Hopkinton, MA, United States) as previously described (Zhang et al., 2013). Luminescence imaging was performed with an open filter for 5 min.

Sandwich Enzyme-Linked Immunosorbant Assay (ELISA)

Levels of IL-6 produced were determined using the corresponding mouse duoset or Quantikine kits (R&D Systems, Minneapolis, MN, United States). A multiplex ELISA to measure an immune panel of cytokines was used to identify the pattern of expression of other inflammatory and associated cytokines. This measurement of protein levels of cytokines and chemokines were performed once with a 20plex immune array kit (Thermo Fisher Scientific, Waltham, MA, United States). Analytes included in the panel: FGFβ, IL-1β, IL-10, IL-13, IL-6, IL12, IL-17, MIP-1α, GMCSF, MCP-1, IL-5, VEGF, IL-1α, IFNγ, TNFα, IL-2, IP-10 (CXCL10), MIG, KC, IL-4. Those analytes not shown in **Supplementary Figure S2** were detected at low levels or below the level of detection of the assay. All assays were performed according the manufacturer's supplied protocol. All ELISA measurements (except for the multiplex immune panel) were repeated 2–3 times from independently conducted experiments and representative data shown. Standard curves were generated and used to determine the concentrations of individual cytokines or chemokines in the sample.

Microbiome Analysis

Single stool pellets from 23 wild type C3H/HeOuJ mice: 11 that received (Neo/PMB) or 12 that received control water for 8 weeks were processed by the UCSF Colitis and Crohn's Disease Microbiome Research Core Facility as previously described (Fujimura et al., 2016; Mar et al., 2016). Briefly, 46 mouse fecal samples (23 from the week prior to starting antibiotic/control water and 23 from the week prior to lung IR surgery) were processed for DNA extraction, PCR amplification of the V4 hypervariable region of the 16S rRNA gene, and DNA sequencing on the Illumina NextSeq. DNA was extracted from all samples using a modified CTAB extraction protocol. Each DNA sample was PCR amplified in triplicate using primers that (1) targeted the V4 hypervariable region of the 16S rRNA gene, (2) contained a unique barcode sequence to enable demultiplexing of pooled samples, and (3) contained an adapter sequence that enables the amplicon to bind to the NextSeq flow cell. Successful amplicons were pooled in equimolar concentrations and sequenced on the Illumina NextSeq.

Downstream analysis: Merged sequencing read pairs containing less than two expected errors were binned into OTUs (operational taxonomic units) using a 97% sequence similarity threshold. OTUs determined to be chimeric or not of bacterial origin were discarded. Additionally, OTUs known to be common contaminants observed in greater than 50% of extraction controls were discarded and the maximum read count of each remaining OTU in any single extraction control was subtracted from the reads counts of that OTU for all sample. Read counts for OTUs which summed across all samples that were less than 1/1000th of a percent of the total read count for the entire dataset were discarded to minimize noise. Sample read numbers were representatively rarefied to 17,444 reads resulting in a rarefied OTU table. Forty-five of 46 (98%) samples had quality filtered read numbers above the specified rarefying threshold and were included in the downstream analyses.

DNA Extraction

Individual murine fecal samples were placed into lysing matrix E (LME) tubes pre-aliquoted with 500 of hexadecyltrimethylammonium bromide (CTAB) DNA extraction buffer and incubated at 65°C for 15 min. An

equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube and samples were homogenized in a Fast Prep-24 homogenizer at 5.5 m/s for 30 s. Tubes were centrifuged for 5 min at 16,000 × *g* and the aqueous phase was transferred to individual wells of a deep-well 96-well plate. An additional 500 μl of CTAB buffer was added to the LME tubes, the previous steps were repeated, and the aqueous phases were combined. An equal volume of chloroform was mixed with each sample, followed by centrifugation at 3000 × *g* for 10 min to remove excess phenol. The aqueous phase (600 μl) was transferred to a deep-well 96-well plate, combined with 2 volume-equivalents of polyethylene glycol (PEG) and stored overnight at 4°C to precipitate DNA. Plates were centrifuged for 60 min at 3000 × *g* to pellet DNA and the PEG solution was removed. DNA pellets were washed twice with 300 μl of 70% ethanol, air-dried for 10 min and suspended in 100 μl of sterile water. DNA samples were quantitated using the Qubit dsDNA HS Assay Kit and diluted to 10 ng/μl.

DNA Amplification and Sequencing

The V4 region of the 16S rRNA gene was amplified in triplicate as previously described (see citation below). Triplicate reactions were combined and purified using the SequalPrep Normalization Plate Kit (Invitrogen) according to manufacturer's specifications. Purified amplicons were quantitated using the Qubit dsDNA HS Assay Kit and pooled at equimolar concentrations. The amplicon library was concentrated using the Agencourt AMPure XP system (Beckman-Coulter) quantitated using the KAPA Library Quantification Kit (KAPA Biosystems) and diluted to 2 nM. Equimolar PhiX was added at 40% final volume to the amplicon library and sequenced on the Illumina NextSeq 500 Platform on a 153 bp × 153 bp sequencing run.

OTU Table Generation

Raw sequence data was converted from bcl to fastq format using bcl2fastq v2.16.0.10. Paired sequencing reads with a minimum overlap of 25 bp were merged using FLASH v1.2.11. Index sequences were extracted from successfully merged reads and demultiplexed in the absence of quality filtering in QIIME (Quantitative Insights Into Microbial Ecology, v1.9.1), and reads with more than two expected errors were removed using USEARCH's fastq filter (v7.0.1001). Remaining reads were de-replicated at 100% identity, clustered into operational taxonomic units (OTUs) at 97% sequence identity, filtered to remove chimeric sequences, and mapped back to OTUs using USEARCH v8.0.1623. Taxonomy was assigned using the Greengenes database (May 2013). OTUs detected in Negative Extraction Controls (NECs) were considered potential contaminants and filtered as follows: any known common contaminant OTU present in more than half of the NECs for this study was removed from all samples; the maximum read count for any OTU found in fewer than half of the NECs was subtracted from all samples; and any remaining OTU with a total read count less than 0.001% of the total read count across all samples was removed.

Alpha-Diversity

Alpha-diversity indices were computed in QIIME. Comparisons between mouse genotypes were assessed using the Kruskal–Wallis one-way analysis of variance test. Results with a p-value of < 0.05 were considered statistically significant.

Beta-Diversity

Beta-diversity dissimilarity matrices (Bray-Curtis, Canberra, weighted and unweighted uniFrac distances) were generated in QIIME. Variables were assessed for their relationship to bacterial beta-diversity by permutational analysis of variance (PERMANOVA) using the Adonis function (vegan package) in the R environment; variables of $p < 0.05$ were considered statistically significant.

Taxonomic Differences

Enriched taxa were identified using a "three model" approach where Poisson, negative-binomial, and zero-inflated negative-binomial models were applied to each taxon individually, and the model that minimized the Akaike information criterion value (AIC) was selected for each taxon. Before applying the models, the OTU table was de-noised by removing taxa that were present in fewer than 25% of the samples. To adjust for multiple-testing, the false-discovery rate was calculated for each taxon; a q-value of < 0.20 was considered significant.

Statistical Analysis

Data in the figures are expressed as mean ±SD. Data from *in vivo* studies comparing two conditions were analyzed using two-tailed unpaired non-parametric Mann–Whitney analyses. Data from *in vitro* studies comparing two conditions were analyzed using two-tailed unpaired parametric t test with Welch's correction. GraphPad Prism was used for statistical analyses (GraphPad Software, La Jolla, CA, United States). For all *in vivo* and *in vitro* experiments, p values < 0.05 were considered statistically significant. *P*-values are represented as follows in the figures: * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001. For multiple comparisons, one-way ANOVA was used, with p-values represented as follows: α < 0.05; αα < 0.01; ααα < 0.001; αααα < 0.0001. When comparing treatment conditions against an untreated or control condition (indicated in figure legends), two-tailed unpaired parametric t-test with Welch's correction was used, with p-values represented as follows: δ < 0.05; δδ < 0.01; δδδ < 0.001; δδδδ < 0.0001. Experiments were repeated two or more times, as indicated in the figure legends.

Study Approval

All mouse studies were approved from an ethical and methodological standpoint by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco and followed the ARRIVE guidelines.

RESULTS

Colonic Lumen Filtrate (CLF) Is Pro-inflammatory and Contains Lipopolysaccharide (LPS)

After previously reporting that antibiotic treatment led to diminished inflammatory changes in the mouse lung following lung ischemia reperfusion (Prakash et al., 2015), we tested whether colon contents of mice contained inflammation-inducing factors. CLF from wild-type C57BL/6 mice was prepared (**Figure 1A**) and used to stimulate human endothelial cells (HUVEC). Similar to LPS, CLF challenge resulted in dose-dependent IL-6 production (**Figure 1B**). Interestingly, CLFs pro-inflammatory effects on human cells were poorly blocked by polymyxin B (PMB – binds and inactivates LPS) (**Supplementary Figure S1**). However, in murine alveolar macrophages (AMs), PMB was able to block the majority of CLF inflammatory effects (**Figure 1C**). Additionally, like LPS, CLF effects were also blocked both in HUVEC and AMs by histone deacetylase (HDAC) inhibition both by high dose SCFA (butyrate) as well as trichostatin A (TSA) (**Supplementary Figure S2**). Taken together, CLF likely contains both LPS as well as other inflammatory agents produced or shed by the gut commensal microbiome.

CLF From Antibiotic-Treated Mice Is Less Inflammatory and Contains Less LPS

Since antibiotic-treated mice displayed blunted inflammatory responses to sterile lung injury and diminished alveolar macrophage responses to LPS (Prakash et al., 2015), we investigated the effects of CLF from these mice. CLF from antibiotic-treated mice (2-week treatment) generated less inflammation from both HUVEC (**Figure 2A**) and murine AMs (**Figure 2B**). Consistently, antibiotic CLF contained greatly reduced levels of LPS (**Figure 2C**). Furthermore, while control CLF was blocked by PMB, antibiotic CLF was not (**Figure 2B**). Overall, these data suggest that antibiotic treatment altered the composition of CLF and reduced its stimulatory effect partially due to a significant reduction in LPS levels. This is consistent with the fact that the antibiotics chosen have primarily excellent gram negative coverage.

Short-Chain Fatty Acid (SCFA) and Amino Acid Metabolomic Analysis of CLF Reveals Antibiotic-Induced Changes in Acetate and Propionate Levels

While comparing the effects of LPS and CLF, we noted the similarities in responses to HDAC inhibition by millimolar levels of the SCFA, butyrate, as described earlier. However, we also noted that lower dose (micromolar) butyrate displayed a paradoxical effect in enhancing LPS mediated and CLF-mediated inflammation for IL-6 and other specific inflammatory genes (**Supplementary Figure S3**). This paradoxical effect was more pronounced during stimulation with lower doses of LPS (data not shown). Furthermore, when we challenged HUVEC to increasing levels of antibiotic CLF, it appeared that antibiotic CLF contained inhibitory factors in contrast to control CLF (**Figure 3A**). Since SCFAs could alter the inflammatory potential of LPS, we hypothesized that perhaps

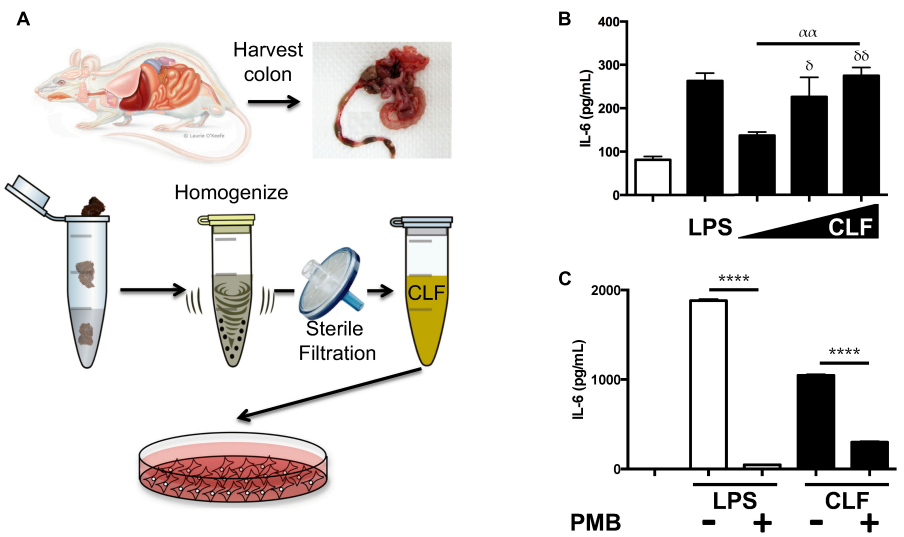

FIGURE 1 | Colonic Lumen Filtrate stimulates (CLF) cells similarly to LPS and contains LPS. **(A)** Schematic for preparation of CLF from mouse stool. **(B)** Human endothelial cells (EC) were stimulated with LPS or CLF (increasing doses from 1 to 100 mg/mL) and IL-6 measured. **(C)** LPS or CLF stimulation of mouse alveolar macrophages (AM) were both inhibited with polymyxin B (PMB) treatment. *P*-values are represented as follows: * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001. For multiple comparisons using one-way ANOVA, *p*-values are represented as follows: α < 0.05; αα < 0.01; ααα < 0.001; αααα < 0.0001. For comparisons against an untreated or control condition, *p*-values are represented as follows: δ < 0.05; δδ < 0.01; δδδ < 0.001; δδδδ < 0.0001. All experiments were conducted at least twice and representative data are shown.

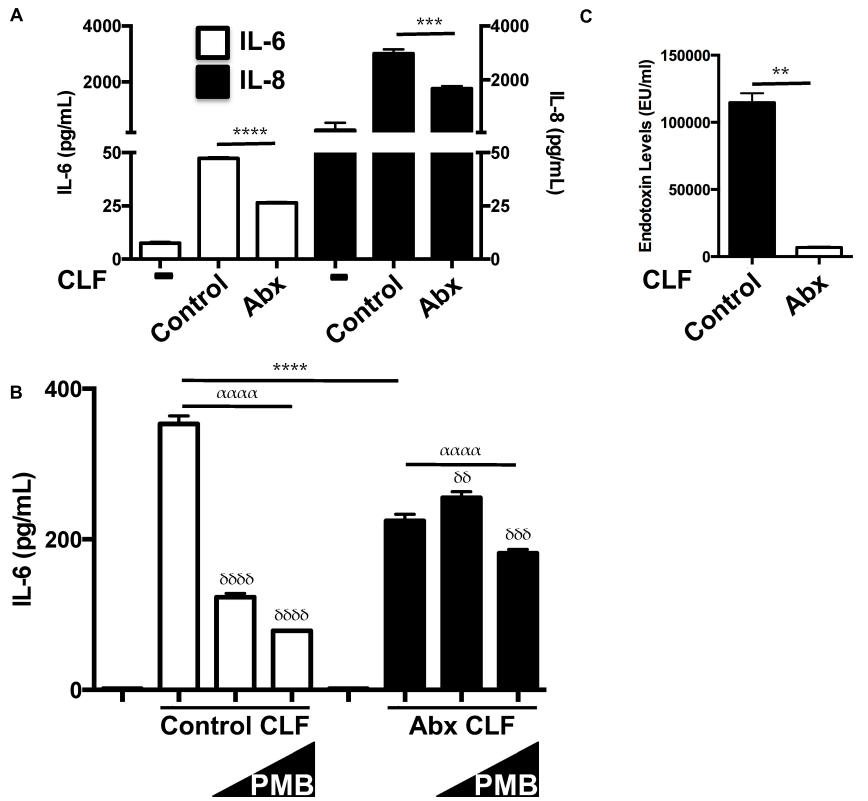

FIGURE 2 | CLF from antibiotic-treated mice is less inflammatory and contains less LPS. **(A)** HUVEC (EC) respond less to antibiotic-exposed CLF (50 mcg/mL) vs. control CLF (50 mcg/mL) vis-à-vis IL-6 and IL-8 production. **(B)** Mouse alveolar macrophages (AM) treated with antibiotic-exposed CLF (100 mcg/ml) respond differently to polymyxin B (PMB; 10 ng/mL and 10 mcg/mL) inhibition compared to control CLF (100 mcg/mL) vis-à-vis IL-6 production. **(C)** Endotoxin levels in control vs. antibiotic-treated mouse stool measured by LAL assay. *P*-values are represented as follows: * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001. For multiple comparisons using one-way ANOVA, *p*-values are represented as follows: $\alpha < 0.05$; $\alpha\alpha < 0.01$; $\alpha\alpha\alpha < 0.001$; $\alpha\alpha\alpha\alpha < 0.0001$. For comparisons against an untreated or control condition [e.g., in **(B)** CLF treatment without any polymyxin B], *p*-values are represented as follows: $\delta < 0.05$; $\delta\delta < 0.01$; $\delta\delta\delta < 0.001$; $\delta\delta\delta\delta < 0.0001$. All experiments were conducted at least twice and representative data are shown.

high levels of an inhibitory SCFA were present in antibiotic CLF that could reconcile with the corresponding diminished lung inflammation we had previously observed *in vivo* (Prakash et al., 2015). CLF itself contains SCFAs as well as other metabolites, so we measured SCFA and amino acid levels present in CLF samples used thus far (Wild-type C57BL/6 CLF, Control C3H/HeOuJ CLF, Antibiotic C3H/HeOuJ CLF) (**Supplementary Table ST1**). Focusing on three important SCFAs, namely, acetate, propionate and butyrate, we noted that antibiotic treatment did not change butyrate levels, but instead drastically altered the propionate:acetate ratio within CLF (**Figure 3B**). Similarly to butyrate, propionate can also act as an HDAC inhibitor at millimolar concentrations (Chang et al., 2014).

Propionate Effects on LPS-Exposed Cells *in vitro* Also Depend on Concentration and Requires LPS Sensing by TLR4

We next tested the effects of low- and high-dose propionate on LPS responses in mouse AMs and ECs. We observed that low and high-dose propionate effects on ECs and AM LPS responses largely mirrored the effects of low and high-dose butyrate (**Figures 4A,B**), namely, low-dose propionate was able to augment LPS inflammatory responses and high-dose blunted LPS responses. This effect was dependent on TLR4 and not caspase-11 sensing of LPS (**Supplementary Figure S4**). TLR2 ligands' inflammatory effects could also be similarly modulated by low dose and high dose SCFAs (**Supplementary Figure S5**). When we used caspase-11 and TLR4 mutant ECs to study CLF inflammatory effects in the presence of low and high-dose SCFAs, we noted that SCFA effects were largely dependent on the ability of cells to recognize extracellular LPS through TLR4 (data not shown). This indicated to us that LPS (and/or other gut microbiome-derived TLR4 ligands) is likely the major bacterial ligand present in our CLFs.

Propionate:Acetate Ratios Can Result in Augmentation or Reduction of LPS Inflammatory Responses *in vitro*

SCFAs are not uniformly diluted into the systemic circulation after absorption in the gut. In fact, various groups have estimated the levels absorbed by the transit organs between the colonic

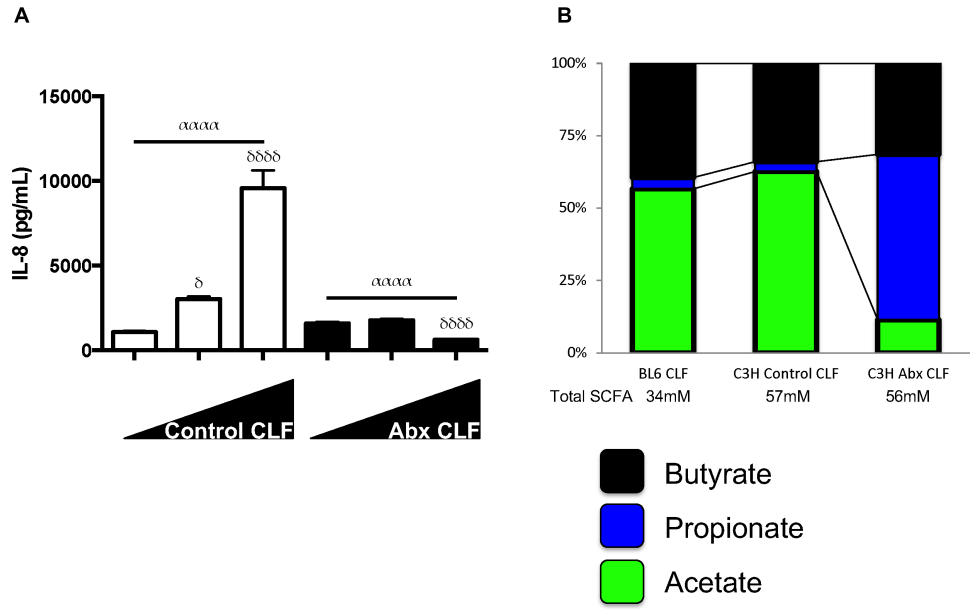

FIGURE 3 | SCFA levels can effect highly divergent inflammatory responses and CLF from control mice and antibiotic-treated mice have different propionate:acetate ratios. **(A)** Increasing dosage of CLF from control or antibiotic-treated animal stool stimulates or inhibits HUVEC inflammatory cytokine (IL-8) production. HUVECs (EC) were treated with increasing amounts of control or antibiotic-exposed CLF (10 mcg/mL, 50 mcg/mL, and 100 mcg/mL) and IL-8 levels measured by ELISA. **(B)** SCFA levels were measured in WT C57BL/6 and WT C3H/HeOuJ mouse stool collected from cages of cohoused mice (5 mice for WT C57BL/6 and 15 mice for control and antibiotic-treated C3H/HeOuJ mice) receiving either control or antibiotic water and pooled before CLF was prepared. Figure shows only on acetate, propionate and butyrate levels. For multiple comparisons using one-way ANOVA, *p*-values are represented as follows: $\alpha < 0.05$; $\alpha\alpha < 0.01$; $\alpha\alpha\alpha < 0.001$; $\alpha\alpha\alpha\alpha < 0.0001$. For comparisons against an untreated or control condition (e.g., 10 mcg/mL CLF treatment), *p*-values are represented as follows: $\delta < 0.05$; $\delta\delta < 0.01$; $\delta\delta\delta < 0.001$; $\delta\delta\delta\delta < 0.0001$.

contents and systemic circulation (Cummings et al., 1979, 1987; Boets et al., 2015). Based on the estimates for intestinal and liver absorption reported by Boets et al. (2015), namely 95% butyrate absorption and 90% propionate, respectively, we estimated the propionate:acetate ratio in the peripheral circulation for control and antibiotic-treated mice (**Supplementary Figure S6**). To examine the inflammation modulating effects of these SCFAs in circulation and in the lung, we exposed mouse lung macrophages *in vitro* to LPS in the presence of varying propionate:acetate ratios. We hypothesized that low propionate:acetate ratios would augment the LPS responses while high ratios would inhibit inflammation. After subjecting AMs to LPS in conjunction with inverse ratios of propionate and acetate, we observed exactly that (**Figure 4C**). Therefore, these *in vitro* data strikingly recapitulated our *in vivo* observations (Prakash et al., 2015) that antibiotic-treatment in mice that targeted mostly gram-negative bacteria within the gut microbiome and resulted in a switch from low to high propionate:acetate ratios within CLF, which in turn, similarly altered lung inflammatory responses *in vivo* and *in vitro*.

In vivo Lung Pretreatment With High Dose Propionate Results in Worsening of *S. aureus* Pneumonia

Acetate levels within the peripheral circulation likely remain constant given their use and production by most gut microbiome species and the lack of major absorption by the intestine and liver (in contrast to propionate and butyrate). Therefore to establish *in vivo* significance to our *in vitro* propionate findings, we chose to address the question of whether altering propionate levels in the lung could affect lung immune responses. We pretreated C57BL/6 wild type mice with high and low dose propionate [1 and 0.1 mM, intratracheally (IT)] and 2 h later subjected them to a *S. aureus* pneumonia with 10^8 CFU (IT). We had previously examined the kinetics of this infection and found that maximal infection was detected 6 h after inoculation and at 24 h the infection had resolved (data not shown). IVIS® *in vivo* imaging was used to image the luminescent *S. aureus* within the lungs *in vivo* (**Figure 5A**) and *ex vivo* (**Figure 5B**) 6 h after infection. Low dose propionate pre-treatment did not alter the levels of *S. aureus* present at 6 h as compared to control mice. However, the high dose propionate group displayed two- to three-fold greater levels of bacteria within the lungs (**Figure 5C**), suggesting that SCFA-mediated attenuation of lung inflammation prior to and during infection diminished the control of the bacterial pneumonia.

Propionate-Producing Bacteria Are Enriched in Gut Microbiomes of Mice With Attenuated Lung Inflammatory Responses

To attempt to correlate physiologic/pathologic responses to lung IR with specific composition of resident gut microbiota within

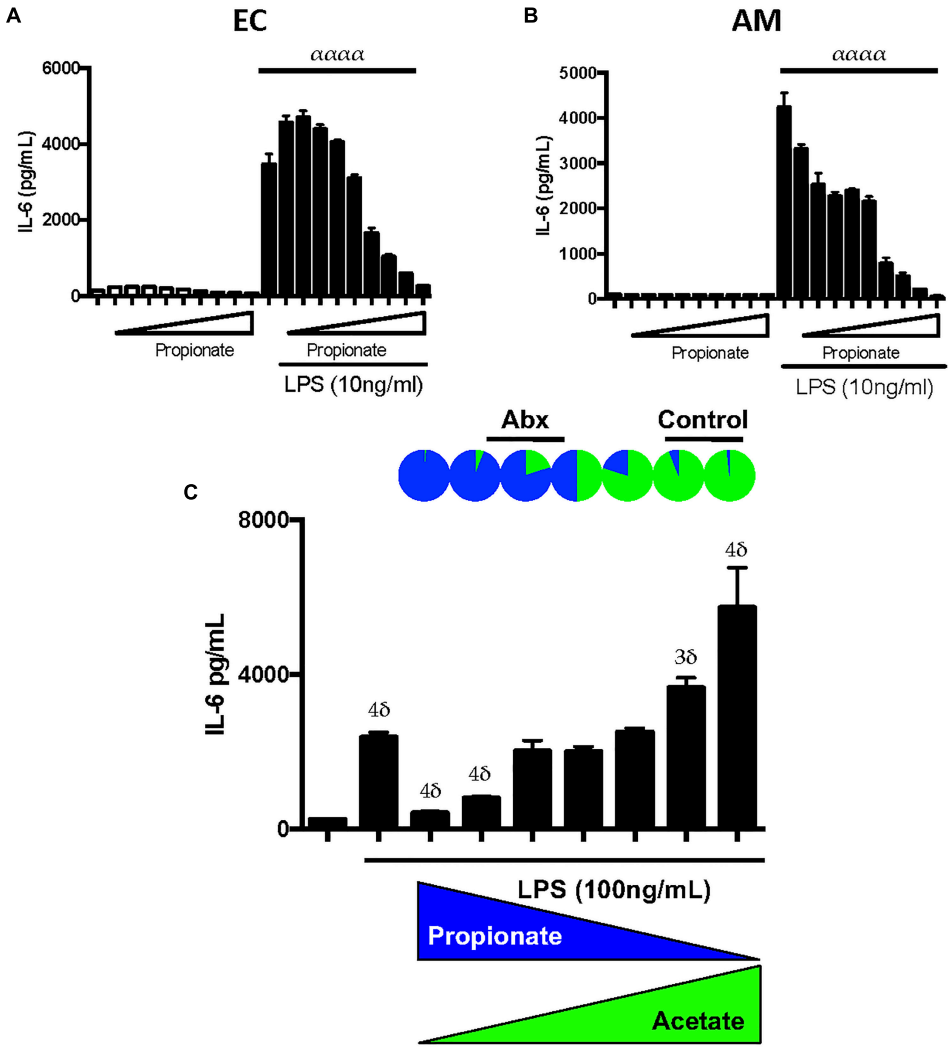

FIGURE 4 | Propionate can have paradoxical effects on LPS inflammatory responses similar to butyrate and propionate:acetate ratios found in control and antibiotic-exposed CLF augment and inhibit LPS-mediated inflammation, respectively. EOMA Endothelial cells (EC) **(A)**, and MH-S Alveolar Macrophage (AM) **(B)** cell lines were treated with a wide dose range of propionate (25 μM–6.4 mM) either in the presence or absence of LPS (10 ng/mL) and overnight production of IL-6 by ELISA was measured. **(C)** AMs were challenged with LPS and inverse levels propionate:acetate, ranging from 4 0mM:10 μM (rightmost) to 10 μM:40 mM (leftmost); therefore, from left to right, propionate was 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 mM, 160, 80, 40, 20, 10 μM, and acetate vice versa. The relative proportions propionate:acetate in control CLF is represented at top as a pie chart and approximate ranges of ratios that correspond to antibiotic CLF and control CLF are noted: Control CLF ratios of propionate:acetate (1:19) as well as estimated ratios in peripheral circulation (1:99; see **Supplementary Figure S7**) and antibiotic CLF ratios of propionate:acetate (5:1) as well as estimated ratios in peripheral circulation (5:4; see **Supplementary Figure S7**). IL-6 levels were measured in the supernatant by ELISA after overnight treatment. For multiple comparisons using one-way ANOVA **(A,B)**, *p*-values are represented as follows: $\alpha < 0.05$; $\alpha\alpha < 0.01$; $\alpha\alpha\alpha < 0.001$; $\alpha\alpha\alpha\alpha < 0.0001$. For comparisons against an untreated or control condition (e.g., LPS only treatment, **C**), *p*-values are represented as follows: $\delta < 0.05$; $2\delta < 0.01$; $3\delta < 0.001$; $4\delta < 0.0001$. All experiments were conducted at least twice and representative data are shown.

a given mouse, we subjected C57BL/6 wild type mice to an 8-week course of either control water or antibiotics (Neo/PMB) and collected stool samples weekly. At the end of 8 weeks, the mice underwent lung IR injury and lung injury was assessed by histology. Surprisingly, we observed greater than expected variation in the mice from each group vis-à-vis their lung injury (**Supplementary Figure S7** and **Supplementary Table ST2**). To understand the source of this variation as well as the effects of the antibiotic treatment on the gut microbiome, we analyzed stool samples after 7 weeks of antibiotic-treatment and prior to lung IR surgery by 16S sequencing. Antibiotic treatment resulted in significantly reduced alpha diversity (increased evenness with reduced richness and alpha diversity by the Faith phylogenetic diversity index but not by Shannon and Simpson phylogenetic diversity indices) (**Supplementary Figure S8**). Firmicutes dominated the microbiome of the control group, while levels of Verrucomicrobia and Bacteriodetes were enriched after the course of antibiotics (**Supplementary Figure S9**). In mice that had a higher lung injury score, alpha diversity was significantly reduced (increased evenness with reduced richness and alpha

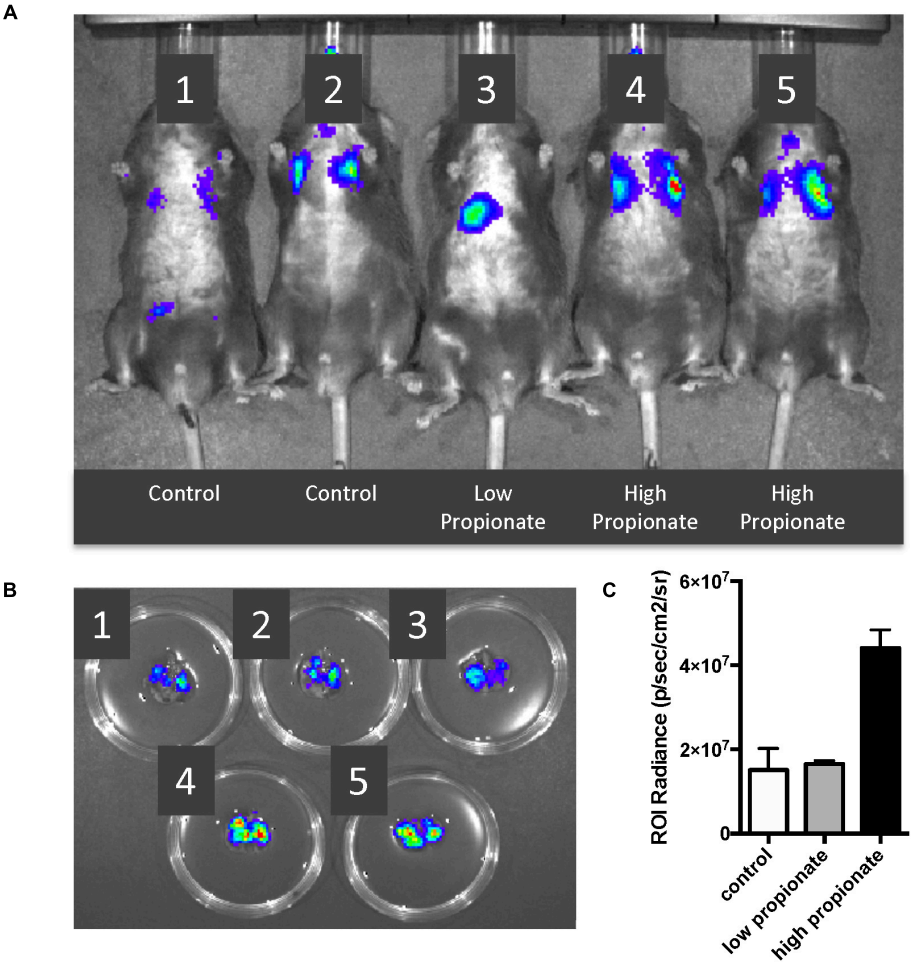

FIGURE 5 | High dose propionate pre-treatment negatively impacts control of *S. aureus* pneumonia. C57BL/6 wild-type mice were pre-treated with high (1 mM) or low (0.1 mM) dose propionate for 2 h, following which they were infected with 10^8 CFU of luminescent *S. aureus*. **(A)** IVIS® *in vivo* imaging of control (Shreiner et al., 2015; Sender et al., 2016), low dose (Alverdy and Krezalek, 2017), and high dose (Cho and Blaser, 2012; Young, 2017) propionate pre-treated mice, 6 h after *S. aureus* infection. **(B)** Immediately after imaging in **(A)**, mice were euthanized and lungs harvested and imaged using IVIS®. **(C)** Levels of luminescence quantified by measurement of luminescence [Region of interest (ROI) radiance]. This experiment as performed twice and combined data are shown in **(C)**.

diversity by the Faith and Simpson phylogenetic diversity indices) (**Supplementary Figure S10** and data not shown). Beta diversity was also significantly different between the control and antibiotic treatment groups as well as between those mice that had a higher vs. lower lung injury score. Differences in bacterial composition was estimated to account for ∼15–20% of the observed lung injury differences (data not shown). Additionally, differences in bacterial community composition were significantly influenced by phylogenetic relatedness, bacterial/presence absence, relative abundance and presence of more and lesser abundant taxa (data not shown). Interestingly, specific OTUs (operational taxonomic units) were enriched (by a Three Model Approach including Negative Binomial Regression, Zero-inflated Negative Binomial Regression, and Poisson Regression with a false discovery rate corrected p-value < 0.2) in mice with *lower lung injury scores*. Eighty percent of the significantly enriched OTUs were members of the phylum Firmicutes, and the orders Bacteriodales and Clostridiales, with the family *Lachnospiraceae* strongly represented (**Supplementary Figure S10**). We observed that low lung injury correlated with higher levels of *Lachnospiraceae* (**Figure 6A**), and this correlation was significant (r^2 0.2123, **Figure 6B**). Coincidently, Bacteriodales and Clostridiales (specifically *Lachnospiraceae*) are the primary producers of propionate within the gut microbiome (Reichardt et al., 2014; Salonen et al., 2014; Louis and Flint, 2017). We also confirmed by metabolomic SCFA analysis that stool of mice with the highest levels of *Lachnospiraceae* (9-fold higher *Lachnospiraceae* than the comparison group) contained ∼150-fold greater levels of propionate (45 mM vs. 300 μM, data not shown).

DISCUSSION

The main findings of these studies indicate that levels of microbiome-derived metabolites, specifically propionate, can influence lung immune and inflammatory responses *in vivo*

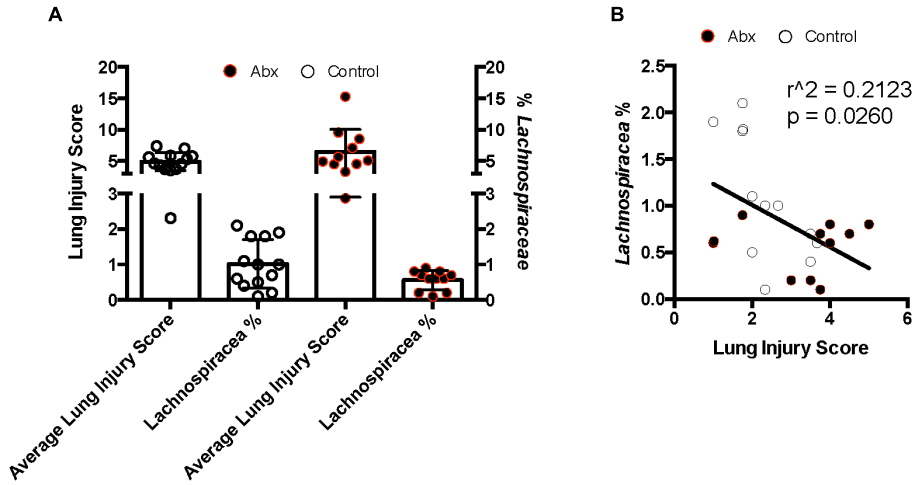

FIGURE 6 | Lung inflammation after ischemia reperfusion (IR) sterile injury negatively correlates with enrichment in *Lachnospiraceae* in both control and antibiotic-treated mice. **(A)** Comparison of average lung injury score by semiquantitative lung injury scoring with percentage of *Lachnospiraceae* present as determined by 16S sequencing and taxonomic OTU determination. **(B)** Correlation of lung injury score with *Lachnospiraceae* enrichment. Pearson correlation coefficient calculated; two-tailed *p*-value presented. Control mice denoted by white circles and antibiotic treated mice by black circles.

and *in vitro*. We began by focusing on colonic lumen filtrate (CLF) from control mice or from mice that received antibiotics given that we had previously showed that antibiotic treatment caused significant alterations in *in vivo* and *ex vivo* lung inflammation to sterile injury (Prakash et al., 2015). First, we provided evidence that LPS was one key component of CLF that resulted in immune activation of cells, and that antibiotic CLF contained less LPS and caused less immune stimulation. We observed an unexpected proinflammatory effect of micromolar concentrations of propionate and butyrate on LPS responses *in vitro*. Compared to control mice, we observed a significant enrichment of CLF propionate concentration in antibiotic-treated mice relative to acetate. Speculating that high propionate:acetate ratios *in vivo* might explain observed attenuated inflammatory responses after lung IR in antibiotic-treated mice, we exposed lung cells *in vitro* to low and high propionate:acetate ratios and reproduced pro- and anti-inflammatory modulation of LPS responses, respectively. To confirm the anti-inflammatory effect of high propionate *in vivo*, we pretreated mouse lungs with high dose propionate, which resulted in worse control of a *S. aureus* pneumonia (unlike low dose propionate). Finally, we found gut microbiome enrichment of high propionate-producing *Lachnospiraceae* among mice with reduced lung inflammation phenotypes. Collectively, the experimental data presented here strongly support a gut-lung immune axis model in which gut-derived SCFAs in combination with CAMPs in circulation profoundly influence lung physiology and immunity.

Evidence in currently published studies support the concept that gut microbiota can influence non-gut disease states. Germ-free mice often have local and systemic physiologic perturbations (reviewed in Erturk-Hasdemir and Kasper, 2013). However, these differences have largely been attributed to altered development of the immune system in the absence of early establishment and maturation of the gut commensal microbiome. Seemingly in conflict with this microbiome-driven immune development concept, other studies have shown that repopulating or altering the gut microbiome in adult mice can rapidly alter their physiology and disease processes (Shreiner et al., 2015). This suggests that highly accessible and circulating factors from the gut microbiome have the capacity to change immune responses in remote host locations (Vieira et al., 2015). Candidate factors that fit this factor profile well include microbially released or dietary metabolites. In fact, segmented filamentous bacteria which can ferment non-digestible starches to produce butyrate and propionate have been observed to confer protection in a number of disease models in mice (Gaboriau-Routhiau et al., 2009 and reviewed in Meyerholz et al., 2002; Ericsson et al., 2014).

Metabolites have been reported to have strong immunomodulatory effects on the host (Arpaia et al., 2013; Chang et al., 2014). Specific metabolites used and produced by the commensal microbiome include short, medium, and long-chain fatty acids, indoles, carbohydrates, gylcolipids, bile acids, vitamins, and other co-factors (reviewed in Shapiro et al., 2014; Levy et al., 2015). These metabolites can regulate different aspects of cell function based on engaging cognate receptors as well as epigenetically. SCFAs, namely acetate, propionate, and butyrate, have known roles as sources of energy (butyrate), effectors of epigenetic changes (propionate and butyrate), and signal transduction (acetate, propionate, and butyrate). At millimolar concentrations, both butyrate and propionate can act as histone deacetylase (HDAC) inhibitors, which silence the transcription of specific inflammatory genes (Chang et al., 2014). Butyrate, in its role as an energy source for colonocytes, can also form part of the switch that converts cells from a metabolic program based on oxidative phosphorylation to one based on glycolysis (i.e., the Warburg effect) (reviewed in Burgess, 2012; Donohoe et al., 2012). At micromolar concentrations, SCFAs

engage and signal through free fatty acid receptors (FFARs) and this engagement has been shown to be important not only locally but also for immune responses in niches not directly in contact with the gut microbiome, such as synovial joints (Vieira et al., 2015) and distal lung airways (Trompette et al., 2014). Other roles, yet to be discovered, are also likely.

We propose that pulmonary immune responses may be calibrated by the levels of commensal-derived SCFAs and CAMPs that transit through the lung and result in immune priming or dampening. In support of this concept, an *in vitro* study has suggested a similar priming phenotype to low-dose SCFA that we report here (Mirmonsef et al., 2012). Other examples of low-dose SCFA effects on immune responses exist, including one in which low levels of circulating acetate specifically promote intestinal IgA responses to microbiota through FFAR2 (GPR43) (Wu et al., 2017). We further propose that low propionate:acetate levels in the lungs create immune priming to support proinflammatory responses. This priming may thus contribute to the generation of healthy baseline immune tone in the homeostatic lung. Conversely, switching to high propionate:acetate levels through gut dysbiosis may reprogram the lung and invoke pathologic or abnormal responses to sterile and infectious challenges.

Studies in mice describe how the gut microbiome is protective against pneumococcal and *S. aureus* pneumonia (Clarke et al., 2010; Gauguet et al., 2015; Brown et al., 2017). Therefore, direct and indirect mechanisms by which the gut commensal microbiome communicates with the lung are of great interest. Some key questions that still need to be answered include the identity of the free-fatty acid receptors (FFARs) that are important for these immune effects of SCFAs and whether or not those FFARs are druggable. Our data that propionate-producing *Lachnospiraceae* are significantly associated with a low lung inflammation phenotype *in vivo* confirm our *in vitro* cell culture data. Since acetate is widely produced by most if not all commensal bacteria and its levels are largely stable in circulation (Reichardt et al., 2014; Louis and Flint, 2017), alterations of propionate production, say by antibiotic exposure, may therefore significantly alter lung immune responses. We have also shown that limiting lung inflammation downstream of lung sterile reperfusion injury can result in a disseminated experimental pneumonia in mice (Tian et al., 2017). Therefore, caution must be exercised when manipulating gut microbiome lest dampening or augmenting lung inflammatory responses cause unintended consequences.

This study has its limitations. SCFAs are just one of many metabolite classes that may have physiologic and pathologic effects, and these include long-chain fatty acids, bile acids, succinate, lactate, and aromatic amino acids (Krishnan et al., 2015) – none of which were studied here. We used the SCFA composition of the stool to estimate SCFA levels in the lung and performed experiments directly administering propionate to the mouse lung. However, direct measurement of SCFA levels in mouse tissue and plasma, while technically challenging, would be more definitive. Our *in vitro* approach to study the effects of SCFAs on lung immune cell and endothelial cell responses will need *in vivo* correlation perhaps through the use of lung-specific FFAR2 and FFAR3 conditional knockout mice. We used human cells early in our studies to confirm that the effects we observed were not limited to mouse cell lines and could be translatable, but further correlation with human data is clearly required. The pulmonary microbiome contributes to lung immunity in disease states such as COPD and though unlikely may also do so in healthy lungs (Dickson et al., 2016). Finally, our work does not exclude the possibility of other members of the microbiome (besides *Lachnospiraceae*) playing important roles as well through their metabolites or released factors that could also modulate lung immunity.

We conclude that specific SCFA metabolites, namely propionate and possibly acetate, are important contributors to the gut-lung immune axis of communication that may augment and suppress lung immune responses. Specifically, high propionate:acetate levels in the lung may be beneficial in situations where lung inflammation suppression is the goal, such as following lung transplant when ischemia reperfusion injury is a threat to graft survival. On the other hand, high propionate:acetate levels that may result from poor antibiotic stewardship and resulting gut microbiome dysbiosis could adversely affect the course of bacterial pneumonia. Propionate may be an unusual non-native compound (believed to be only derivable from the diet or gut microbiome) that has perhaps been adapted for specialized functions making it a fascinating topic for further investigation. Overall, we believe that SCFA levels achieved in the pulmonary parenchyma may be critical for "healthy" or "normal" primed immune responses to lung injury and as such determine the establishment of an overall homeostatic resting lung immune tone. The identification of simple but powerful metabolites and the microbiota from which they originate as important controllers of the gut-lung immune axis may help explain the vast pathophysiologic diversity of human lung injury responses as well as the ever expanding contribution to human heath of diet, lifestyle, environment, immune history, antibiotic and medication use, and hospital-setting exposure. By expanding this knowledge base and understanding, we hope to pave the way toward devising strategies to positively modulate lung immune responses within diverse clinical scenarios.

ETHICS STATEMENT

All mouse studies were approved from an ethical and methodological standpoint by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco and followed the ARRIVE guidelines.

AUTHOR CONTRIBUTIONS

XT performed all *in vitro* and some *in vivo* experiments, analyzed data, and edited manuscript. JH assisted with experimental design, analyzed data, and edited manuscript. AH, HC, and KF engineered and developed the bacterial strains used in the study. AP designed all the experiments, performed mouse surgeries, analyzed data, and wrote and edited manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00159/full#supplementary-material

REFERENCES

Alverdy, J. C., and Krezalek, M. A. (2017). Collapse of the microbiome, emergence of the pathobiome, and the immunopathology of sepsis. *Crit. Care Med.* 45, 337–347. doi: 10.1097/CCM.0000000000002172

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., et al. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504, 451–455. doi: 10.1038/nature12726

Boets, E., Deroover, L., Houben, E., Vermeulen, K., Gomand, S., Delcour, J., et al. (2015). Quantification of *in Vivo* colonic short chain fatty acid production from inulin. *Nutrients* 7, 8916–8929. doi: 10.3390/nu7115440

Brown, R. L., Sequeira, R. P., and Clarke, T. B. (2017). The microbiota protects against respiratory infection via GM-CSF signaling. *Nat. Commun.* 8:1512. doi: 10.1038/s41467-017-01803-x

Budden, K. F., Gellatly, S. L., Wood, D. L. A., Cooper, M. A., Morrison, M., Hugenholtz, P., et al. (2017). Emerging pathogenic links between microbiota and the gut–lung axis. *Nat. Rev. Microbiol.* 15, 55–63. doi: 10.1038/nrmicro.2016.142

Burgess, D. J. (2012). Metabolism: warburg behind the butyrate paradox? *Nat. Rev. Cancer* 12:798. doi: 10.1038/nrc3401

Chang, P. V., Hao, L., Offermanns, S., and Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2247–2252. doi: 10.1073/pnas.1322269111

Cho, I., and Blaser, M. J. (2012). The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/nrg3182

Clarke, T. B., Davis, K. M., Lysenko, E. S., Zhou, A. Y., Yu, Y., and Weiser, J. N. (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16, 228–231. doi: 10.1038/nm.2087

Cummings, J. H., Hill, M. J., Bone, E. S., Branch, W. J., and Jenkins, D. J. (1979). The effect of meat protein and dietary fiber on colonic function and metabolism. II Bacterial metabolites in feces and urine. *Am. Clin. J. Nutr.* 32, 2094–2101. doi: 10.1093/ajcn/32.10.2094

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P., and Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28, 1221–1227. doi: 10.1136/gut.28.10.1221

Dickson, R. P., Erb-Downward, J. R., Martinez, F. J., and Huffnagle, G. B. (2016). The Microbiome and the respiratory tract. *Annu. Rev. Physiol.* 78, 481–504. doi: 10.1146/annurev-physiol-021115-105238

Donohoe, D. R., Collins, L. B., Wali, A., Bigler, R., Sun, W., and Bultman, S. J. (2012). The warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. *Mol. Cell* 48, 612–626. doi: 10.1016/j.molcel.2012.08.033

Ericsson, A. C., Hagan, C. E., Davis, D. J., and Franklin, C. L. (2014). Segmented filamentous bacteria: commensal microbes with potential effects on research. *Comp. Med.* 64, 90–98.

Erturk-Hasdemir, D., and Kasper, D. L. (2013). Resident commensals shaping immunity. *Curr. Opin. Immunol.* 25, 450–455. doi: 10.1016/j.coi.2013.06.001

Fujimura, K. E., Sitarik, A. R., Havstad, S., Lin, D. L., Levan, S., and Fadrosh, D. (2016). Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. *Nat. Med.* 22, 1187–1191. doi: 10.1038/nm.4176

Gaboriau-Routhiau, V., Rakotobe, S., Lécuyer, E., Mulder, I., Lan, A., Bridonneau, C., et al. (2009). The key role of segmented filamentous bacteria in the coordinated maturation of gut helper t cell responses. *Immunity* 31, 677–689. doi: 10.1016/j.immuni.2009.08.020

Gauguet, S., D'Ortona, S., Ahnger-Pier, K., Duan, B., Surana, N. K., Lu, R., et al. (2015). Intestinal microbiota of mice influences resistance to *Staphylococcus aureus* pneumonia. *Infect. Immun.* 83, 4003–4014. doi: 10.1128/IAI.00037-15

Krishnan, S., Alden, N., and Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. *Curr. Opin. Biotechnol.* 36, 137–145. doi: 10.1016/j.copbio.2015.08.015

Levy, M., Thaiss, C. A., Zeevi, D., Dohnalová, L., Zilberman-Schapira, G., Mahdi, J. A., et al. (2015). Microbiota-Modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. *Cell* 163, 1428–1443. doi: 10.1016/j.cell.2015.10.048

Lloyd, C. M., and Marsland, B. J. (2017). Lung homeostasis: influence of age, microbes, and the immune system. *Immunity* 46, 549–561. doi: 10.1016/j.immuni.2017.04.005

Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* 19, 29–41. doi: 10.1111/1462-2920.13589

Mar, J. S., LaMere, B. J., Lin, D. L., Levan, S., Nazareth, M., Mahadevan, U., et al. (2016). Disease severity and immune activity relate to distinct interkingdom gut microbiome states in ethnically distinct ulcerative colitis patients. *mBio* 7:e1072-16. doi: 10.1128/mBio.01072-16

Marsland, B. J., Trompette, A., and Gollwitzer, E. S. (2015). The gut-lung axis in respiratory disease. *Ann. Am. Thorac. Soc.* 12, S150–S156. doi: 10.1513/AnnalsATS.201503-133AW

Meyerholz, D. K., Stabel, T. J., and Cheville, N. F. (2002). Segmented filamentous bacteria interact with intraepithelial mononuclear cells. *Infect. Immun.* 70, 3277–3280. doi: 10.1128/IAI.70.6.3277-3280.2002

Mirmonsef, P., Zariffard, M. R., Gilbert, D., Makinde, H., Landay, A. L., and Spear, G. T. (2012). Short-chain fatty acids induce pro-inflammatory cytokine production alone and in combination with toll-like receptor ligands. *Am. Reprod. J. Immunol.* 67, 391–400. doi: 10.1111/j.1600-0897.2011.01089.x

Prakash, A., Mesa, K. R. R., Wilhelmsen, K., Xu, F., Dodd-O, J. M. M., and Hellman, J. (2012). Alveolar macrophages and toll-like receptor 4 mediate ventilated lung ischemia reperfusion injury in mice. *Anesthesiology* 117, 822–835. doi: 10.1097/ALN.0b013e31826a4ae3

Prakash, A., Sundar, S. V., Zhu, Y. G., Tran, A., Lee, J. W., Lowell, C., et al. (2015). Lung Ischemia Reperfusion (IR) is a sterile inflammatory process influenced

by commensal microbiota in mice. *Shock* 44, 272–279. doi: 10.1097/SHK.0000000000000415

Reichardt, N., Duncan, S. H., Young, P., Belenguer, A., McWilliam Leitch, C., Scott, K. P., et al. (2014). Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *ISME J.* 8, 1323–1335. doi: 10.1038/ismej.2014.14

Salonen, A., Lahti, L., Salojärvi, J., Holtrop, G., Korpela, K., Duncan, S. H., et al. (2014). Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *ISME J.* 8, 2218–2230. doi: 10.1038/ismej.2014.63

Samuelson, D. R., Welsh, D. A., and Shellito, J. E. (2015). Regulation of lung immunity and host defense by the intestinal microbiota. *Front. Microbiol.* 6:1085. doi: 10.3389/fmicb.2015.01085

Sender, R., Fuchs, S., and Milo, R. (2016). Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol.* 14:e1002533. doi: 10.1371/journal.pbio.1002533

Shapiro, H., Thaiss, C. A., Levy, M., and Elinav, E. (2014). The cross talk between microbiota and the immune system: metabolites take center stage. *Curr. Opin. Immunol.* 30, 54–62. doi: 10.1016/j.coi.2014.07.003

Shreiner, A. B., Kao, J. Y., and Young, V. B. (2015). The gut microbiome in health and in disease. *Curr. Opin. Gastroenterol.* 31, 69–75. doi: 10.1097/MOG.0000000000000139

Tian, X., Sun, H., Casbon, A. J., Lim, E., Francis, K. P., Hellman, J., et al. (2017). NLRP3 inflammasome mediates dormant neutrophil recruitment following sterile lung injury and protects against subsequent bacterial pneumonia in mice. *Front. Immunol.* 8:1337. doi: 10.3389/fimmu.2017.01337

Trompette, A., Gollwitzer, E. S., Yadava, K., Sichelstiel, A. K., Sprenger, N., Ngom-Bru, C., et al. (2014). Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat. Med.* 20, 159–166. doi: 10.1038/nm.3444

Vieira, A. T., Macia, L., Galvão, I., Martins, F. S., Canesso, M. C., Amaral, F. A., et al. (2015). A role for gut microbiota and the metabolite-sensing receptor GPR43 in a murine model of gout. *Arthritis Rheumatol.* 67, 1646–1656. doi: 10.1002/art.39107

Vieira, A. T., Rocha, V. M., Tavares, L., Garcia, C. C., Teixeira, M. M., Oliveira, S. C., et al. (2016). Control of *Klebsiella pneumoniae* pulmonary infection and immunomodulation by oral treatment with the commensal probiotic Bifidobacterium longum 5(1A). *Microbes Infect.* 18, 180–189. doi: 10.1016/j.micinf.2015.10.008

Wu, W., Sun, M., Chen, F., Cao, A. T., Liu, H., Zhao, Y., et al. (2017). Microbiota metabolite short-chain fatty acid acetate promotes intestinal IgA response to microbiota which is mediated by GPR43. *Mucosal Immunol.* 10, 946–956. doi: 10.1038/mi.2016.114

Young, V. B. (2017). The role of the microbiome in human health and disease: an introduction for clinicians. *BMJ* 356:j831. doi: 10.1136/bmj.j831

Zhang, N., Francis, K. P., Prakash, A., and Ansaldi, D. (2013). Enhanced detection of myeloperoxidase activity in deep tissues through luminescent excitation of near-infrared nanoparticles. *Nat. Med.* 19, 500–505. doi: 10.1038/nm.3110

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LL37 Inhibits *Aspergillus fumigatus* Infection via Directly Binding to the Fungus and Preventing Excessive Inflammation

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The incidence of *Aspergillus fumigatus* infection and the rate of resistance to antifungal drugs have sharply increased in recent years. LL37 has been reported as a host defense peptide with broad-spectrum antibacterial activities. However, the role of LL37 during *A. fumigatus* infection remains unclear. Here, we examined the interaction between LL37 and *A. fumigatus* and found that synthetic LL37 could directly bind to the surface of *A. fumigatus*, disrupting the integrity of the cell wall *in vitro*. LL37 inhibited mycelial growth in a concentration-dependent manner, rather than fungicidal effect even at high concentration (e.g., 20 μM). Interestingly, low concentrations of LL37 (e.g., 4 μM) significantly attenuated mycelial adhesion and prevented the invasion and destruction of epithelial cells. Following LL37 treatment, the levels of proinflammatory cytokines released by *A. fumigatus*-stimulated macrophages decreased significantly, accompanied by downregulation of M1 type markers. In a mouse model of pulmonary *A. fumigatus* infection, LL37-treated mice showed lower amounts of fungi load, moderate pathological damage, and reduced proinflammatory cytokines. Further, LL37 transgenic mice (LL37+/+) were examined to investigate the effects of endogenous LL37 in an *A. fumigatus* infection model and showed lower susceptibility to *A. fumigatus* infection in comparison with wild-type mice. In addition, LL37 also played a protective role in an immunosuppressed mouse model of *A. fumigatus* infection. Thus, LL37 inhibits *A. fumigatus* infection via directly binding to mycelia and reducing excessive inflammation. LL37 or its analogs may therefore constitute potential drug components for *A. fumigatus* infection.

Keywords: LL37, *Aspergillus fumigatus*, mycelium, inflammation, adhesion

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INTRODUCTION

Aspergillus fumigatus, an opportunistic pathogen widely distributed in nature, is the leading cause of pulmonary Aspergillosis (1). Pulmonary Aspergillosis includes three subtypes—specifically, chronic pulmonary aspergillosis (CPA), allergic bronchopulmonary aspergillosis (ABPA) and invasive pulmonary aspergillosis (IPA)—which are associated with different immune statuses of susceptible hosts. The morbidity and mortality of IPA have sharply increased in recent decades

due to a rise in immunocompromised individuals, such as patients receiving organ transplants or chemotherapy (2). Furthermore, the emergence of antifungal drug resistance limits the effectiveness of clinical treatment (3–5). Even worse, excessive inflammation and severe tissue damage can deteriorate a patient's condition and increase treatment difficulty (6).

LL37, a short fragment composed of 37 amino acids, is the unique member of human cathelicidin antimicrobial peptides (CAMPs). It presents with broad-spectrum antimicrobial activity against various pathogens, including prokaryotic, and fungal organisms (7, 8). Its positive charge allows it to bind to negatively charged phospholipid membranes of prokaryotic cells, prompting membrane penetration, the formation of transmembrane pores, and bacterial lysis (9). On the other hand, LL37 possesses diverse modulating properties on immune system such as the recruitment of inflammatory cells and the release of inflammatory factors, showing both proinflammatory and anti-inflammatory effects (10). LL37 is expressed in a variety of immune cells and epithelial cells. In different types of cells and tissues, LL37 has different physiological concentrations and often increases during infection.

Recently, a few studies have shown that LL37 expression is significantly upregulated in corneal epithelium and nasal tissue in response to *A. fumigatus* (11, 12), suggesting that LL37 may play an important role in *A. fumigatus* infection. Therefore, in the present investigation, we explored the possible effects of LL37 against *A. fumigatus* infection. We tested whether or not LL37 could bind to *A. fumigatus*, destroy cell wall structures and inhibit mycelium growth and adhesion *in vitro*. To test the immunomodulatory effects of LL37, the release of tumor necrosis factor alpha (TNF-α) and interleukin (IL-6) from *A. fumigatus*-stimulated bone marrow-derived macrophages (BMDMs) after LL37 treatment was evaluated. Subsequently, exogenous LL37-treated mice and LL37+/+ mice were involved, respectively to determine the *in vivo* effects of exogenous and endogenous LL37 on fungi clearance, pathological injury, neutrophil infiltration, and cytokine production during *A. fumigatus* infection. Overall, this study demonstrates that LL37 not only directly inhibits *A. fumigatus* hyphae growth and adhesion but also prevents *A. fumigatus*-induced excessive inflammation, thus providing new evidence for the dual therapeutic value of LL37 against *A. fumigatus* infection.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 mice and FVB mice breeding pairs were purchased from the SLAC Laboratory Animal Center (Shanghai, China). LL37+/+ mice were produced via the microinjection of linearized plasmids expressing hCAP18/LL37 into fertilized eggs of mice bred with an FVB genetic background (**Figures S1A,B** in Supplementary Material). All of the mouse strains were housed in specific pathogen-free conditions within an animal care facility (Center of Laboratory Animal, Tongji University, Shanghai, China) until the day of sacrifice. All of the animal experiments were performed under the guidance and with approval from the Institutional Animal Care and Use Committee of Tongji University (Permit Number: TJLAC-015-002).

Reagents

Human cathelicidin LL37 (LLGDFFRKSKEKIGKEFKRIVQRIK DFFRNLVPRTES) with a purity of 95% was purchased from Rockland Immunochemicals (Pottstown, PA, USA). Scrambled form sL37 (RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVK IL) with a purity of 95% was synthesized by GL Biochem (Shanghai, China). Anti-cathelicidin antibody was purchased from Abcam (Cambridge, UK).

A. fumigatus Strains and Culture

Conidia (*A. fumigatus* train, Af293) harvest and growth into swollen conidia and hyphae were performed as described previously (13). Briefly, fungi were inoculated on Sabouraud Dextrose Agar slant and cultured at 37°C for 7 days. Conidia were collected with phosphate-buffered saline (PBS), filtered through a 40-μm nylon mesh, then stored at 4°C for use. To obtain swollen conidia (SC) and hyphae, resting conidia (RC) were incubated in Roswell Park Memorial Institute (RPMI)-1640 media at 37°C for 8 h to achieve swelling and for an additional 2 h to achieve germination.

Cell Culture

BMDMs from mice were prepared as previously described (14). Bone marrow was extracted from the femur and tibia of 6–8-week-old female C57BL/6 mice. Cells were centrifuged following the removal of erythrocytes and then were differentiated into BMDMs in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum, 30% L929 supernatant, 1% antibiotic-antimycotic, and 0.1% β-Mercaptoethanol.

Transmission Electron Microscopy (TEM)

Aspergillus fumigatus conidia were treated with 4 μM LL37 or sL37 and incubated in RIPM-1640 medium for 24 h. Subsequently, the mycelia were pelleted by centrifugation and prefixed in a solution of 5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h. Samples were then washed three times with 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide for 3 h and were dehydrated with increasing concentrations of ethanol or acetone (i.e., 50, 70, 90, 100%), respectively. Following embedding and fixation, samples were cut into ultrathin sections using an ultramicrotome. After staining with uranyl acetate and lead citrate, the ultrathin sections were viewed under a JEOL JEM-1230 (80KV) transmission electron microscope.

Hyphae Growth Inhibition Assay

Aspergillus fumigatus conidia were incubated in RPMI-1640 medium with different concentrations of LL37 or sL37 at 37°C for 12 h. Hyphae length was then measured under microscopy. Each group included at least 10 visual fields containing ≥50 hyphae.

Adhesion Assay

Adhesion assay was performed as previous described (15). *A. fumigatus* conidia were incubated in a 96-well plate with RPMI-1640 media in the presence of different concentrations of LL37 or sL37 at 37°C for 24 h. The supernatant was removed and then the wells were washed three times with PBS. Adhesive capacity was estimated by staining the biofilms that had not been

washed off with 0.5% crystal violet for 15 min. Then excess stain was washed with PBS for three times. Afterwards, the biofilms were decolorized with 95% ethanol. The density of the biofilms was analyzed by determining the absorbance of the decolorized solution at 570 nm. At the same time, the wells both before and after washing were photographed under a microscope.

Epithelial Cell Damage Assay

A mouse alveolar epithelial cell line (MLE12) were plated on a 48-well plate in a monolayer formation and infected with *A. fumigatus* conidia in the presence of LL37 or sL37. Following incubation for 16 h, the supernatant was collected and transferred to a 96-well plate. The LDH released in the supernatant was detected using the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega Corp., Madison, WI, USA) according to the manufactures' instructions. Then the corrected values in the following formula were used to compute percent cytotoxicity:

$$\text{Percent cytotoxicity} = 100 \times \frac{\text{Experimental LDH Release (OD 490)}}{\text{Maximum LDH Release (OD 490)}}$$

The Maximum LDH Release means a positive control (i.e., treatment with detergent).

Binding of LL37 and *A. fumigatus* Analysis

Aspergillus fumigatus RC, SC or hyphae were incubated with or without 4 μM of LL37 dissolved in PBS for 30 min at room temperature. The samples were then washed three times with PBS and fixed in 4% paraformaldehyde for an hour before being blocked with 5% fetal bovine serum albumin (BSA) for an hour. Following washing with PBS for three times, samples were incubated with anti-LL37 antibody overnight at 4°C. Following washed with PBS for three times, samples were incubated with a FITC-labeled secondary antibody for an hour at room temperature. The RC samples were analyzed using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) while SC and hyphae samples were visualized and imaged using a confocal microscope (Nikon Inc., Tokyo, Japan).

Cytokines Secretion

The 3×10^5 BMDMs were plated on a 48-well plate in a monolayer and infected with 1.5×10^6 UV-killed *A. fumigatus* swell conidia or 3×10^5 hyphae in the presence of different concentrations of LL37 or sL37. After stimulation for 16 h, the supernatant was collected. The concentration of TNF-α and IL-6 in the supernatant was detected using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufactures' instructions.

In vitro Killing Assay

Here, the 3×10^6 BMDMs were plated on a 12-well plate in a monolayer and infected with 6×10^5 viable *A. fumigatus* conidia in the presence or absence of 4 μM LL37 or sL37. After incubation for 6 h at 37°C, the fungi were scraped off and diluted with sterile PBS. Afterwards, SDA plates were inoculated with diluted solution and cultured at 37°C for 48 h. The killing capacity was estimated by counting the number of fungi colonies.

Phagocytosis Assay

A phagocytosis assay was based on and developed as described previously(16). Briefly, *A. fumigatus* conidia were pretreated with a Fluoro Tag™ FITC Conjugation Kit (Sigma-Aldrich, St. Louis, MO, USA) and peritoneal macrophages were isolated from C57/BL6 mice as described previously (17). Then peritoneal macrophages were cultured on a 12-well plate in a monolayer and each well was inoculated with FITC-labeled *A. fumigatus* conidia to achieve a multiplicity of infection (MOI) equal to 10. After 1 h of coincubation at 37°C with 5% CO_2, the wells were washed with PBS before digested by 0.25% trypsin to collect the adherent cells. Then, the collected macrophages were labeled with anti-CD11b PerCP cy5.5 (M1/70) (eBioscience, San Diego, CA, USA) and samples were analyzed using a BD flow cytometer. The percentages of phagocytosis were calculated by the ratio of $FITC^+$ macrophages to all macrophages.

Murine Models of Pulmonary Aspergillus Infection

Mice were anesthetized by isoflurane inhalation, and then $(2\text{-}10) \times 10^6$ *A. fumigatus* conidia in 35 μl of PBS were instilled into the trachea by pressing tongue intratracheal instillation. For the immunosuppressed model, mice were administered with 40 mg/kg of the corticosteroid, triamcinolone acetonide (TargetMol, Boston, MA, USA) injected subcutaneously 1 day prior to infection, as previously described (18). At the indicated times, mice were killed and lung tissues were isolated for detection of CFU, inflammatory cells, and cytokines as well as histopathological analysis.

Fungal Burden Analysis

Mice were killed at the indicated times after infection and lungs were dissected carefully, excised, and homogenized in PBS. For CFU, the homogenates were serially diluted and spread onto SDA plates. After incubated at 37°C overnight, the colonies were counted and normalized to lung weights.

Histopathological Analysis

Lung tissues isolated from mice were fixed in 10% buffer formalin, dehydrated, and embedded in paraffin. Then the lungs were cut into sections and stained with hematoxylin & eosin (HE) or Gomori's methenamine silver stain (GMS) according to standard staining procedures at pathology platform of Servicebio Technology, Wuhan, China.

Flow Cytometry Analysis

Lung tissues from mice were digested with collagenase to establish a single-cell suspension as described previously (13). Briefly, lungs were dissected and cut into very small pieces before incubated in collagenase digestion solution (10 mg collagenase type II in 10 ml PBS) for 1 h at 37°C. Next, the digested lungs were sieved through 40 mm sieve and treated with RBC lysis buffer. Then, cells were washed and stained with the following monoclonal antibodies: anti-mouse CD45-FITC (30-F11), anti-mouse Ly6G-BV421 (1A8), anti-mouse CD11b-PerCP cy5.5 (M1/70) (eBioscience, San Diego, CA, USA), and anti-mouse CD11c-APC (HL3), anti-mouse SiglecF-PE (E50-2440)

(BD Biosciences, San Jose, CA, USA), after which the cells were analyzed by a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Next, the data were analyzed with the Flow-Jo software: First, all of the cells were defined via FCS-A and SSA-A and then single cells were determined via SSC-A and FCS-W. Afterwards, we defined $CD45^+$ cells as immune cells in lung tissue, from which $Ly6G^+CD11c^+$ cells were gated as neutrophils and $SiglecF^+CD11c^+$ cells were gated as macrophages, respectively (**Figure S2** in Supplementary Material).

RNA Extraction and Real-Time Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Takara, Kusatsu, Japan). Reverse transcription was performed by utilizing a Primescript RT reagent kit (Takara, Kusatsu, Japan). Real-time quantitative PCR measurement was performed using SYBR Green reagent kit (Vazyme, Nanjing, China) as per the manufacturer's instructions, and the ABI 7500 sequence detection system. The amounts of transcript were normalized to GAPDH. Relative mRNA expression was plotted as fold changes calculated using the $\Delta\Delta$Ct method. The primers used for real-time RT-PCR were as follows: iNOS (forward: 5′- ACATCGA CCCGTCCACAGTAT−3′; reverse: 5′- CAGAGGGGTAGGCT TGTCTC−3′); CXCL9 (forward: 5′- ATCTCCGTTCTTCAGT GTAGCAATG-3′; reverse: 5′- ACAAATCCCTCAAAGACCTC AAACAG-3′); CXCL10 (forward: 5′- AGGGGAGTGATGGAG AGAGG-3′; reverse: 5′- TGAAAGCGTTTAGCCAAAAAAGG-3′); GAPDH (forward: 5′- AGGTCGGTGTGAACGGATTTG-3′; reverse: 5′- TGTAGACCATGTAGTTGAGGTCA-3′).

Statistical Analysis

Statistical analysis was performed using GraphPad Prim 5.0 software. The graph represents at least three dependent experiments and data were presented as mean ± Standard Deviation (SD). Log-rank testing was used to evaluate the equality of survival curves. The other statistical differences were determined by the two-tailed unpaired *t*-test and one-way ANOVA. It was considered statistically significant when $p < 0.05$.

RESULTS

LL37 Binds Directly to *A. fumigatus* and Destroys Cell Wall Integrity

To investigate the direct antimicrobial activity of LL37 against *A. fumigatus*, we initially performed immunofluorescence staining of LL37. The direct binding of LL37 to the surface of *A. fumigatus* resting conidia was proved by flow cytometer after 30 min of coincubation, as well as swell conidia and hyphae observed under confocal microscopy (**Figures 1A–C**). Furthermore, to assess the effect of LL37 on the structure of *A. fumigatus*, mycelia grown from conidia in the presence of 4 μM LL37 were visualized by transmission electron microscope. The images revealed obvious alterations of cell wall integrity prompted by LL37—that is, the cell walls were more likely to aggregate into clumps and the apical plasma membrane to separate from the cell wall to form folds (**Figure 1D**). Together, these data indicate that LL37 binds directly to *A. fumigatus* and destroys cell wall integrity.

LL37 Inhibits *A. fumigatus* Hyphae Growth and Adhesion *in vitro*

To explore the effect of LL37 on the biological activity of *A. fumigatus*, we measured the hyphal length of *A. fumigatus* following 10 h of incubation with LL37 or sL37 at concentration gradients. Our results showed that hyphal growth was significantly inhibited by LL37 in a concentration-dependent manner (**Figure 2A**). Unexpectedly, we noticed that *A. fumigatus* were still viable even at high concentration of 20 μM LL37, which indicates that LL37 plays a role in inhibiting the growth of mycelium rather than sterilization. Furthermore, this inhibiting effect on hyphae growth resulted in impaired biofilm formation (**Figure 2B**). Crystal violet staining was applied to quantify the adherent biofilm density and it was shown that adherent biofilm formation was markedly attenuated even at a low dose of LL37 treatment ($\leq$ 4 μM) (**Figure 2C**). To further exam whether or not LL37 impacts *A. fumigatus* invasion and destruction of epithelial cells, alveolar epithelial cells were incubated with *A. fumigatus* in the presence of LL37 or sL37 for 16 h, and then the secretion of LDH in the supernatant was measured to identify the cell death rate. The results indicated that LL37 attenuated *A. fumigatus* invasion and cytotoxicity on epithelial cells (**Figure 2D**). Overall, these findings suggest that LL37 inhibited *A. fumigatus* hyphae growth and adhesion *in vitro*.

LL37 Inhibits *A. fumigatus*-Induced Pro-Inflammatory Cytokine Production in Macrophages

To assess the influence of LL37 on inflammatory activity in *A. fumigatus*-infected macrophage, BMDMs were stimulated with ulraviolet-killed or heat-killed *A. fumigatus* hyphae or swollen conidia in combination with different concentrations of LL37 or sL37 for 16 h, after which point, TNF-α and IL-6 in the supernatant were detected. In comparison with sL37 treatment, LL37 significantly reduced the *A. fumigatus*-induced TNF-α and IL-6 production in macrophages and it performed as a dose-dependent manner in ulreaviolet-killed groups (**Figures 3A–F**).

Meanwhile, we examined the transcriptional levels of macrophage polarization-related markers. Consistent with the reduced production of proinflammatory cytokines, LL37 also downregulated the mRNA expressions of iNOS, CXCL9 and CXCL10 (**Figures 3G–I**), which related to M1-type macrophages. However, the levels of M2-type macrophage—related markers, such as arginase and Fizz, were very low in all of groups following *A. fumigatus* stimulation (data not shown).

In addition, we wondered whether LL37 affected macrophage phagocytosis and killing capacity on *A. fumigatus* as it decreased inflammatory cytokines production, so we incubated *A. fumigatus* conidia with macrophages in the presence of LL37 or sL37. However, both LL37 and sL37 did not attenuate the phagocytosis or killing capacity of macrophages against *A. fumigatus* (**Figures 3J,K**). Together, those data suggest that LL37 inhibits *A. fumigatus*-induced proinflammatory cytokine production from macrophages and downregulates M1-type markers without affecting macrophage phagocytosis or killing capacity.

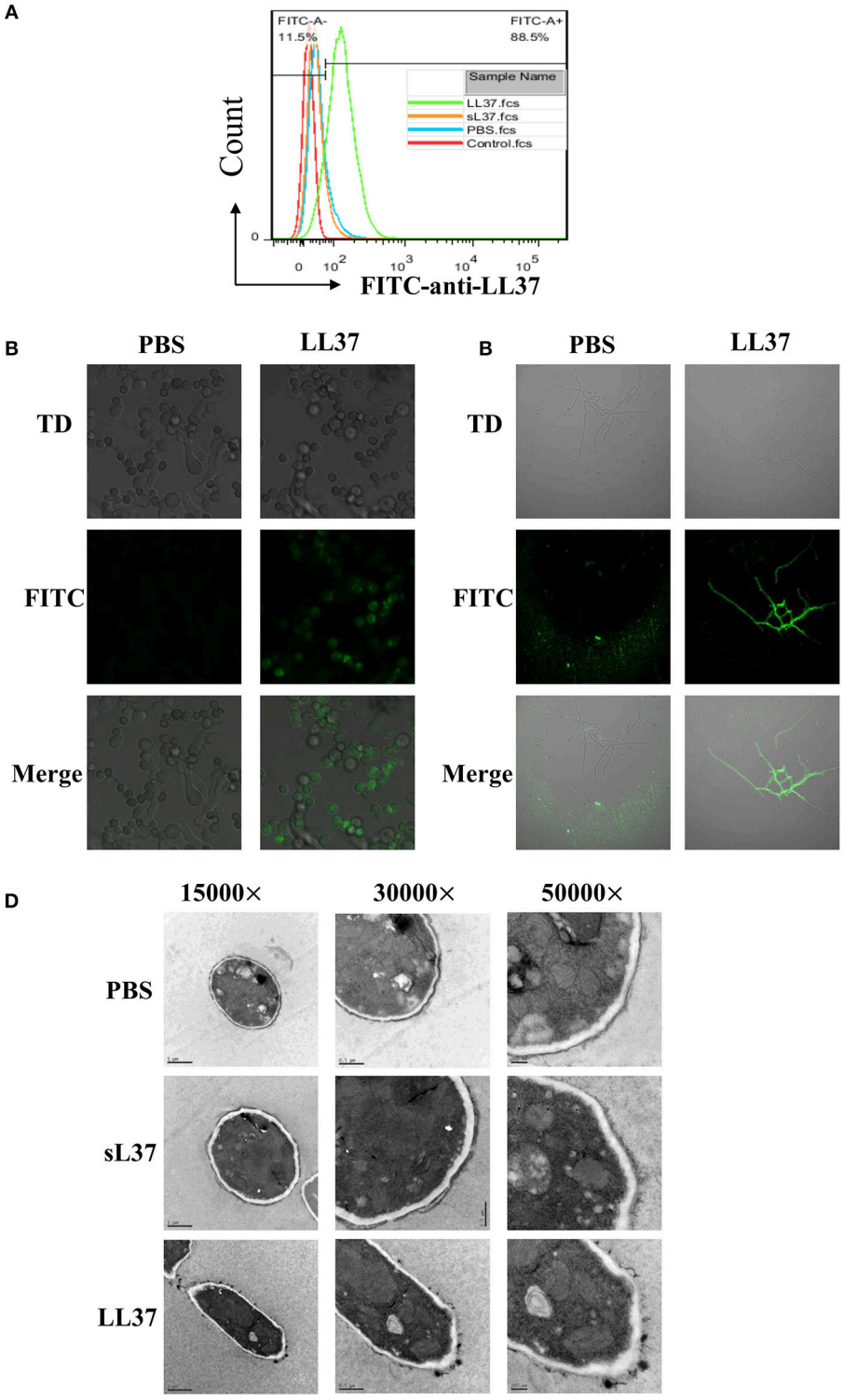

FIGURE 1 | The direct interaction between LL37 and *A. fumigatus*. *A. fumigatus* resting conidia, swell conidia and hyphae were incubated with or without LL37 dissolved in PBS at room temperature for 30 min. After being washed and fixed, samples were stained with anti-LL37 antibody and FITC-labeled secondary antibody. Resting conidia **(A)** was analyzed by flow cytometer. Swell conidia **(B)** and hyphae **(C)** were visualized and imaged using a Nikon confocal microscope. **(D)** *A. fumigatus* conidia were incubated with LL37, sL37, or PBS for 24 h to grow into hyphae state. Following fixation and embedding, samples were cut into ultrathin sections and analyzed by transmission electron microscope. The data are representative of three independent experiments. PBS, phosphate buffer saline.

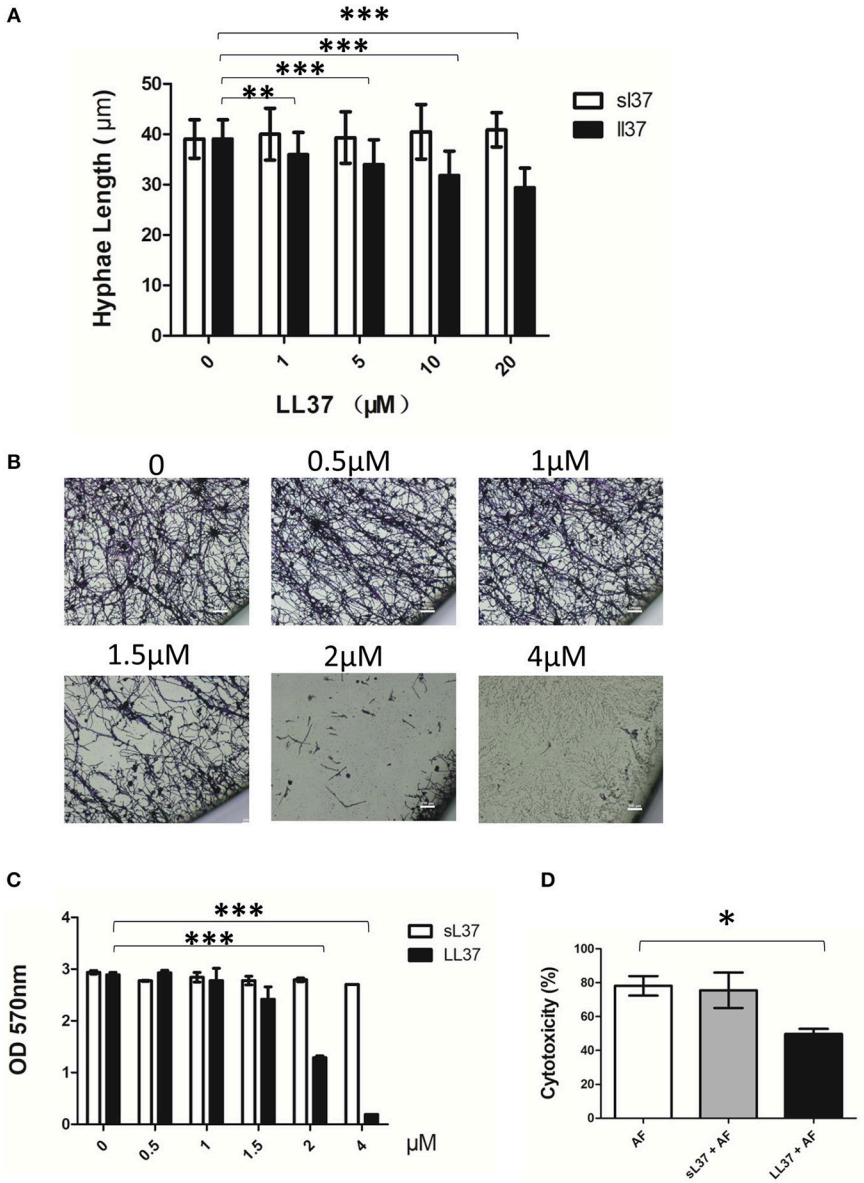

FIGURE 2 | Effects of LL37 on *A. fumigatus* hyphal growth and adhesion. **(A)** *A. fumigatus* were incubated with different concentration of LL37 or sL37(0, 1, 5, 10, 20 μM) at 37°C for 12 h. Hyphal lengths were determined under a microscope. For each variable, at least 50 hyphal measurements were recorded. **(B,C)** *A. fumigatus* were incubated on polystyrene plates with different concentration of LL37 or sL37(0, 0.5, 1, 1.5, 2, 4 μM) at 37°C for 24 h. The wells were washed with PBS and stained with crystal violet. Then the wells were photographed under a microscope and the absorbance of decolorized solution at 570 nm after decoloring by ethanol was determined. **(D)** Alveolar epithelial cells (1×10^5) were incubated with *A. fumigatus* (1×10^6) in the presence or absence of 4 μM LL37 or sL37 at 37°C for 16 h. Cell viability was analyzed by the LDH released in the supernatant. The bars represent the mean values and the standard deviation. The data are representative of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. AF, *Aspergillus fumigatus*. sL37, scrambled-LL37.

LL37 Promotes Fungi Clearance and Alleviates Lung Pathological Injury in an *A. fumigatus*-Infected Mouse Model

The findings described above indicate that LL37 inhibits *A. fumigatus in vitro*, however, the role of LL37 in the *A. fumigatus* infection *in vivo* is not clear. To investigate this, we infected mice via intratracheal administration of 2×10^7 *A. fumigatus* conidia followed by LL37 peptide solution or PBS instillation. As compared with mice in the control group, LL37-treated mice infected with *A. fumigatus* showed significantly lower fungi burdens in the lungs at both 2 and 7 days (**Figures 4A,B**; **Figure S3** in Supplementary Material). Subsequently, we performed histological analysis of lung tissues from mice at 7 days after *A. fumigatus* infection. PASM staining was used to visualize the fungal distribution and revealed that the majority of fungi were confined around the trachea and

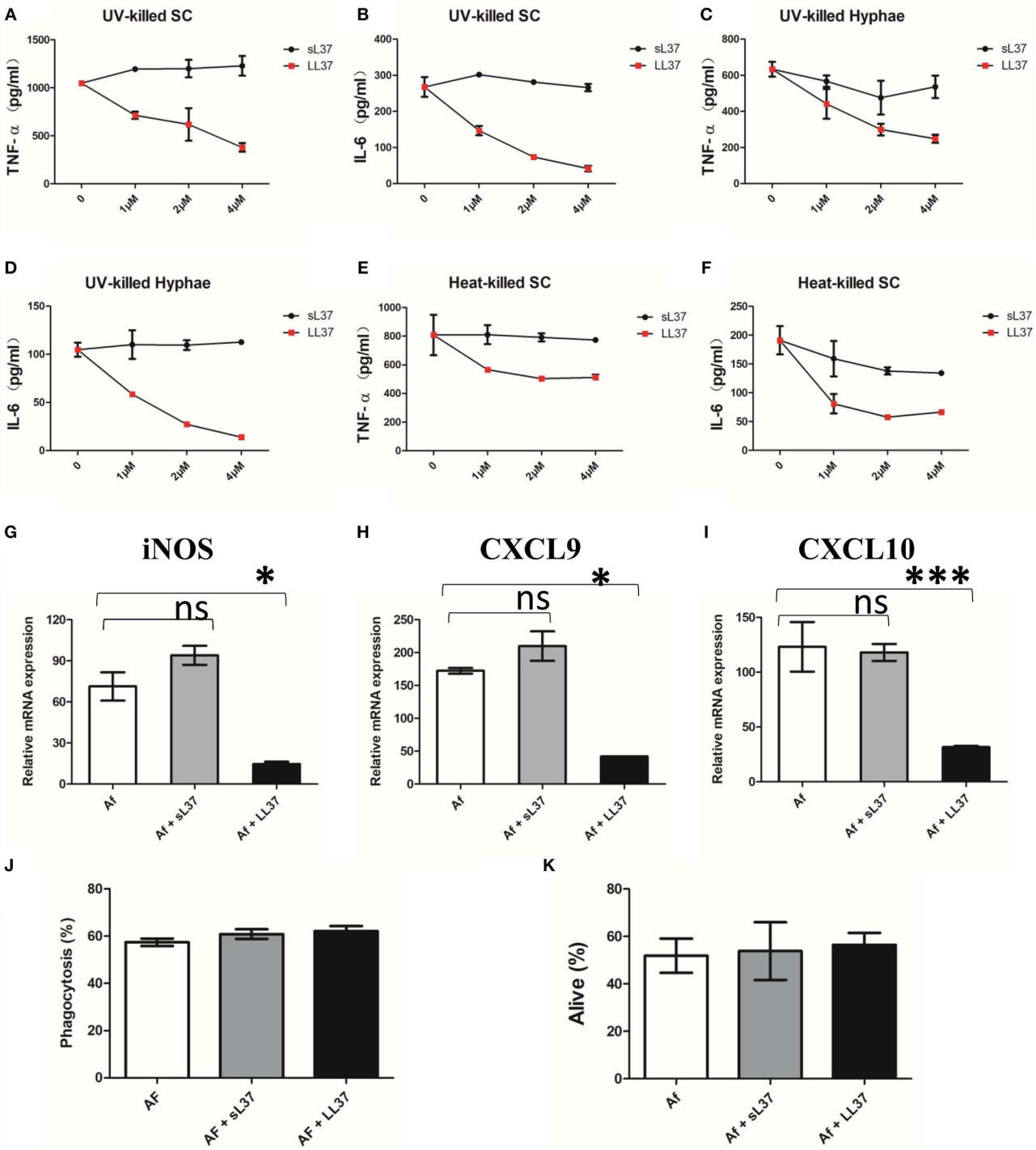

FIGURE 3 | Effects of LL37 on the activation of *A. fumigatus*-infected macrophages. **(A–F)** Macrophages were incubated with ultraviolet-killed or heat-killed *A. fumigatus* swell conidia (MOI = 5:1) or hyphae (MOI = 1:1) in the presence of LL37 or sL37(0, 1, 2, 4 μM). TNF-α and IL-6 levels were determined at 16 h after stimulation. **(G–I)** Macrophages were incubated with ultraviolet-killed *A. fumigatus* swell conidia (MOI = 5:1) in the presence or absence of 4 μM LL37 or sL37. The relative mRNA expression of iNOS, CXCL9, CXCL10 were determined at 12 h after stimulation. **(J)** Macrophages were cocultured with FITC-labeled *A. fumigatus* conidia (MOI = 10) in the presence or absence of 4 μM LL37 or sL37 for 1 h. Then the macrophages were stained with anti-CD11b PerCP cy5.5 before being analyzed using a BD flow cytometer. The percentages of phagocytosis were calculated by the ratio of $FITC^+$ macrophages to all macrophages. **(K)** Macrophages were incubated with viable *A. fumigatus* conidia (MOI = 1:5) in the presence or absence of 4 μM LL37 or sL37 for 6 h. The fungi were scraped off and diluted with PBS and then cultured on SDA plates to count colonies. The bars represent the mean values and standard errors of the means. The data are presented as the mean ± SD and representative of three independent experiments. $^*P < 0.05$; $^{***}P < 0.001$. TNF-α, tumor necrosis factor-α. IL-6, interleukin-6. SC, swell conidia. UV, ultraviolet. iNOS, inducible nitric oxide synthase. CXCL, Chemokine (C-X-C motif) ligand. sL37, scrambled-LL37.

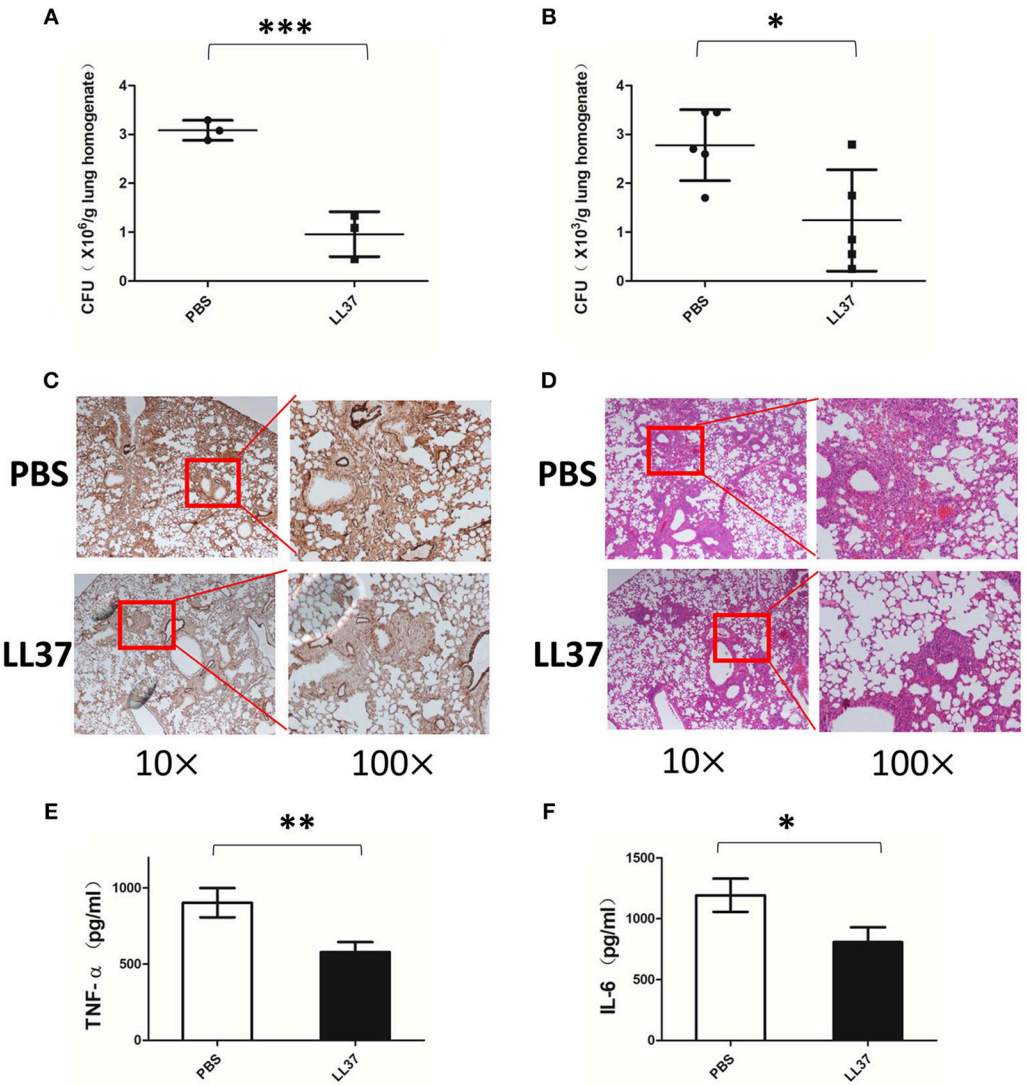

FIGURE 4 | Exogenous LL37 acts on controlling *A. fumigatus* infection *in vivo*. C57 BL/6 mice were infected via intratracheal administration of 2×10^7 *A. fumigatus* conidia, followed by supplement with 35 μL LL37(0.1 mg/mL) or PBS. **(A,B)** The fungal burden in lung tissues were determined at 2 days and 7 days after *A. fumigatus* infection, respectively. **(C,D)** Histopathological examination by PASM and HandE staining of mouse lungs were conducted at 7 days after *A. fumigatus* infection. The levels of TNF-α **(E)** and IL-6 **(F)** in lung homogenates were determined after 2 days of *A. fumigatus* infection. The data are representative of three independent experiments. The bars represent the mean values and standard deviation. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. TNF-α, tumor necrosis factor-α. IL-6, interleukin-6. PBS, phosphate buffer saline.

bronchi in the lungs of LL37-treated mice, whereas *A. fumigatus* hyphae had spread into the alveolar tissues of the lungs of the control group (**Figure 4C**). Additionally, hematoxylin and eosin (H&E) staining of lung tissues revealed that a greater number of immune cells accumulated and the more severe phenomenon of congestion and structural damage happened in the mice of the control group vs. LL37-treated mice (**Figure 4D**). These results collectively indicated that LL37 prompted *A. fumigatus* clearance and controlled the hyphae invasion, which alleviated pathological damage.

LL37 Inhibits *A. fumigatus*-Induced Pro-inflammatory Cytokines *in vivo*

Since LL37 inhibits *A. fumigatus*-induced pro-inflammatory cytokines *in vitro*, we sought to investigate whether the anti-inflammatory effects of LL37 were also available *in vivo*. The cytokine levels in lung homogenate were analyzed at 2 days after *A. fumigatus* infection. In line with our hypothesis, the release of TNF-α and IL-6 from the lungs of LL37-treated mice was significantly lower than that from the control group mice (**Figures 4E,F**).

Endogenous LL37 Plays a Protective Role in *A. fumigatus* Infection

Considering the complex interactions among molecules in the immune system, we constructed LL37 transgenic mice to explore whether endogenous LL37 played a protective role in *A. fumigatus* infection. We infected LL37+/+ and wild-type mice via intratracheal administration of 2×10^7 *A. fumigatus* conidia. Both 2 days and 7 days after infection, the fungi load showed that the LL37+/+ mice had significantly smaller number of *A. fumigatus* in the lungs than did the wild-type mice (**Figures 5A,B**). At 7 days after infection, histopathological analysis was performed. PMSF staining revealed that wild-type mice had significantly more *A. fumigatus* invading lesions vs. the LL37+/+ mice (**Figure 5C**). H&E staining suggested that the lungs of wild-type mice were heavily infiltrated by inflammatory cells and the congestion and tissue destruction were more obvious in these mice vs. in the LL37+/+ mice (**Figure 5D**).

Endogenous LL37 Alleviates Inflammation Caused by *A. fumigatus* Infection

To examine the effects of endogenous LL37 on the inflammatory response mediated by *A. fumigatus* infection, we analyzed inflammatory cells and inflammatory cytokines in mouse lung. The results showed that the lung tissues of LL37+/+ mice released less TNF-α and IL-6 than did those of wild-type mice after 2 days of infection (**Figures 5E,F**). Using flow cytometry, we additionally observed that LL37+/+ mice performed significantly reduced number and percentage of neutrophils in the lung vs. mice of the control group, who showed plenty of neutrophils (i.e., over 40% of total cells) recruited to the lung (**Figures 6A–C**). In contrast, there was little difference in the number or percentage of macrophages between LL37+/+ mice and control group mice, which both showed an obvious descent of macrophages after *A. fumigatus* infection (**Figures 6D–F**).

LL37 Plays a Protective Role in an *A. fumigatus*-Infected Immunosuppressed Mouse Model

Considering the data above, we still wondered whether the protective effects of LL37 *in vivo* were directly implemented on the fungus or indirectly enacted by reducing the associated inflammation. Further, we examined the role of LL37 during infections in corticosteroid-treated mice, which imitated the effects of immunosuppression. Under these conditions, LL37-treated mice infected with *A. fumigatus* for 2 days showed obviously lower fungi burdens in the lungs as compared with mice in the control group (**Figure 7A**). Furthermore, the survival rate curve suggested that LL37-treated mice were significantly less susceptible to *A. fumigatus* infection vs. control group ones (**Figure 7B**). These results indicated that the direct inhibition of LL37 on *A. fumigatus* contributed to its protective role *in vivo*.

DISCUSSION

LL37, as a host defense peptide, is known to have a broad antibacterial spectrum, including against both Gram-negative and Gram-positive bacteria (10). Meanwhile, the fungicidal activity of LL37 against *Candida albicans* (19–21) and CAMP from other species against *Cryptococcus neoformans* have also been reported recently (22). Therefore, our interest was piqued regarding exploring the biological effects of human-derived LL37 on *A. fumigatus*, which is the most important pathogen of aspergillosis. The current study provided evidence that LL37 bind to *A. fumigatus* and destroyed its architecture, resulting in inhibiting hyphae growth and adhesion *in vitro*. Meanwhile, LL37 prevented *A. fumigatus*-induced aggressive macrophages activation. Further, both exogenous and endogenous LL37 prompted the elimination and prevented the invasion of fungi *in vivo*, and also reduced pathological damage and inflammation.

As mentioned above, the bactericidal activity of LL37 has been described as the model of peptide-membrane interaction. In contrast to the structure and composition of the bacterial cell membrane, fungi show thicker cell walls and different polysaccharides that contain more zwitterionic phospholipids and sterols (23, 24). Thus, LL37 may exert different mechanism against fungi. Regarding another common fungal pathogen, *C. albicans*, studies have reported that LL37 performs its fungicidal effect through membrane permeability as well as by affecting fungal structural integrity and altering cell wall composition (20, 21, 25). Our results suggested that LL37 could bind to the surface of *A. fumigatus,* which was confirmed by fluorescence confocal microscopy. Further, we observed that LL37 disrupted the cell wall structure and caused the cytoplasmic membrane at the apical side to separate from the cell wall to form a wavy structure under the electron microscope. However, the mechanism of LL37 acting on *A. fumigatus* directly via activating glycanase or forming pores on cell wall/membrane, or indirectly via triggering stress response pathways, remains to be further studied.

Our experiments additionally suggested LL37 inhibited mycelial growth in a dose-dependent manner, but we noted that *A. fumigatus* could still grow into hyphae that were just a little shorter than the normal at high concentration of LL37 (e.g., 20 μM). This is consistent with the findings of previous literature reports that indicated the minimum inhibitory concentrations of five CAMP peptides derived from cattle, sheep and pigs against *Candida* and *Cryptococcus* are in the range of 0.5–32 μM, while filamentous fungi, such as *Aspergillus*, are less susceptible to these peptides (22).

Interestingly, while the mycelial growth of *A. fumigatus* was almost unaffected at low concentrations of LL37 (e.g., 4 μM), its adhesion ability in comparison was significantly diminished. Therefore, the primary function of LL37 on *A. fumigatus* may tend to inhibit invasion rather than act in a fungicidal manner, which is not the same effects as on bacteria. This is in line with our *in vivo* experiments wherein pathological foci showed that *A. fumigatus* in the lungs of LL37 transgenic mice was mainly confined around trachea and bronchi but disseminated to the alveolar and pulmonary vessels in wild-type mice. As with *A. fumigatus*, LL37 significantly inhibited the adhesion of *C. albicans*, which is related to the elevation of β-1,3-exoglucanase activity (26). However, further research is needed to investigate the mechanism(s) how LL37 inhibits adhesion and invasion of *A. fumigatus*.

Other studies have demonstrated that LL37 inhibits inflammation by neutralizing LPS and inhibiting TLR4 activation

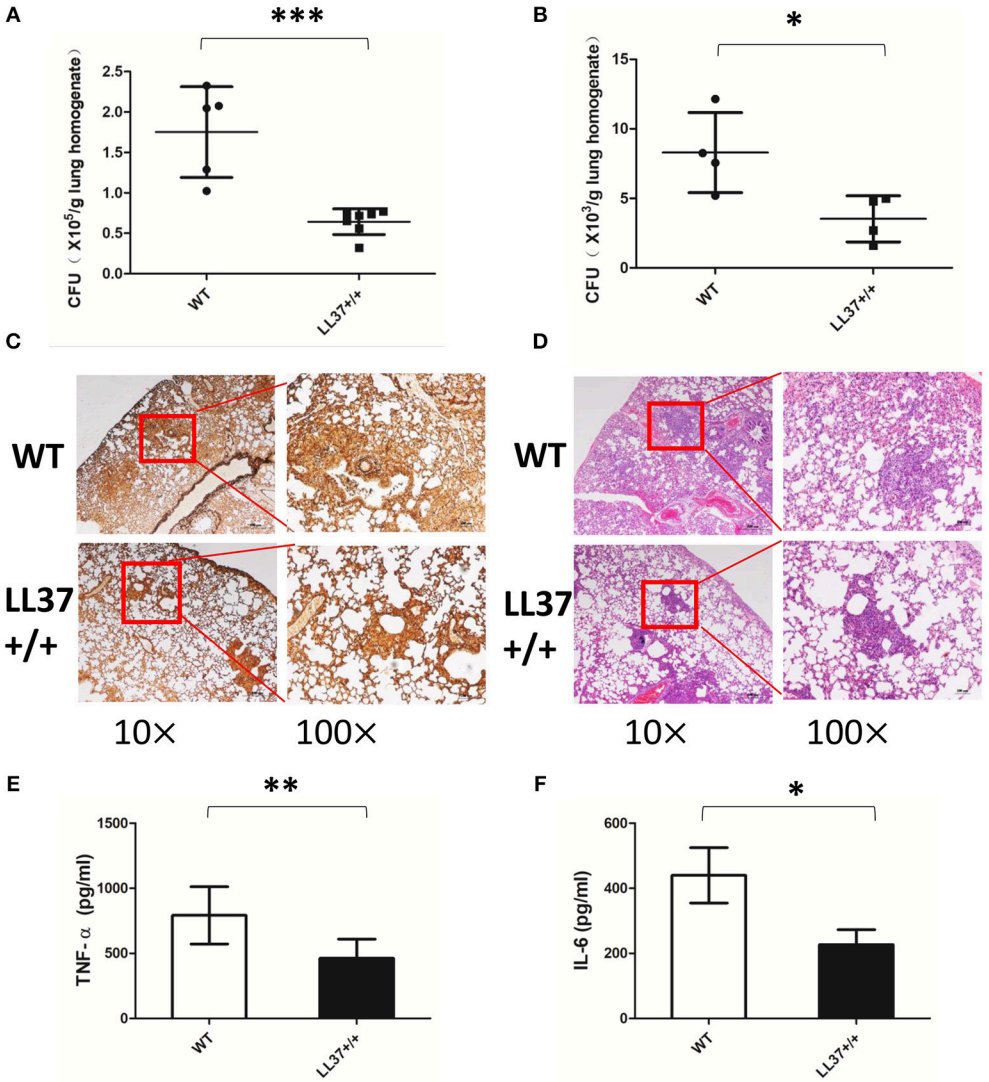

FIGURE 5 | Endogenous LL37 promotes fungi eradication and alleviates pathological injury. Wild-type mice and LL37+/+ mice were infected by way of intratracheal administration of 2×10^7 *A. fumigatus* conidia. After 2 days **(A)** and 7 days **(B)**, the fungal burden in the lungs were analyzed. **(C,D)** PASM and H&E staining outcomes of lungs tissues were analyzed after 7 days. TNF-α **(E)** and IL-6 **(F)** in lung homogenates were determined after 2 days. The bars represent the mean values and standard deviation. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. WT, wild type. TNF-α, tumor necrosis factor-α. IL-6, interleukin-6.

in bacterial infections (27–30). The present investigation suggested that TNF-α and IL-6 levels from *A. fumigatus*-stimulated macrophages incubating with LL37 also decreased. Given that cell wall components often serve as PAMPs to trigger pro-inflammatory responses in innate cells such as macrophages, there is reason to speculate that impaired cell wall integrity by LL37 accounts, at least partly, for reduced production of pro-inflammatory cytokines. However, there was no change of TNF-α and IL-6 production in LL37 treated-macrophages in the absence of infection vs. in untreated group, neither in macrophages infected by Aspergillus pre-treated with LL37 or sL37 for an hour vs. by untreated *A. fumigatus* (data not shown). In order to distinguish the direct fungistatic and immunomodulatory effects of LL37, we treated *A. fumigatus* by ultraviolet inactivation and heat inactivation treatment, followed by stimulation. The results showed that LL37 could still significantly inhibit the production of TNF-α and IL-6 both in ultraviolet- and heat- killed groups, which indicated LL37 mainly acted by immunomodulation rather than via direct fungistatic effects to reduce inflammation in the situation we set.

To explore how LL37 regulates inflammation in *A. fumigatus*-infected macrophages, we examined the expression of macrophage polarization-related makers and found that LL37 downregulated M1 type markers, such as iNOS, CXCL9 and CXCL10. It has been reported that LL37 can directly induce macrophages to differentiate into M1 type with

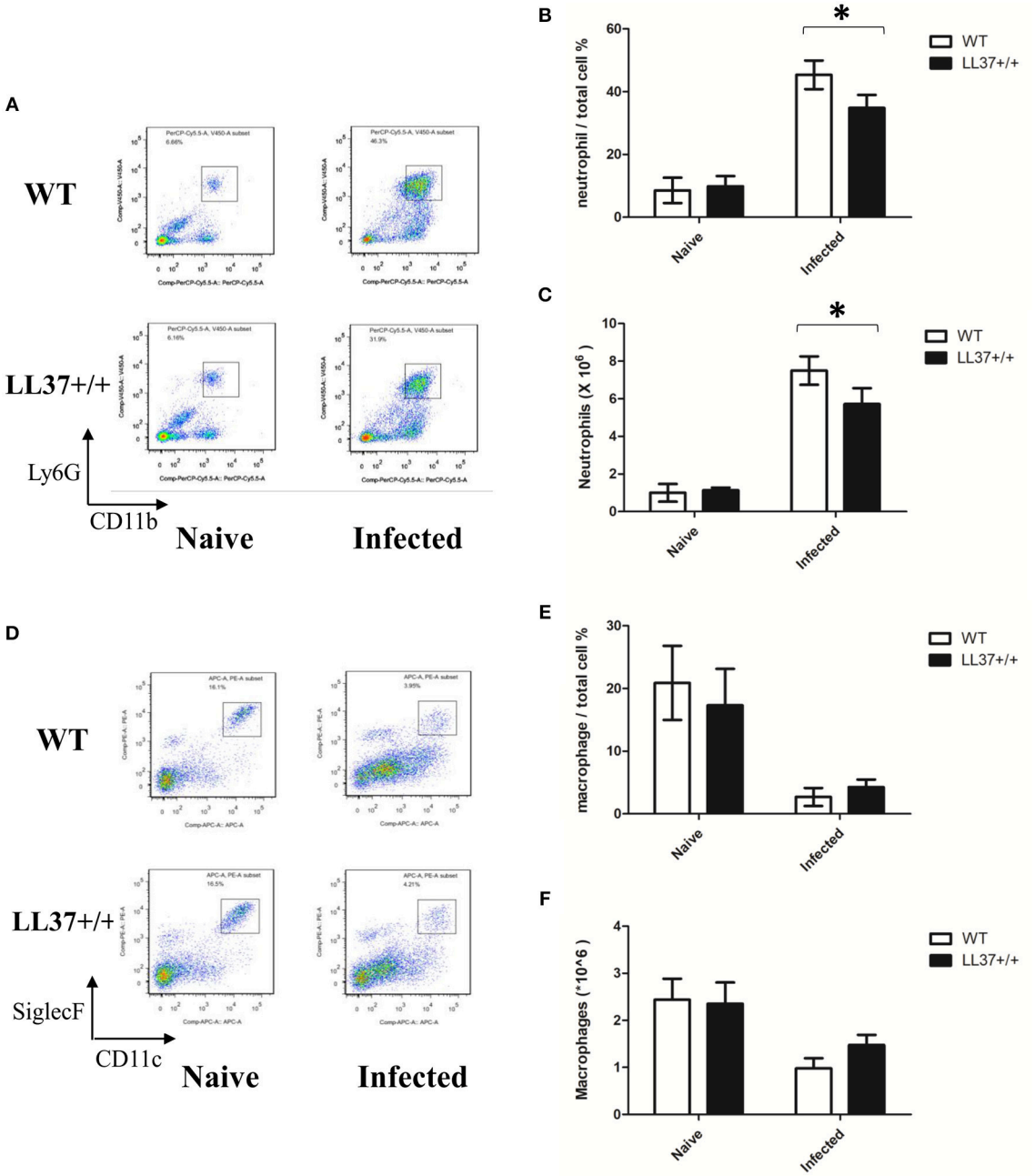

FIGURE 6 | Effects of endogenous LL37 on neutrophils and macrophage infiltration in the lung infected by *A. fumigatus*. **(A–F)** Wild-type mice and LL37+/+ mice were infected via intratracheal administration of 2×10^7 *A. fumigatus* conidia. After 2 days, the number of total cells in the lungs was counted, while the percentage of neutrophil and macrophage percentages were analyzed by flow cytometry. The data are representative of three independent experiments. The bars represent the mean values and standard deviation. *$P < 0.05$. WT, wild type.

proinflammatory effects (31). Therefore, we can assume that the immunomodulatory effect of LL37 is closely related with the type of pathogens.

Inflammatory response is a reasonable manifestation of a host's defense against invasive pathogens; however, excessive inflammation can cause damage to the host tissues and even adversely affect the pathogen clearance (6, 32, 33). As seen in our animal experiments, the percentage of neutrophils in the lungs of wild-type mice infected with *A. fumigatus* was as high as 40%, which resulted in severe edema, congestion and destruction of lung tissue. In contrast, the lungs of LL37+/+ mice experienced a relatively lesser degree of inflammatory cells infiltration, more effective pathogen clearance, and reduced lung damage. Although *A. fumigatus* is clinically susceptible to immunosuppressed patients, it also occurs in patients with high levels of inflammation such as chronic granulomatous disease or cystic fibrosis, who show host damage and impaired fungi eradication (34, 35). Therefore, for the treatment of aspergillosis,

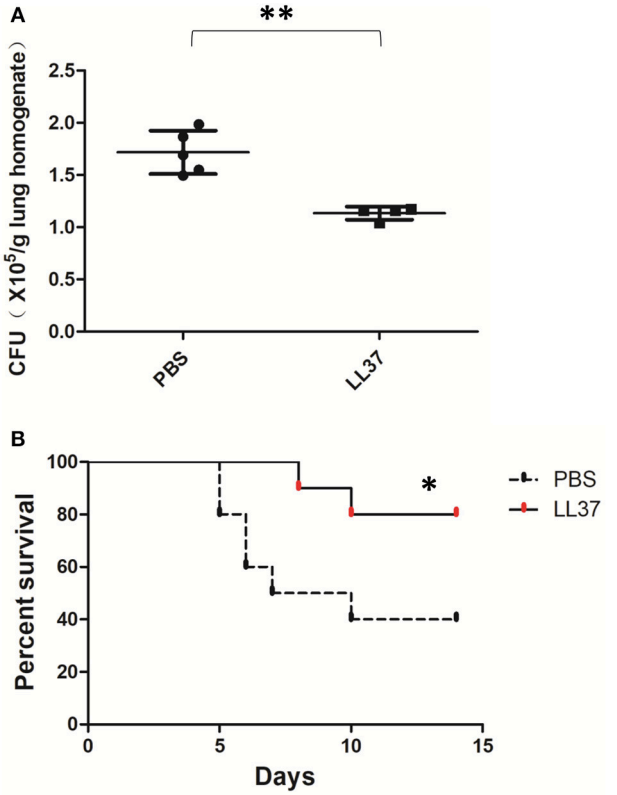

FIGURE 7 | Effects of LL37 in an *A. fumigatus*-infected immunosuppressed mouse model. Corticosteroid-treated mice were infected via intratracheal administration of *A. fumigatus* conidia (2×10^6 for fungal burden, 5×10^6 for survival), followed by supplement of 35 μL LL37 (0.1 mg/mL) or PBS. **(A)** The fungal burden in lung tissues was determined after 2 days of *A. fumigatus* infection. **(B)** Survival was monitored for 2 weeks. $^{**}P < 0.05$. $^{*}P < 0.05$.

in addition to considering the killing of fungi, the promotion of protective immune response regulation is also crucial.

Furthermore, our data demonstrated that LL37 still reduced the degree of susceptibility to *A. fumigatus* in immunosuppressive mouse models and suggested that the direct inhibition of LL37 on *A. fumigatus* contributed to its protective role *in vivo*. We suppose that LL37 significantly inhibits mycelium adhesion and invasion *in vivo* as shown in the *in vitro* experiments.

However, *in vitro* experiments cannot explain the role of LL37 in different microenvironments *in vivo* (36, 37). Even if exogenously synthesized LL37 was used in mouse experiments, it could not fully reflect the complex interactions that may occur in the innate immune system. In this study, we established LL37 transgenic mice by microinjection of linearized plasmids expressing hCAP18/LL37 into fertilized eggs and demonstrated that endogenous LL37 had a protective effect against *A. fumigatus* infection. As this transgenic mouse overexpressed LL37 systemically, further experiments are needed to identify the alveolar epithelium or neutrophils where the LL37 derived from that play a major role.

It is worth noting that a recent study reported that LL37 promoted the growth of *A. fumigatus* (38). However, in addition to the differences between fungal strains we used (Af293 vs. ATCC strain), the authors were mainly concerned about the effects of much lower levels of LL37 (i.e., 5 μg/ml, approximately 1 μM) than us at mostly higher than 1 μM. In growth experiments, their lower concentrations of LL37 (7.8–1.9 μg/ml) were more conducive to *A. fumigatus* growth, while we focused on the inhibition of hyphal length and adhesion by larger doses of LL37. As the authors explained in the discussion, a low level of stress may activate compensatory growth pathways in *A. fumigatus*. Therefore, it is necessary to carry out further rigorous experiments to determine the LL37 concentration threshold that causes completely opposite effects before LL37 can be used to treat *A. fumigatus* infection in the future.

In conclusion, LL37 directly inhibits *A. fumigatus* mycelial growth and its adhesion and invasion ability. Meanwhile, there is beneficial immunomodulatory effect—LL37 attenuates the *A. fumigatus*-induced excessive inflammatory response. Taken together, it can be surmised that LL37 exerts a dual protective effect in the treatment of *A. fumigatus* infection and it and its analogs may be potential drug components for use against *A. fumigatus* infection.

AUTHOR CONTRIBUTIONS

X-LL designed, performed, and analyzed the experiments and wrote the manuscript. J-XL designed, performed and analyzed the experiments. J-FX and X-MJ designed, analyzed and provided overall guidance for the experiments, wrote and revised the manuscript. H-RH helped with the experiments and interpretation of the data. J-LD, R-XD, R-JT, LY, and JH contributed to the experiments.

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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.2019.00283/full#supplementary-material

REFERENCES

1. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. *Sci Transl Med.* (2012) 4:165rv113. doi: 10.1126/scitranslmed.3004404
2. Segal BH. Aspergillosis. *N Engl J Med.* (2009) 360:1870–84. doi: 10.1056/NEJMra0808853
3. Verweij PE, Mellado E, Melchers WJ. Multiple-triazole-resistant aspergillosis. *N Engl J Med.* (2007) 356:1481–3. doi: 10.1056/NEJMc061720
4. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, et al. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. *Emerg Infect Dis.* (2011) 17:1846–54. doi: 10.3201/eid1710.110226
5. Vermeulen E, Lagrou K, Verweij PE. Azole resistance in *Aspergillus fumigatus*: a growing public health concern. *Curr Opin Infect Dis.* (2013) 26:493–500. doi: 10.1097/QCO.0000000000000005
6. Carvalho A, Cunha C, Iannitti RG, De Luca A, Giovannini G, Bistoni F, et al. Inflammation in aspergillosis: the good, the bad, and the therapeutic. *Ann N Y Acad Sci.* (2012) 1273:52–9. doi: 10.1111/j.1749-6632.2012.06754.x
7. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol.* (2004) 75:39–48. doi: 10.1189/jlb.0403147
8. Torrent M, Pulido D, Rivas L, Andreu D. Antimicrobial peptide action on parasites. *Curr Drug Targets* (2012) 13:1138–47. doi: 10.2174/138945012802002393
9. Yin LM, Edwards MA, Li J, Yip CM, Deber CM. Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions. *J Biol Chem.* (2012) 287:7738–45. doi: 10.1074/jbc.M111.303602
10. Kahlenberg JM, Kaplan MJ. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol.* (2013) 191:4895–901. doi: 10.4049/jimmunol.1302005
11. Ooi EH, Wormald PJ, Carney AS, James CL, Tan LW. Fungal allergens induce cathelicidin LL-37 expression in chronic rhinosinusitis patients in a nasal explant model. *Am J Rhinol.* (2007) 21:367–72. doi: 10.2500/ajr.2007.21.3025
12. Zhang Y, Wu J, Xin Z, Wu X. *Aspergillus fumigatus* triggers innate immune response via NOD1 signaling in human corneal epithelial cells. *Exp Eye Res.* (2014) 127:170–8. doi: 10.1016/j.exer.2014.07.025
13. Xu X, Xu JF, Zheng G, Lu HW, Duan JL, Rui W, et al. CARD9(S12N) facilitates the production of IL-5 by alveolar macrophages for the induction of type 2 immune responses. *Nat Immunol.* (2018) 19:547–60. doi: 10.1038/s41590-018-0112-4
14. Huang HR, Li F, Han H, Xu X, Li N, Wang S, et al. Dectin-3 Recognizes Glucuronoxylomannan of *Cryptococcus neoformans* Serotype AD and *Cryptococcus gattii* Serotype B to Initiate Host Defense Against Cryptococcosis. *Front Immunol.* (2018) 9:1781. doi: 10.3389/fimmu.2018.01781
15. Lin CJ, Sasse C, Gerke J, Valerius O, Irmer H, Frauendorf H, et al. Transcription factor SomA is required for adhesion, development and virulence of the human pathogen *Aspergillus fumigatus*. *PLoS Pathog* (2015) 11:e1005205. doi: 10.1371/journal.ppat.1005205
16. Herbst S, Shah A, Mazon Moya M, Marzola V, Jensen B, Reed A, et al. Phagocytosis-dependent activation of a TLR9-BTK-calcineurin-NFAT pathway co-ordinates innate immunity to *Aspergillus fumigatus*. *EMBO Mol Med.* (2015) 7:240–58. doi: 10.15252/emmm.201404556
17. Zhu QY, Liu Q, Chen JX, Lan K, Ge BX. MicroRNA-101 targets MAPK phosphatase-1 to regulate the activation of MAPKs in macrophages. *J Immunol.* (2010) 185:7435–42. doi: 10.4049/jimmunol.1000798
18. Shepardson KM, Jhingran A, Caffrey A, Obar JJ, Suratt BT, Berwin BL, et al. Myeloid derived hypoxia inducible factor 1-alpha is required for protection against pulmonary *Aspergillus fumigatus* infection. *PLoS Pathog.* (2014) 10:e1004378. doi: 10.1371/journal.ppat.1004378
19. Wong JH, Ng TB, Legowska A, Rolka K, Hui M, Cho CH. Antifungal action of human cathelicidin fragment (LL13-37) on *Candida albicans*. *Peptides* (2011) 32:1996–2002. doi: 10.1016/j.peptides.2011.08.018
20. Ordonez SR, Amarullah IH, Wubbolts RW, Veldhuizen EJ, Haagsman HP. Fungicidal mechanisms of cathelicidins LL-37 and CATH-2 revealed by live-cell imaging. *Antimicrob Agents Chemother* (2014) 58:2240–8. doi: 10.1128/AAC.01670-13
21. Tsai PW, Cheng YL, Hsieh WP, Lan CY. Responses of *Candida albicans* to the human antimicrobial peptide LL-37. *J Microbiol.* (2014) 52:581–9. doi: 10.1007/s12275-014-3630-2
22. Benincasa M, Scocchi M, Pacor S, Tossi A, Nobili D, Basaglia G, et al. Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts. *J Antimicrob Chemother* (2006) 58:950–9. doi: 10.1093/jac/dkl382
23. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev.* (2003) 55:27–55. doi: 10.1124/pr.55.1.2
24. Peckys DB, Mazur P, Gould KL, de Jonge N. Fully hydrated yeast cells imaged with electron microscopy. *Biophys J.* (2011) 100:2522–9. doi: 10.1016/j.bpj.2011.03.045
25. Tsai PW, Yang CY, Chang HT, Lan CY. Human antimicrobial peptide LL-37 inhibits adhesion of *Candida albicans* by interacting with yeast cell-wall carbohydrates. *PLoS ONE* (2011) 6:e17755. doi: 10.1371/journal.pone.0017755
26. Chang HT, Tsai PW, Huang HH, Liu YS, Chien TS, Lan CY. LL37 and hBD-3 elevate the beta-1,3-exoglucanase activity of Candida albicans Xog1p, resulting in reduced fungal adhesion to plastic. *Biochem J.* (2012) 441:963–70. doi: 10.1042/BJ20111454
27. Rosenfeld Y, Papo N, Shai Y. Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action. *J Biol Chem.* (2006) 281:1636–43. doi: 10.1074/jbc.M504327200
28. Di Nardo A, Braff MH, Taylor KR, Na C, Granstein RD, McInturff JE, et al. Cathelicidin antimicrobial peptides block dendritic cell TLR4 activation and allergic contact sensitization. *J Immunol.* (2007) 178:1829–34. doi: 10.4049/jimmunol.178.3.1829
29. Brown KL, Poon GF, Birkenhead D, Pena OM, Falsafi R, Dahlgren C, et al. Host defense peptide LL-37 selectively reduces proinflammatory macrophage responses. *J Immunol.* (2011) 186:5497–505. doi: 10.4049/jimmunol.1002508
30. Coorens M, Schneider VAF, de Groot AM, van Dijk A, Meijerink M, Wells JM, et al. Cathelicidins Inhibit Escherichia coli-Induced TLR2 and TLR4 Activation in a viability-dependent manner. *J Immunol.* (2017) 199:1418–28. doi: 10.4049/jimmunol.1602164
31. van der Does AM, Beekhuizen H, Ravensbergen B, Vos T, Ottenhoff TH, van Dissel JT, et al. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. *J Immunol.* (2010) 185:1442–9. doi: 10.4049/jimmunol.1000376
32. Romani L, Puccetti P. Protective tolerance to fungi: the role of IL-10 and tryptophan catabolism. *Trends Microbiol.* (2006) 14:183–9. doi: 10.1016/j.tim.2006.02.003
33. Perfect JR. The impact of the host on fungal infections. *Am J Med* (2012) 125:S39–51. doi: 10.1016/j.amjmed.2011.10.010
34. Romani L, Fallarino F, De Luca A, Montagnoli C, D'Angelo C, Zelante T, et al. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature* (2008) 451:211–5. doi: 10.1038/nature06471
35. King J, Brunel SF, Warris A. Aspergillus infections in cystic fibrosis. *J Infect.* (2016) 72 (Suppl.)S50–5. doi: 10.1016/j.jinf.2016.04.022
36. Bals R, Weiner DJ, Meegalla RL, Wilson JM. Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. *J Clin Invest.* (1999) 103:1113–7. doi: 10.1172/JCI6570
37. Bals R, Weiner DJ, Moscioni AD, Meegalla RL, Wilson JM. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect Immun* (1999) 67:6084–9.
38. Sheehan G, Bergsson G, McElvaney NG, Reeves EP, Kavanagh K. The human cathelicidin antimicrobial peptide LL-37 promotes the growth of the pulmonary pathogen *Aspergillus fumigatus*. *Infect Immun.* (2018). doi: 10.1128/IAI.00097-18. [Epub ahead of print].

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GENERAL COMMENTARY
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Commentary: Gut microbiome–mediated bile acid metabolism regulates liver cancer via NKT cells

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Keywords: gut microbiome, bile acid, liver cancer, NKT cells, immune regulation

A Commentary on

Gut microbiome–mediated bile acid metabolism regulates liver cancer via NKT cells
by Ma, C., Han, M., Heinrich, B., Fu, Q., Zhang, Q., Sandhu, M., et al. (2018). Science 360:eaan5931. doi: 10.1126/science.aan5931

A study by Ma et al. (1) showed that gut microbiome composition in mice closely associates with liver cancer by influencing the immune system. This group provided evidence showing that changing commensal gut bacteria in mice affected the accumulation of hepatic $CXCR6^{+}$ natural killer T (NKT) cells through mediation of CXCL16 expression in liver sinusoidal endothelial cells. CXCL16 is the only ligand for the chemokine receptor CXCR6, which mediates NKT cell survival and accumulation in the liver (2, 3). The accumulation of CXCR6 in hepatic NKT cells enhances the production of interferon-γ upon antigen stimulation, which contributes to the inhibition of tumor growth. The accumulation of NKT cells is known to be mainly regulated by a type of *Clostridium* species that metabolizes primary bile acids to secondary bile acids because depletion of *Clostridium* by vancomycin increases hepatic NKT cells and colonization of *C. scindens* induces a rapid decrease in liver NKT cells (1). This evidence highlighted the significant contribution of the gut microbiome to regulating anti-tumor immunity in liver and hepatic cancers.

Human microbiota plays a critical role in maintaining metabolic and immune homeostasis and protecting the host against pathogens (4, 5). The gut microbiota provides a prominent benefit to the host; however, there is also increasing evidence of the involvement of the gut microbiota in human disease (6). The liver is closely linked to the gut because of its anatomical connection via the portal vein. The liver is the first system to acquire nutrient-rich blood via a portal vein from the gastrointestinal tract. Accordingly, the liver is also the first target of metabolites from the gut microbiota, including bile acids, choline, short-chain fatty acids, indole derivatives, and lipopolysaccharides (7). Bile acids can be classified into primary bile acids and secondary bile acids, which are synthesized by the liver and by bacterial metabolism in the colon, respectively. Recently, emerging evidence has also indicated direct associations between obesity, gut microbiota, secondary bile acids, and hepatocellular carcinoma (HCC) (8, 9). Dietary obesity induces a clear expansion of gram-positive gut microbiota, especially *Clostridium* clusters XI and XVIa, in mice with a high-fat diet (8, 9). The elevation in the strains increased the levels of deoxycholic acid (DCA), a secondary bile acid, and lipoteichoic acid (LTA), a major cell wall component in gram-positive bacteria. The accumulation of the two molecules in the livers of HFD mice treated by chemical carcinogen cooperatively enhanced the Toll-like receptor 2 (TLR2)-mediated signals by the upregulation of the receptor, which induced overexpression of cyclooxygenase2 (COX2), catalyzing the production of prostaglandin E_2 (PGE_2). Accumulation of PGE_2 suppressed

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anti-tumor immunity through a PTGER4 receptor on CD8 cells, thereby contributing to HCC progression (9).

Compared with previous studies, this study contributes to advances in the related field in the following ways. First, the results from Ma et al. (1) indicated that altering the gut microbiome caused the accumulation of both CD8 cells and NKT cells; however, depleting CD8 cells alone had minor effects on the tumor inhibition caused by elimination of commensal gut bacteria, and antibiotic treatment of tumor-bearing mice lacking NKT cells did not reduce liver tumor size. These results suggest that NKT cells are critical for effects on hepatic tumor growth induced by alterations in the gut microbiome. Second, Ma et al. (1) also provided evidence that increasing primary bile acids increased hepatic NKT cells and enhanced tumor inhibition but that increasing secondary bile acids had opposing effects. These analyses ascertained the beneficial effect of primary bile acids functioning as a regulator to enhance tumor inhibition. These findings indicate an axis of bile acids and CXCL16, CXCR6, and NKT cells that regulate live cancer. Third, DCA, a secondary bile acid, has always been speculated to be a promoter of liver cancer (10). This study showed that other secondary bile acids also played important roles. For example, ω-muricholic acid (ω-MCA) but not DCA decreased *cxcl16* mRNA expression. Increasing ω-MCA expression by feeding was shown to inhibit the activation of liver sinusoidal endothelial cells. These findings present new knowledge of the function of different secondary bile acids.

The findings from Ma et al. shed light on the prevention and treatment of liver cancer by targeting the gut microbiota in clinical application. The data directly indicated that elimination of gram-positive bacteria by vancomycin from the gut prevents tumorigenesis (1). The data from the study also solidified the evidences of influence of liver health by diets, probiotics, and antibiotics, which affect the composition of the human gut microbiota. This research cautioned that *Clostridium* colonization in gut promotes tumor growth, on the other hand, the commensal *Bifidobacterium* can enhance antitumor immunity and regulate the therapy efficacy by blocking programmed cell death 1 ligand 1 (PD-L1) (11). Because there is individual variability in response to diets, endobiotics, and xenobiotics (12), the studies of precision editing of the gut microbiota are needed to prevent live cancer. Furthermore, the results from Ma et al. also raised the questions on the influence of gut microbiota on the monoclonal antibodies therapies by PD-L1 or PD-1 (programmed cell death 1) blockade. Nivolumab, an anti-PD-1 monoclonal antibody, has been approved by the FDA for liver cancer in 2017 (13). Recent studies showed that gut microbiomes modulate the efficacy of immunotherapies against melanoma and epithelial tumors (14, 15). On the basis of this study, further studies should be performed to assess the effect of gut microbiomes on the immunotherapies to cure liver cancer in clinical trials.

This study provided a comprehensive analysis of the relationship among the gut microbiome, the immune system, and liver cancer. However, this research invokes three related questions. First, the mechanism of bile acids regulating *Cxcl16* expression is still unclear. CXCL16 is a small cytokine with a C-X-C motif with an O-glycosylated mucin-like stalk, a transmembrane helix and a cytoplasmic domain with a potential tyrosine phosphorylation site. These features allow CXCL16 to be expressed as a soluble chemokine as well as a cell surface-bound molecule (16). Further analysis should be performed to elicit if CXCL16 bind bile acids directly or through other molecules. Second, which secondary bile acids did contribute significantly to liver cancer? It has been reported that DCA induced liver cancer and nodules in rats in 1991 (17). Yoshimoto and Loo further showed that DCA was one of the factors facilitating liver cancer development (8, 9). The current research indicated that ω-MCA should be one of the critical players to promote liver cancer. ωMCA is a transformed from primary bile acid βMCA by three strains in a cooperative way, including one *Eubacterium lentum* strain and two *Fusobacterium sp.* strains (18). While DCA is transformed from cholic acid by *Clostridium* clusters XI and XVIa. Then it is critical to elucidate the contributions of different bacteria and secondary bile acids to promote live cancer. Third, can the findings be applied to humans? Approximately 1% of hepatic lymphocytes are NKT cells in humans; however, the cells constitute up to 40% of hepatic lymphocytes in mice. Promisingly, it was shown that primary bile acid CDCA levels in human samples were correlated with *CXCL16* expression, whereas secondary bile acid glycolithocholate (GLCA) levels were inversely correlated. Furthermore, mucosal-associated invariant T (MAIT) cells, which are prevalent in human liver, can also express CXCR6 that can bind CXCL16. This evidence suggests that the current study could be translated into clinical practice. cancer. However, comprehensive analysis of human liver tissue is necessary for clinical application considering the differences between humans and mice.

AUTHOR CONTRIBUTIONS

BJ wrote the commentary.

REFERENCES

1. Ma C, Han M, Heinrich B, Fu Q, Zhang Q, Sandhu S, et al. Gut microbiome-mediated bile acid metabolism regulates liver cancer via NKT cells. *Science* (2018) 360:eaan5931. doi: 10.1126/science.aan5931
2. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, Briskin MJ, et al. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol.* (2005) 3:e113. doi: 10.1371/journal.pbio.0030113
3. Shekhar S, Joyee AG, Yang X. Dynamics of NKT-cell responses to chlamydial infection. *Front Immunol.* (2015) 6:233. doi: 10.3389/fimmu.2015.00233
4. Shekhar S, Schenck K, Petersen FC. Exploring host-commensal interactions in the respiratory tract. *Front Immunol.* (2017) 8:1971. doi: 10.3389/fimmu.2017.01971
5. Thursby E, Juge N. Introduction to the human gut microbiota. *Biochem J.* (2017) 474:1823–36. doi: 10.1042/BCJ20160510

6. Schroeder BO, Backhed F. Signals from the gut microbiota to distant organs in physiology and disease. *Nat Med.* (2016) 22:4185. doi: 10.1038/nm.4185
7. Fu ZD, Cui JY. Remote sensing between liver and intestine: importance of microbial metabolites. *Curr Pharmacol Rep.* (2017) 3:101–13. doi: 10.1007/s40495-017-0087-0
8. Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* (2013) 499:97–101. doi: 10.1038/nature12347
9. Loo TM, Kamachi F, Watanabe Y, Yoshimoto S, Kanda H, Arai Y, et al. Gut microbiota promotes obesity-associated liver cancer through PGE2-mediated suppression of antitumor immunity. *Cancer Discov.* (2017) 7:522–38. doi: 10.1158/2159-8290.cd-16-0932
10. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. Bile acids and the gut microbiome. *Curr Opin Gastroenterol.* (2014) 30:332–8. doi: 10.1097/mog.0000000000000057
11. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal *Bifidobacterium* promotes antitumor immunity and facilitates anti–PD-L1 efficacy. *Science* (2015) 350:1084–9. doi: 10.1126/science.aac4255
12. Jetten MJ, Claessen SM, Dejong CH, Lahoz A, Castell JV, Van Delft JH, et al. Interindividual variation in response to xenobiotic exposure established in precision-cut human liver slices. *Toxicology* (2014) 323:61–9. doi: 10.1016/j.tox.2014.06.007
13. Finkelmeier F, Waidmann O, Trojan J. Nivolumab for the treatment of hepatocellular carcinoma. *Expert Rev Anticancer Ther.* (2018) 18:1169–75. doi: 10.1080/14737140.2018.1535315
14. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiomemodulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* (2018) 359:97–103. doi: 10.1126/science.aan4236
15. Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillere R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* (2018) 359:91–7. doi: 10.1126/science.aan3706
16. Abel S, Hundhausen C, Mentlein R, Schulte A, Berkhout TA, Broadway N, et al. The transmembrane CXC-chemokine ligand 16 is induced by IFN-gamma and TNF-alpha and shed by the activity of the disintegrin-like metalloproteinase ADAM10. *J Immunol.* (2004) 172:6362–72. doi: 10.4049/jimmunol.172.10.6362
17. Armstrong D, Cameron RG. Comparison of liver cancer and nodules induced in rats by deoxycholic acid diet with or without prior initiation. *Cancer Lett.* (1991) 57:153–7.
18. Eyssen H, De Pauw G, Stragier J, Verhulst A. Cooperative formation of omega-muricholic acid by intestinal microorganisms. *Appl Environ Microbiol.* (1983) 45:141–7.

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TLR4 May Be Involved in the Regulation of Colonic Mucosal Microbiota by Vitamin A

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Objectives: To investigate the specific role of Toll-like receptor 4 (TLR4) in the regulation of the intestinal mucosa-associated microbiota by vitamin A (VA).

Methods: Both TLR4$^{-/-}$ (knockout, KO) and wild-type (WT) female mice were randomly fed a VA normal (VAN) or VA deficient (VAD) diet for 4 weeks to establish the following four mouse model groups: TLR4$^{-/-}$ mice fed a VAN diet (KO VAN), TLR4$^{-/-}$ mice fed a VAD diet (KO VAD), WT mice fed a VAN diet (WT VAN), and WT mice fed a VAD diet (WT VAD). Then, the mice from each experimental group were mated with male mice with the same genetic background. The pups in the KO VAD and WT VAD groups were subsequently fed the VAD diet after weaning, while the pups in the KO VAN and WT VAN groups were fed the VAN diet continuously after weaning. The serum retinol levels of 7-week-old offspring were determined using high-performance liquid chromatography, and colons were collected from mice in each group and analyzed via 16S rRNA gene sequencing using an Illumina MiSeq platform to characterize the overall microbiota of the samples.

Results: The abundance and evenness of the colon mucosa-associated microbiota were unaffected by dietary VA and TLR4 KO. VAD decreased the abundance of *Anaerotruncus* (*Firmicutes*), *Oscillibacter* (*Firmicutes*), *Lachnospiraceae _NK4A136 _group* (*Firmicutes*) and *Mucispirillum* (*Deferribacteres*) and increased the abundance of *Parasutterella* (*Proteobacteria*). TLR4 KO decreased the abundance of *Bacteroides* (*Bacteroidetes*) and *Alloprevotella* (*Bacteroidetes*). However, the abundance of *Allobaculum* (*Firmicutes*), *Ruminiclostridium_9* (*Firmicutes*), *Alistipes* (*Bacteroidetes*), and *Rikenellaceae_RC9* (*Bacteroidetes*) impacted the interaction between VA and TLR4.

Conclusion: TLR4 may play a pivotal role in regulation of the intestinal mucosa-associated microbiota by VA to maintain the intestinal microecology.

Keywords: toll-like receptor 4 (TLR4), vitamin A normal (VAN), vitamin A deficiency (VAD), intestinal mucosa-associated microbiota, intestinal innate immunity

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Abbreviations: HPLC, high-performance liquid chromatography; IBD, inflammatory bowel disease; Mean ± SEM, mean ± standard error of mean; OTU, operational taxonomic units; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RARβ, retinoic acid receptor β; TLR4$^{-/-}$, Toll-like receptor 4 knockout; TLR4, Toll-like receptor 4; VA, vitamin A; VAD, vitamin A deficiency; VAN, vitamin A normal; WT, wild-type; ZO-2, zonula-occludens 2.

INTRODUCTION

Vitamin A is an essential fat-soluble vitamin that maintains normal growth and development, participates in the immune response, promotes reproduction, and maintains visual function (Stephensen, 2001; Clagett-Dame and DeLuca, 2002). VAD remains a significant public health concern in many regions of the world (World Health Organization [WHO], 1995). Children experiencing VAD are especially prone to gastrointestinal (GI) tract infections (Thornton et al., 2014). Studies by us and others have shown that VAD can decrease gut integrity and impact the immune response of the GI tract in humans and animals (Quadro et al., 2000; Liu et al., 2014). The diversity and balance of the gut microbiota is important for maintaining the normal biological barrier function of the intestine (Topping and Clifton, 2001; Round et al., 2010). Recent studies have shown that the VA nutritional status can affect the total amount of bacteria in the GI tract and alter the intestinal microflora (Amit-Romach et al., 2009). Our preliminary study found that VAD impacts the structural segregation of the gut microbiota in children with persistent diarrhea (Lv et al., 2016).

Toll-like receptors (TLRs) are membrane-anchored proteins that are expressed on immune cells and enterocytes (Takeda et al., 2003). TLRs act as pathogen recognition receptors (PRRs), identifying microbe-associated molecular patterns (MAMPs) to activate specific signaling pathways (Frosali et al., 2015). A total of 10 TLRs are expressed in humans, and TLR4 is the best characterized PRR. Recognition of MAMPs by TLR4 is involved in protective innate immune response mechanisms against bacterial invasion (Furuta et al., 2006). In addition, TLR4$^{-/-}$ mice exhibited a striking reduction in acute inflammatory cells, impaired epithelial cell proliferation and marked bacterial translocation during injury compared with WT mice (Fukata et al., 2005). Moreover, mouse epithelial cells overexpressing the TLR4 signaling pathway exhibited increased bacterial density in the colonic mucosa and increased bacterial translocation (Dheer et al., 2016). In our previous study, we confirmed that RARβ enhanced ZO-2 expression by regulating TLR4 to improve intestinal epithelial barrier function both *in vivo* and *in vitro* (Li et al., 2017). However, the role of TLR4 in regulation of the gut microbiota by VA is unclear.

Therefore, the purpose of this study was to determine the effect of TLR4 on the intestinal mucosal microbiota associated with VA nutritional levels. In the present study, TLR4$^{-/-}$ and WT mice were acquired to establish both VAN and VAD mouse models. 16S rRNA deep sequencing was used to examine the distribution and structural characteristics of the intestinal mucosa-associated microbiota.

MATERIALS AND METHODS

Animals, Diets and Sample Collection

This study was approved by the Animal Experimentation Ethics Committee of Chongqing Medical University (Chongqing, China) and was conducted in accordance with the guidelines of the Animal Care Committee of Chongqing Medical University. TLR4$^{-/-}$ (knockout, KO) and WT mice obtained from Jackson laboratories (Maine, United States) were purchased from the Model Animal Research Center of Nanjing University (MARC). The TLR4$^{-/-}$ mouse strain was C57BL/10ScNJNju, which is based on the C57BL/10JNju mouse strain (WT). The mice were housed in the same room with a constant airflow system, controlled temperature (22–24°C), and a 12-h light/dark cycle. The VAN and VAD animal models were constructed according to methods described previously (Liu et al., 2014). Half of the female KO and WT mice (3 weeks of age), which were randomly selected, were fed a VAD-inducing diet comprising 400 IU/kg VA for 4 weeks to establish a TLR4$^{-/-}$ mouse model with VAD (KO VAD) and a WT mouse model with VAD (WT VAD), and the other half received a VAN diet containing 6,500 IU/kg VA for 4 weeks to establish a VAN TLR4$^{-/-}$ mouse model (KO VAN) and a VAN WT mouse model (WT VAN). Then, the (female) mice from each experimental group were mated with the corresponding male mice with the same genetic background. Pregnant mice were fed either the VAD or VAN diet during both gestation and lactation to maintain stable serum retinol levels. Once the pups had weaned, their mothers were sacrificed, and blood was collected from the eyeballs. The serum retinol levels of the maternal VAN mice increased to 1.05 μmol/L, and those of the maternal VAD mice decreased to 0.7 μmol/L. The offspring were used for subsequent experiments. The pups in the KO VAD and WT VAD groups were subsequently fed the VAD diet continuously for 4 weeks, while the pups in the KO VAN and WT VAN groups were fed the VAN diet continuously for the same time period. Next, the mice were sacrificed, and blood was immediately harvested from the eyeball. The colons were extracted from the mice in each group, and after cleaning with 0.01 M PBS, the colons were stored at −80°C until further study.

Serum Retinol Detection

The serum retinol levels in the collected mouse blood were determined using HPLC. VA standard curve preparation and testing methods were modified slightly following methods described previously (Li et al., 2017), and VA standard compound was purchased from Sigma (R7632, United States). Briefly, 200 μL of serum was deproteinized with the same volume of anhydrous ethanol. Then, 1000 μL of hexane was used to extract the retinol from the serum, and the hexane was evaporated using nitrogen gas. The retinol residue was dissolved in 100 μL of the mobile phase mixture (methanol:water = 97:3). Finally, the prepared sample was measured using an HPLC apparatus (DGU-20As, Shimadzu Corporation, Japan). The retinoids were separated by chromatography on an analytical column (Hypersil phenyl 120 A 5 mm, 250 mm × 4.6 mm, Phenomenex, United States) via gradient elution of the mobile phase in a liquid chromatograph equipped with a 315-nm ultraviolet photodiode array detector.

DNA Extraction and PCR Amplification

Microbial DNA was extracted from colon samples using an OMEGA DNA Kit (Omega Bio-Tek, United States) according to the manufacturer's protocol. The final concentration of the

purified DNA was determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States), and DNA quality was checked via 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Xu et al., 2016) using a PCR thermocycler system (GeneAmp 9700, ABI, United States). The PCRs were conducted using the following program: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C; and a final extension at 72°C for 10 min. The PCRs were performed in triplicate in 20-μL mixtures containing 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel, further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified using QuantiFluor^{TM}-ST (Promega, United States) according to the manufacturer's protocol.

Illumina MiSeq Sequencing

Colon samples were collected from 40 mice from 4 groups: KO VAN group, KO VAD group, WT VAN group, and WT VAD group, with 10 samples per group. After DNA extraction and PCR amplification, the target band size and concentration of the samples to be sequenced were correct. Purified amplicons were pooled in equimolar amounts, and paired-end (2 × 300) sequencing was performed on an Illumina MiSeq platform (TruSeq^{TM} DNA Sample Prep Kit, United States) according to the standard protocols recommended by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP: 158355).

Processing of Sequencing Data

Raw fastq files were demultiplexed, quality-filtered with Trimmomatic and merged using FLASH with the following criteria: (i) The reads were truncated at any site that received an average quality score <20 over a 50-bp sliding window. (ii) Primers were exactly matched, allowing 2-nucleotide mismatches, and reads containing ambiguous bases were removed. (iii) Sequences with overlaps longer than 10 bp were merged at the overlap sequence.

Operational taxonomic units were clustered with a 97% similarity cutoff using UPARSE (version 7.1[1]), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm[2] compared against the Silva (SSU128) 16S rRNA database using a confidence threshold of 70%.

[1]http://drive5.com/uparse/

[2]http://rdp.cme.msu.edu/

Microbial Analysis

Sets of sequences with 97% identity were clustered into OTUs using USEARCH (version 7.0[3]). OTUs that reached 97% similarity levels were used for community richness (Chao, ACE), community diversity (Shannon, Simpson), and rarefaction curve analyses. The β-diversity was estimated by computing unweighted UniFrac distances and visualized with principal coordinate analysis. To effectively distinguish between the four groups, a partial least squares discriminant analysis (PLS-DA) was conducted. In addition, linear discriminant analysis (LDA) effect size (LEfSe) was determined using LEfSe software to determine the community or species that most influenced the group division. After features that were significantly different at various bacterial taxonomic levels were identified by LEfSe, the nonparametric factorial Kruskal-Wallis (KW) sum-rank test and LDA were performed to determine whether these features were consistent with the expected behaviors of the different bacterial taxonomic levels; genera with LDA scores greater than three were defined as having a significant impact on the group.

Statistical Analyses

All data were obtained from ten biological replicates and are presented as the mean ± SEM. Significant differences were calculated via two-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test using the GraphPad Prism version 5.0 software package. The interaction between the effect of the different VA nutrition levels and the effect of TLR4 deletion was investigated with a Bonferroni *post hoc* test. When there was a statistically significant interaction, all the experimental groups were compared using a Bonferroni *post hoc* test. However, when no interaction was observed, the effect of the different VA nutrition levels or TLR4 deletion was assessed using Student's *t*-test. Only the relevant comparisons of the combined groups are presented in the Results section. Significance was accepted at $P < 0.05$.

RESULTS

The VAD Diet Decreased Serum Retinol Levels in Mice

To explore whether TLR4 participates in regulation of intestinal microbial homeostasis by VA, we established VAN and VAD mouse models in both TLR4$^{-/-}$ (KO VAN and KO VAD) and WT mice (WT VAN and WT VAD). As shown in **Figure 1A**, the serum retinol levels of offspring in WT mice fed the VAD diet (0.393 ± 0.027 μmol/L) were significantly lower than those in WT mice fed the VAN diet (1.027 ± 0.067 μmol/L) ($P < 0.001$). Similar results were observed for the TLR4$^{-/-}$ mice, and the serum retinol levels were significantly repressed in the KO VAD group (0.328 ± 0.054 μmol/L) compared with those in the KO VAN group (1.171 ± 0.104 μmol/L). However, there was no significant interaction between the effects of VA and TLR4, determined using two-way ANOVA with a Bonferroni

[3]http://drive5.com/uparse/

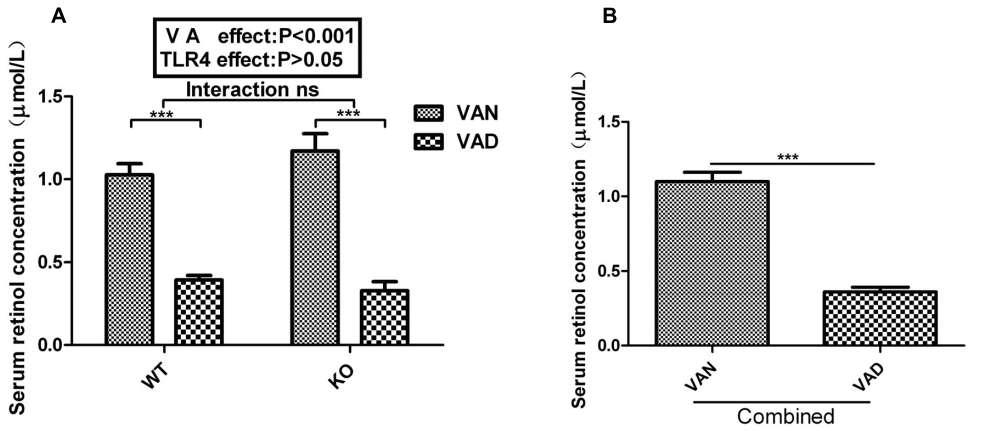

FIGURE 1 | Effects of different VA nutritional levels and TLR4 deletion on the serum retinol levels of seven-week-old offspring mice. **(A)** Changes in the serum retinol levels of the WT and TLR4$^{-/-}$ mice fed VAN or VAD diets (n = 10 for each group). **(B)** The main effect of VA, independent of TLR4$^{-/-}$ challenge, on the differences in serum retinol levels between the combined VAN and VAD groups (n = 20). The values are the means ± SEMs; "Interaction" indicates an effect of the different VA nutritional levels in the TLR4 knockout vs. WT mice; ***P < 0.001. VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

post hoc test, but differences in VA nutritional levels had an effect on the serum retinol levels (P < 0.001, **Figure 1A**). After combining the data for the WT and KO groups, we found that the serum retinol levels in the combined VAD group were markedly lower than those in the combined VAN group (P < 0.001) (**Figure 1B**). The above data demonstrate that the VAD diet was an important factor associated with the decreased serum retinol levels in mice, providing us with a solid foundation for subsequent studies.

Rarefaction Curves and Alpha Diversity Index

After optimization, a total of 1,487,466 high-quality sequences were obtained from 40 samples, and there were 37,187 high-quality sequences per sample on average according to MiSeq sequencing. We acquired numerous OTUs from valid sequences that exhibited 97% similarity for further statistical analyses. Along with an increase in the number of reads, the rarefaction curves for all the samples shown in **Figure 2A** exhibited smooth increasing trends and approached saturation plateaus, demonstrating that the sequencing data volume acquired was suitable for the present study.

In general, Chao and ACE diversity indexes reflect the richness of the microbiota, while Shannon and Simpson diversity indexes are considered to be indicators of colony richness and evenness. After two-way ANOVA with a *post hoc* test, no significant interaction was found between the effects of VAD and TLR4 KO on the Chao, ACE, Shannon and Simpson diversity indexes (**Figures 2B–E**). Furthermore, these four indexes were not affected by either VAD or TLR4 KO, even though the Simpson index of the KO VAD group was slightly higher than that of the WT VAD group (P < 0.05, **Figure 2E**) and the Shannon index of the KO VAD group was significantly decreased when compared with that of the WT VAD group (P < 0.05, **Figure 2D**). The above data demonstrate that the abundance and evenness of the colonic mucosa-associated microflora were unaffected by dietary VA and TLR4 KO.

Different VA Levels in the Diet May Affect the Microbial Community Structure of the Colonic Mucosa in Both the TLR4 KO and WT Mice

To further evaluate structural differences in the microbial communities among the four groups, an unweighted UniFrac distance matrix was calculated based on the OTUs of each sample. **Figure 3A** shows that samples from the WT VAN, WT VAD, and KO VAN groups were relatively concentrated compared with the KO VAD group samples. Principle component analysis (PCA) revealed a separation of the TLR4$^{-/-}$ and WT mice fed VAN or VAD diets based on the first two principal component (PC) scores, accounting for 25.5 and 16.77% of explained variances (**Figure 3A**). Meanwhile, ANOSIM analysis showed that the difference among the four groups was significantly greater than the difference within the group (R^2 = 0.3478, P = 0.001, **Figure 3A**), indicating that our grouping was meaningful. The results of the subsequent PLS-DA are shown in **Figure 3B**, and the variance among the KO VAD group samples was greater than that among samples from the other three groups. These data suggest that there may be differences in the distribution of the colonic mucosal microbiota due to the different levels of VA in the diet and due to TLR4 deletion.

Both VA and TLR4 Are Involved in the Community Abundance of the Colonic Mucosal Microbiota at the Phylum Level

Figure 4A shows the composition of the dominant microflora with a relative abundance of more than 1% at the phylum level in the TLR4 KO and WT mice fed the VAN or VAD diet. In the WT VAN group, the predominant phyla were *Firmicutes*

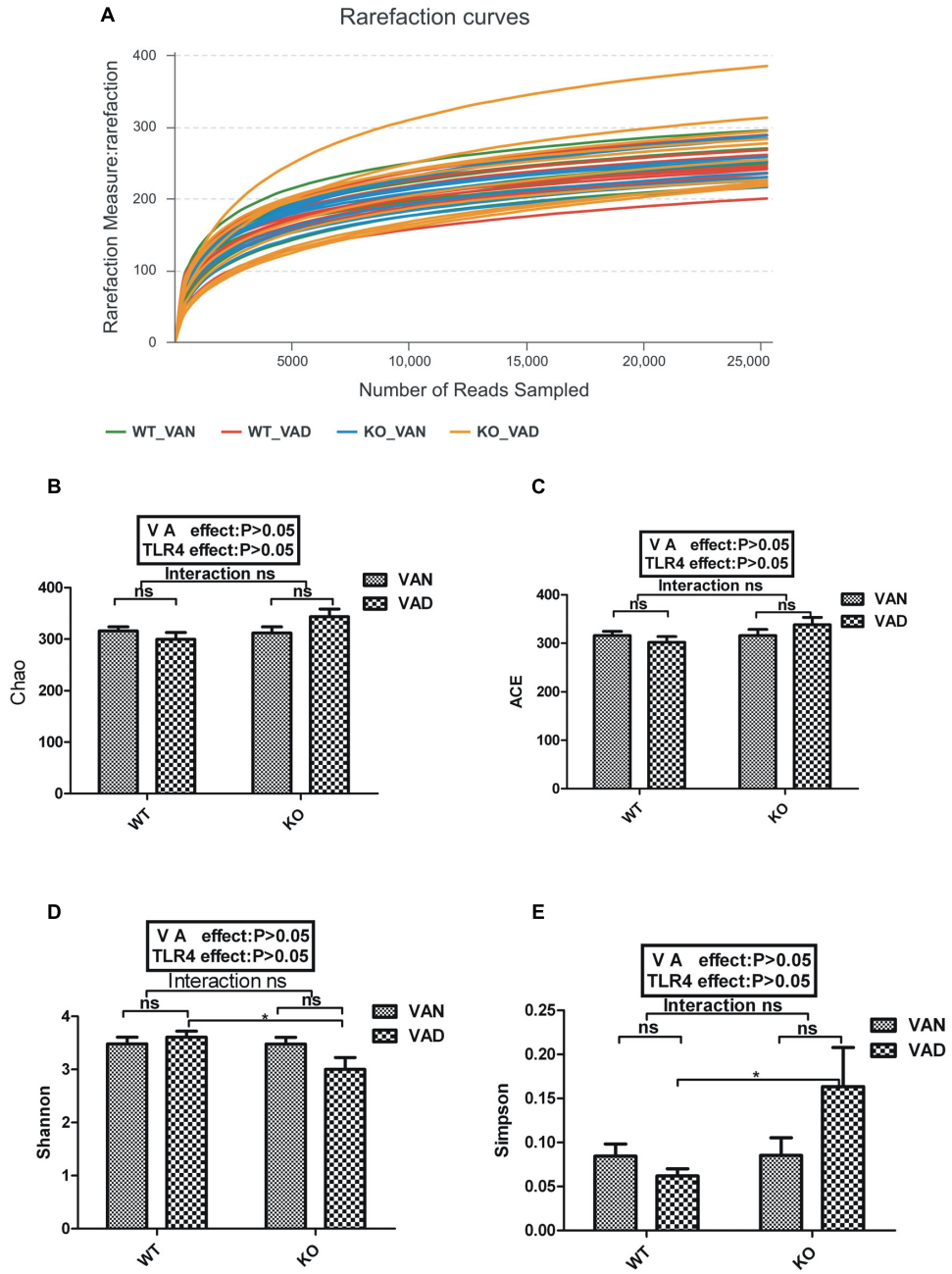

FIGURE 2 | Rarefaction curves and α diversity analysis for WT and TLR4$^{-/-}$ mice fed VAN or VAD diets. **(A)** Rarefaction curves were calculated for OTUs with 97% identity in the gut microbiota in the WT VAN, WT VAD, KO VAN, and KO VAD groups (n = 10). The green curves represent the WT VAN group; red curves represent the WT VAD group; blue curves represent the KO VAN group; yellow curves represent the KO VAD group (n = 10). Comparison of **(B)** Chao index, **(C)** ACE index, **(D)** Shannon index and **(E)** Simpson index among the four groups (n = 10). Mean ± SEM; ns. = not significant. "Interaction" indicates an effect of the different VA nutritional levels in the TLR4 knockout vs. WT mice; *P < 0.05. VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

(34.47%), *Bacteroidetes* (31.34%), *Proteobacteria* (30.62%), and *Deferribacteres* (2.372%); however, the predominant phyla in the WT VAD group were *Bacteroidetes* (37.33%), *Firmicutes* (30.91%), *Proteobacteria* (28.38%), and *Deferribacteres* (1.718%). The most abundant phyla in the KO VAN group, in decreasing order, were *Firmicutes* (40%), *Proteobacteria* (24.66%), *Bacteroidetes* (23.42%), and *Deferribacteres* (8.928%). Notably, the percentage of *Actinobacteria* was highest in the KO VAD group compared with that in the other three groups, and the most abundant phyla, in decreasing order, in the KO VAD group were *Proteobacteria* (46.58%), *Bacteroidetes* (22.92%), *Firmicutes* (22.69%), *Actinobacteria* (3.644%), and *Deferribacteres* (1.357%).

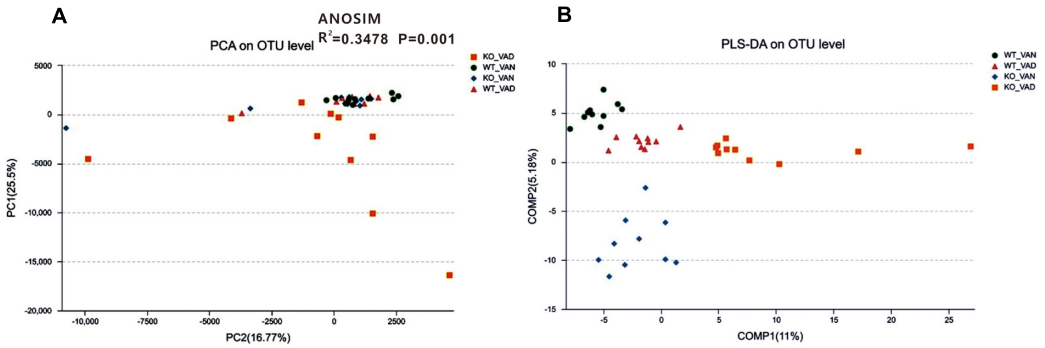

FIGURE 3 | PCA and PLS-DA of samples from the TLR4$^{-/-}$ and WT mice fed VAN or VAD diets. **(A)** PCA scores were plotted based on the relative abundance of the OTUs (n = 10). **(B)** PLS-DA was plotted based on the unweighted UniFrac distance metrics (n = 10). The green circles represent the WT VAN group; red triangles represent the WT VAD group; blue diamonds represent the KO VAN group; orange squares represent the KO VAD group (n = 10). VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

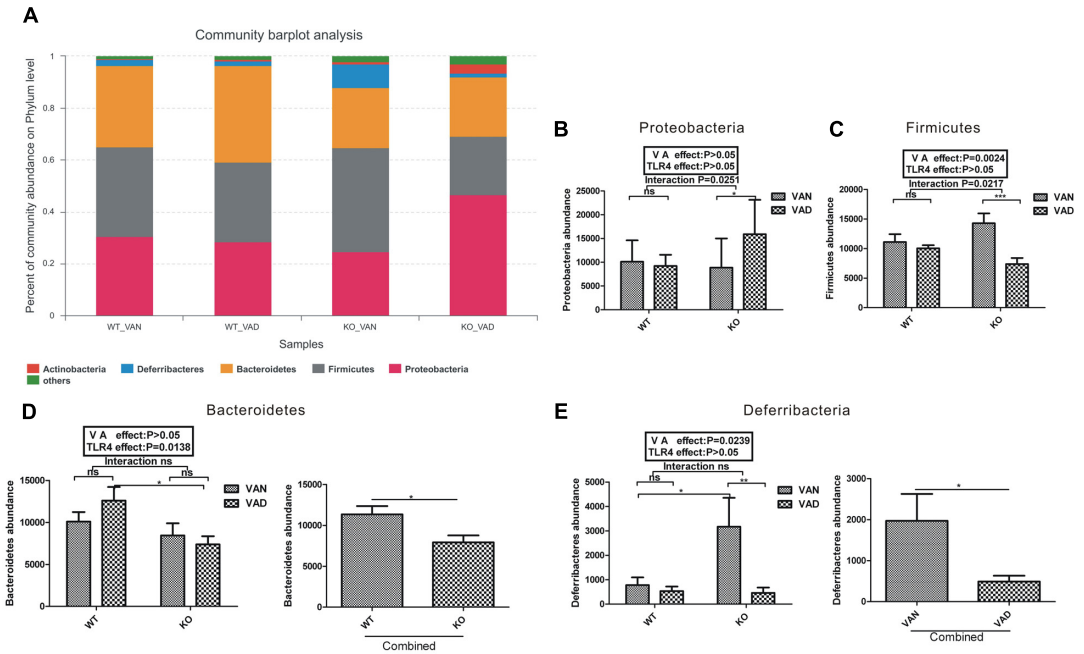

FIGURE 4 | The relative abundances of bacterial phyla in the colonic mucosa of the TLR4$^{-/-}$ and WT mice fed the VAN or VAD diet. **(A)** The dominant bacterial phyla with relative abundances greater than 1% in the four groups (n = 10). The combined effects of the different VA nutritional levels and the TLR4 deletion on the relative abundance of **(B)** *Proteobacteria* and **(C)** *Firmicutes* determined by two-way analysis of variance with a Bonferroni *post hoc* test (n = 10). **(D)** The main effect of TLR4, independent of VA nutritional level, on the relative abundance of *Bacteroidetes* in the four groups (n = 10). **(E)** The main effect of VA, independent of TLR4 deletion, on the relative abundance of *Deferribacteres* in the four groups (n = 10). Mean ± SEM; ns. = not significant. "Interaction" indicates an effect of the different VA nutritional levels in the TLR4 knockout vs. WT mice; *P < 0.05, **P < 0.01, and ***P < 0.001. VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

As shown in **Figure 4B,** although VA and TLR4 had no effect on the abundance of *Proteobacteria* (P > 0.05), the P-value of the interaction between the VA and TLR4$^{-/-}$ challenges was 0.0251 after two-way ANOVA with a Bonferroni *post hoc* test. The *Firmicutes* abundance in the VAD group was lower than that in the VAN group in the TLR4$^{-/-}$ mice (**Figure 4C**). The VA nutritional level had significant effects on the *Firmicutes* abundance (P = 0.0024), and the P-value of the interaction between the VA and TLR4$^{-/-}$ challenges for the *Firmicutes* abundance was 0.0217 according to the *post hoc* test (**Figure 4C**). However, for the abundance of *Bacteroidetes* and *Deferribacteres*, no significant interaction was observed between the VA and TLR4$^{-/-}$ challenges, determined using two-way ANOVA (**Figures 4D,E**). After combining the VAN and VAD

groups, the *Bacteroidetes* abundance in the combined KO group was significantly lower than that in the combined WT group ($P < 0.05$, **Figure 4D**). The *Deferribacteres* abundance in the combined VAD group was significantly lower than that in the combined VAN group ($P < 0.05$, **Figure 4E**). Based on these data, VA and TLR4 have interactive effects on the abundance of *Proteobacteria* and *Firmicutes*; the *Bacteroides* abundance was affected by TLR4 and that of *Deferribacteres* was affected by VA.

VA and TLR4 Altered the Community Structure of the Colonic Microbiota at the Genus Level

To further understand the effect of VA and TLR4 on colonic bacteria, we analyzed the community structure at the genus level in the four groups. **Figure 5** shows the composition of the dominant microflora with relative abundances greater than 2% at the genus level in the $TLR4^{-/-}$ and WT mice fed the VAN or VAD diet. We noticed that the percentage of *Helicobacter* in the KO VAD group was highest compared with the percentage in the other three groups.

Two-way ANOVA was conducted for each strain, as shown in **Figures 6**, **7**. There were five genera of the phylum *Firmicutes* that were affected by VA or both VA and TLR4. A significant interaction was observed between the effects of VA and TLR4 on the abundance of *Allobaculum* and *Ruminiclostridium_9* according to a Bonferroni *post hoc* analysis, and the *P*-values were 0.0125 and 0.0345, respectively (**Figures 6A,B**). On the other hand, the main effects of VA, independent of TLR4, were on the relative abundance of *Anaerotruncus* ($P = 0.0003$), *Lachnospiraceae _NK4A136 _group* ($P = 0.0224$), and *Oscillibacter* ($P = 0.0029$) in the four groups (**Figures 6C–E**). After combining the WT and KO groups, the abundance of *Anaerotruncus* ($P < 0.001$), *Lachnospiraceae_NK4A136_group* ($P < 0.05$), and *Oscillibacter* ($P < 0.01$) was markedly decreased in the combined VAD group compared with that in the combined VAN group (**Figures 6C–E**).

Vitamin A and TLR4 also impacted the community structure at the genus level within the phyla *Bacteroidetes, Proteobacteria, and Deferribacteres*. According to two-way ANOVA, there was a significant interaction between the effects of VA and TLR4 on the abundance of *Alistipes* and *Rikenellaceae_RC9* in the phylum *Bacteroidetes*, and the *P*-values were 0.001 and 0.0026, respectively (**Figures 7A,B**). However, no significant interaction was observed between the effects of VA and TLR4 on the abundance of *Bacteroides* (**Figure 7D**) and *Alloprevotella* (**Figure 7E**). After combining the VAN and VAD groups, the abundance of *Bacteroides* and *Alloprevotella* in the combined KO group was markedly lower than that in the combined WT group. TLR4 seems to be the main factor affecting the abundance of *Bacteroides* and *Alloprevotella* at the *Bacteroidetes* level. However, the *Parasutterella* abundance (*Proteobacteria*) was significantly increased in the combined VAD group compared with that in the combined VAN group, and the abundance of *Mucispirillum* (*Deferribacteres*) was significantly reduced in the combined VAD group compared with that in the combined VAN group.

Based on these data, both VA and TLR4 affected the abundance of *Allobaculum* (*Firmicutes*), *Ruminiclostridium_9* (*Firmicutes*), *Alistipes* (*Bacteroidetes*), and *Rikenellaceae_RC9* (*Bacteroidetes*), while the abundance of *Anaerotruncus* (*Firmicutes*), *Lachnospiraceae_NK4A136_group* (*Firmicutes*), *Oscillibacter* (*Firmicutes*), *Parasutterella* (*Proteobacteria*), and *Mucispirillum* (*Deferribacteres*) was mainly affected by VA, and that of *Bacteroides* (*Bacteroidetes*) and *Alloprevotella* (*Bacteroidetes*) was primarily affected by TLR4.

Key Phylotypes in the $TLR4^{-/-}$ and WT Mice With Different VA Levels

A metagenomic analysis approach (LEfSe) was used to identify the key phylotypes responsible for the differences among the TLR4 KO and WT mice fed the VAN or VAD diet. **Figure 8** shows a comparison of the bacterial populations in the four groups at the genus level. The results indicated that the key genera in the WT VAN group were *Alloprevotella* (LDA = 4.34, $P = 0.003751$), *Lachnospiraceae_NK4A136_group* (LDA = 4.14, $P = 0.000845$), *Clostridium_innocuum_group* (LDA = 3.45, $P = 0.000844$), and *Blautia* (LDA = 3.68, $P = 0.000227$). In the WT VAD group, the key genera were *Aeromicrobium* (LDA = 3.81, $P = 0.004725$), *Escherichia_Shigella* (LDA = 3.63, $P = 0.000193$), *Lactobacillus* (LDA = 3.29, $P = 0.007436$), *Tyzzerella* (LDA = 3.58, $P = 0.005026$), *Rikenellaceae_RC9_gut_group* (LDA = 4.26, $P = 0.000131$), and *Allobaculum* (LDA = 4.13, $P = 0.010607$). *Oleibacter* (LDA = 3.19, $P < 0.003641$), *Pseudomonas* (LDA = 3.00, $P = 0.000502$), *Mucispirillum* (LDA = 4.54, $P = 0.012631$), *Ruegeria* (LDA = 3.51, $P = 0.000837$), *Shewanella* (LDA = 3.52, $P = 0.023659$), and *Pseudoalteromonas* (LDA = 3.07, $P < 0.0001$) were the key genera in the KO VAN group. However, only two genera played key roles in the KO VAD group: *Acetivibrio_ethanolgignens_group* (LDA = 3.07, $P = 0.004929$) and *Eubacterium_coprostanoligenes_group* (LDA = 3.15, $P = 0.022025$).

DISCUSSION

The GI tract harbors a complex community of bacteria in the mucosa, lumen and feces. Feces may primarily contain bacteria that are not adherent (Zoetendal et al., 2002), and the luminal microbiota is more variable than the mucosal microbiota (Li et al., 2015). Therefore, in our study, the mucosal bacteria may better reflect the actual intestinal microbiota (Amit-Romach et al., 2009). Some ecologists have noted that the Chao and ACE diversity indexes reflect the richness of the microbiota. The Shannon and Simpson diversity indexes are considered indicators of colony richness and evenness. The Simpson index is sensitive to dominant species, and the Shannon index is sensitive to rare species (Magurran, 1988). In the present study, TLR4 KO reduced the Shannon index and upregulated the Simpson index in the VAD group. TLR4 KO may decrease the abundance of rare species and increase the dominant intestinal mucosa-associated microbiota in VAD rats. However, VA had no effect on the Shannon and Simpson indexes in WT mice; therefore, these two indexes were not found to be affected by either VA

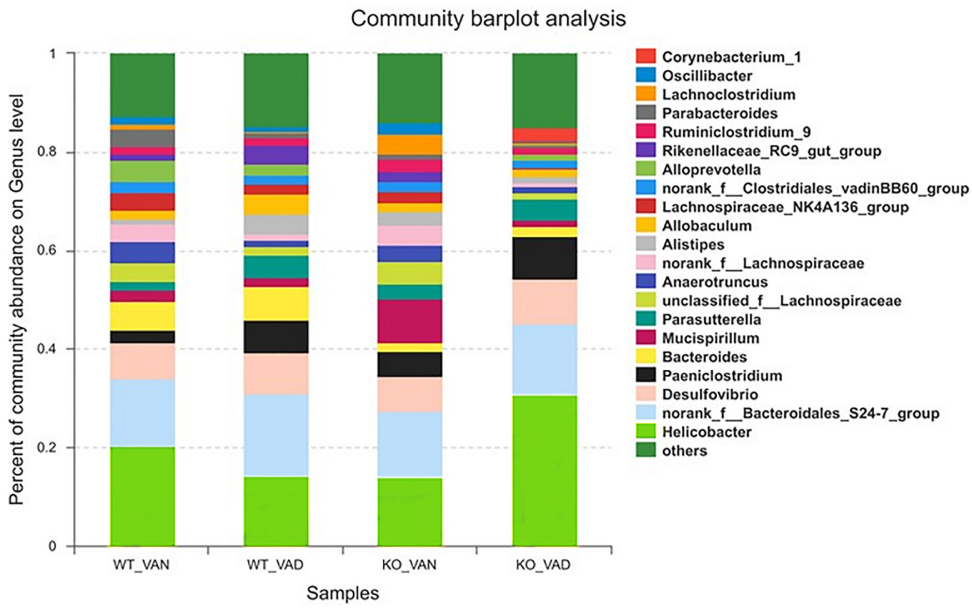

FIGURE 5 | The dominant bacterial genera with relative abundances greater than 2% in the colonic mucosa of TLR4$^{-/-}$ and WT mice fed the VAN or VAD diet (n = 10). VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

or TLR4 after two-way ANOVA with a *post hoc* test. Although VA and TLR4 had no effect on the abundance and evenness of the colonic mucosa-associated microflora, the results of PCA and PLS-DA showed that VA and TLR4 affected the distribution and composition of the colonic mucosa-associated bacteria.

Amit-Romach showed that VAD led to a reduction in the proportion of *Lactobacillus* spp. and resulted in the appearance of pathogenic *Escherichia coli* strains among the mucosa-associated bacteria (Amit-Romach et al., 2009). Matthew C. found that acute VAD had an effect on bacterial community structure, leading to an increase in the abundance of *Bacteroides vulgatus* (Hibberd et al., 2017). Studies on changes in gut microbial diversity have shown varying results. However, in the present study, at the genus level, VAD decreased the abundance of *Anaerotruncus, Oscillibacter, Lachnospiraceae_NK4A136_group,* and *Mucispirillum* and increased the abundance of *Parasutterella.*

Anaerotruncus is a newly described bacterial genus isolated from human stool (Lawson et al., 2004). Currently, *Anaerotruncus colihominis* and *Anaerotruncus massiliensis* are the identified species of this genus (Lau et al., 2006). A. H. Togo isolated *Anaerotruncus massiliensis* from an obese patient after bariatric surgery (Togo et al., 2016). Although *Anaerotruncus* is not well known, studies have shown that *Anaerotruncus* species might be optimal probiotic strains because these species express enzymes that favor the production of butyrate (Polansky et al., 2015). Butyrates are important nutrients for cells lining the mammalian colon. As critical short-chain fatty acid derivatives that regulate colon homeostasis, butyrates participate in colon inflammation (Donohoe et al., 2011). In a study of human gut microflora, *Oscillibacter valericigenes* was identified in a significantly greater number of samples from healthy controls than from patients with Crohn's disease (Man et al., 2011; Mondot et al., 2011). *Anaerotruncus* and *Oscillibacter* appear to play a positive role in maintaining intestinal immune homeostasis. *Lachnospiraceae* species are also associated with maintenance of gut health, and members of this family may protect against colon cancer in humans by producing butyric acid (Tap et al., 2009; Meehan and Beiko, 2014; Liu et al., 2017). Mice administered retinoic acid (RA) orally and then subjected to partial hepatectomy had higher levels of *Lachnospiraceae* than mice in the control group, which were not treated with RA (Liu et al., 2016). In the present study, VAD reduced the abundance of *Anaerotruncus* and *Oscillibacter* in the colonic mucosa, and the abundance of *Lachnospiraceae _NK4A136 _ group* was also lower in the combined VAD group than in the VAN group, while VAD upregulated the abundance of *Parasutterella*. Previously, the *Parasutterella* abundance was found to be increased and the *Lachnospiraceae* abundance was decreased in the submucosal tissues of patients with Crohn's disease (Chiodini et al., 2015). In addition, the *Parasutterella* abundance was increased significantly in rats with hypertriglyceridemia-related acute necrotizing pancreatitis (Huang et al., 2017). These results suggest that VA may be involved in regulation of the intestinal mucosa-associated microbiota.

Mucispirillum is a core member of the laboratory mouse microbiota and can colonize the intestinal tract from the stomach to the colon; this genus is represented by a single species, namely, *Mucispirillum schaedleri* (Robertson et al., 2005). As part of the phylum *Deferribacteres*, *Mucispirillum* has been shown to be associated with both inflammatory markers (El Aidy et al., 2014) and active colitis in a T-bet$^{-/-}$ Rag2$^{-/-}$ mouse model (Berry et al., 2012; Rooks et al., 2014)

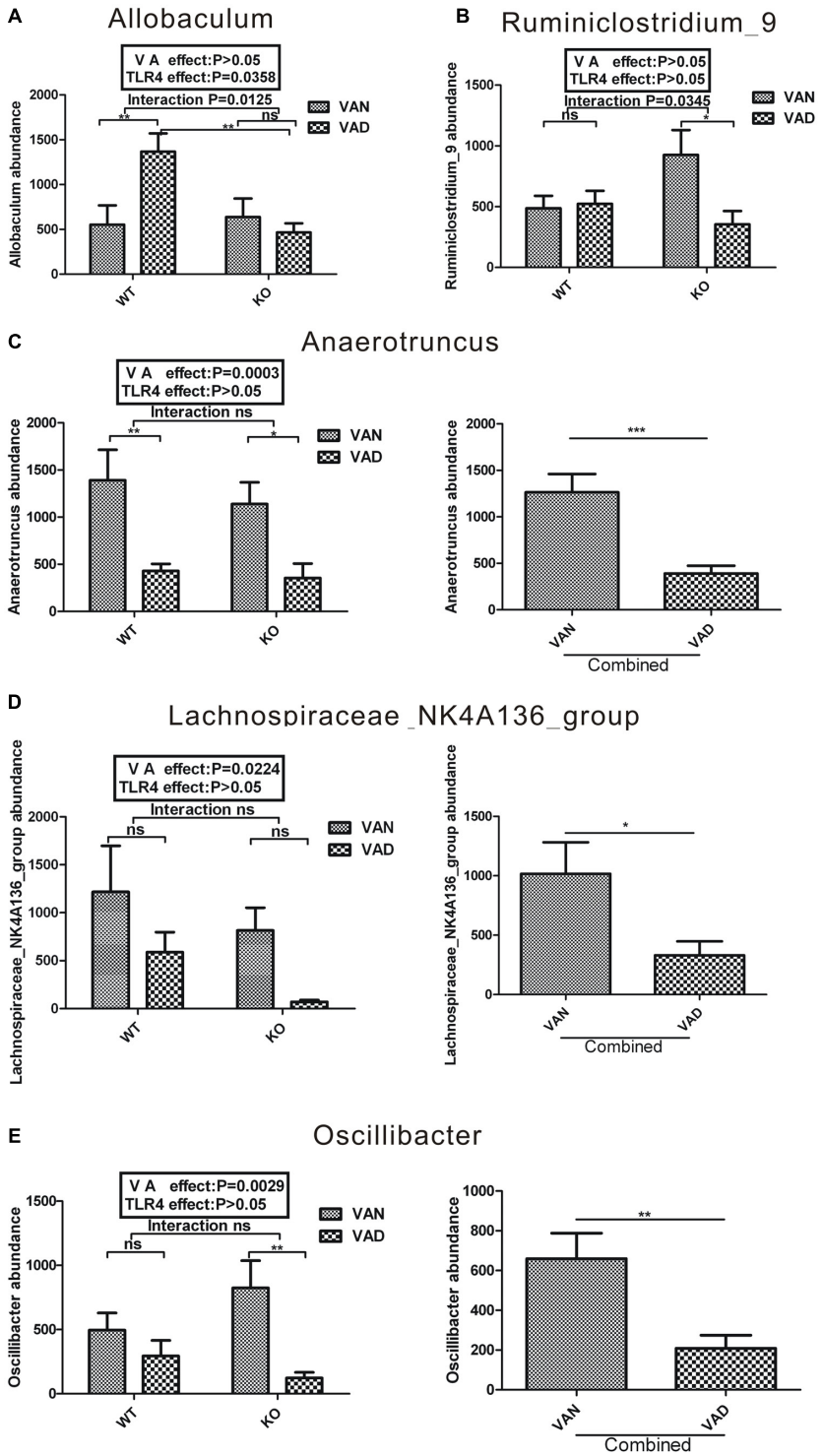

FIGURE 6 | Two-way ANOVA of the five genera with relative abundances greater than 2% from the phylum *Firmicutes* in the colonic mucosa of TLR4$^{-/-}$ and WT mice fed the VAN or VAD diet. The combined effects of the different VA nutritional levels and the TLR4 deletion on the relative abundance of **(A)** *Proteobacteria* and **(B)** *Ruminiclostridium_9* (n = 10). The main effect of VA, independent of TLR4 deletion, on the relative abundance of **(C)** *Anaerotruncus*, **(D)** *Lachnospiraceae_NK4A136_group* and **(E)** *Oscillibacter* in the four groups (n = 10). Mean ± SEM; ns. = not significant. "Interaction" indicates an effect of the different VA nutritional levels in the TLR4 knockout vs. WT mice; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

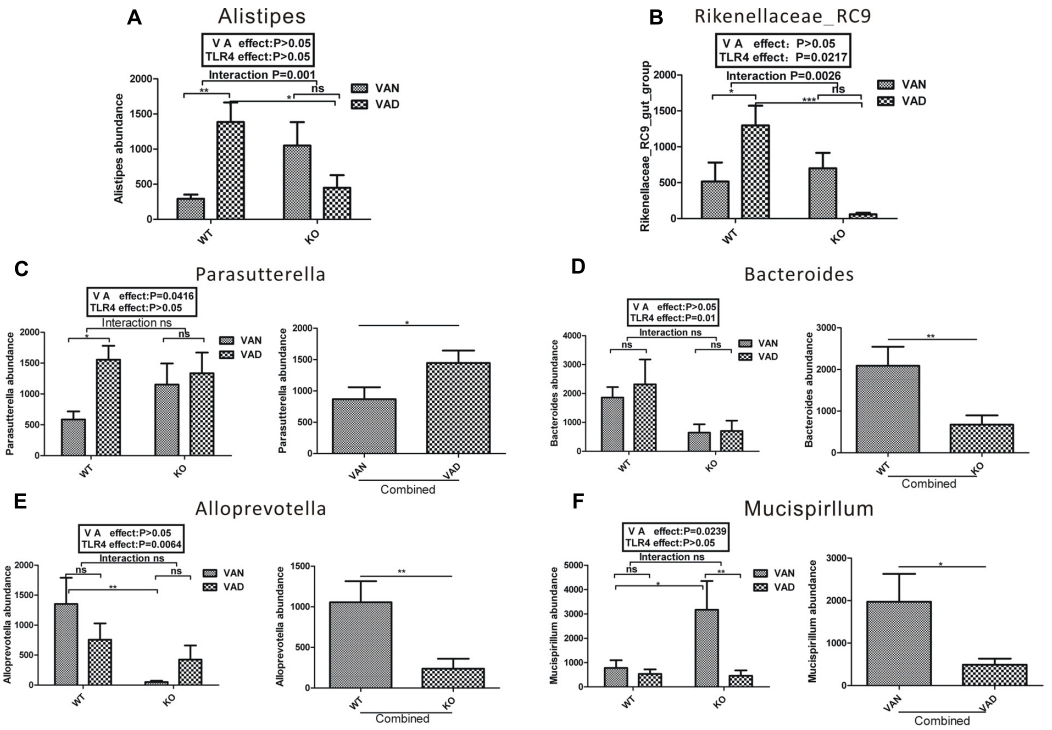

FIGURE 7 | Two-way ANOVA of genera with relative abundances greater than 2% from the phyla *Proteobacteria*, *Bacteroidetes*, and *Deferribacteres* in the colonic mucosa of the TLR4$^{-/-}$ and WT mice fed the VAN or VAD diet. The combined effects of the different VA nutritional levels and TLR4 deletion on the relative abundance of **(A)** *Alistipes* and **(B)** *Rikenellaceae_RC9* (n = 10). The main effect of VA, independent of TLR4 deletion, on the relative abundance of **(C)** *Parasutterella* and **(F)** *Mucispirillum* in the four groups (n = 10). The main effect of TLR4, independent of VA levels, on the relative abundance of **(D)** *Bacteroides* and **(E)** *Alloprevotella* among the four groups (n = 10). Mean ± SEM; ns. = not significant. "Interaction" indicates an effect of the different VA nutritional levels in the TLR4 knockout vs. WT mice; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

and was even associated with *Citrobacter rodentium* infection (Hoffmann et al., 2009). A recent study showed that *M. schaedleri* possesses specialized systems to handle oxidative stress during inflammation (Loy et al., 2017). Interestingly, our data showed that VAD downregulated the abundance of *Mucispirillum*. We speculate that this downregulation may be a self-regulatory effect of the intestinal mucosa-associated microbiota.

Activation of TLRs by commensal microflora is critical for protection against gut injury (Rakoff-Nahoum et al., 2004; Fukata et al., 2005; Fukata et al., 2006). A large amount of research has indicated that TLR4 signaling affects the intestinal microbiota (Anitha et al., 2012). However, the effects of TLR4 on the intestinal mucosa-associated microbiota are complex and remain unclear. Our results suggest that TLR4 KO decreased the abundance of *Bacteroides* and *Alloprevotella*. *Bacteroides*, a commensal bacterium that colonizes the lower digestive tract, can strongly affect the host immune system (Swidsinski et al., 2009). A study by Erin B showed that *Bacteroides* species produce a capsular polysaccharide, polysaccharide A (PSA), to repress proinflammatory cytokines (Troy and Kasper, 2010). Many studies have implicated decreased levels of *Bacteroides* in the development of IBD (Zhou and Zhi, 2016). In our study, TLR4$^{-/-}$ mice exhibited a defective immune response, which may be associated with the marked reduction in *Bacteroides* abundance. *Alloprevotella* is a genus of *Prevotellaceae*, and the clinical significance of this genus remains unclear. Decreased inflammatory cytokine expression in the mouse intestine following interferon tau (IFNT) supplementation led to increased *Alloprevotella* abundance in the colon (Ren et al., 2016), indicating that *Alloprevotella* has positive effects on the intestinal mucosa. Intriguingly, the abundance of *Alloprevotella* and *Bacteroides* was distinctly reduced in the TLR4 KO group, indicating a complex relationship among TLR4, the microbiota, and intestinal immunity.

Notably, at the genus level, VA and TLR4 had a combined effect on the abundance of *Allobaculum* (*Firmicutes*), *Ruminiclostridium_9* (*Firmicutes*), *Alistipes* (*Bacteroidetes*), and *Rikenellaceae_RC9* (*Bacteroidetes*). *Allobaculum* has been shown to prevent dextran sulfate sodium (DSS)-induced inflammation (Wang et al., 2015), and the abundance of this genus was seen to be positively correlated with markers of ileal immunity (Cox et al., 2014). However, in the present study, the abundance of *Allobaculum* in the WT VAD group was significantly higher than that in the WT VAN group, but there was no difference in the abundance of this genus between the VAN and VAD groups of TLR4 KO mice, suggesting that VA might regulate *Allobaculum* via TLR4. *Ruminiclostridium_9* belongs to the *Ruminococcaceae* family and is associated with the

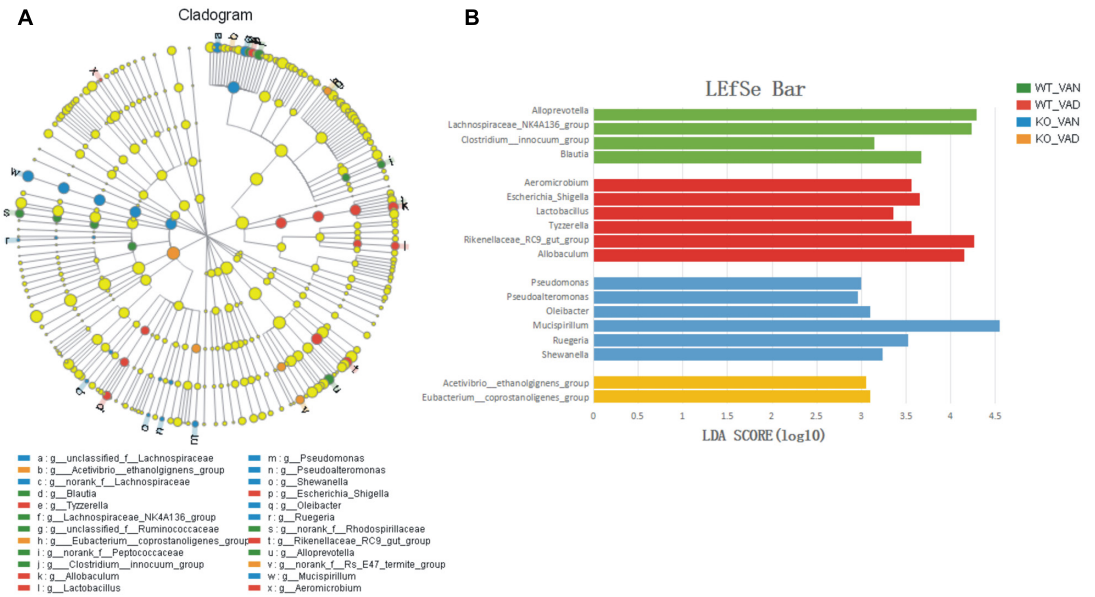

FIGURE 8 | LEfSe analysis of the different structures of the colonic mucosal microbiota in the TLR4 KO and WT mice fed the VAN or VAD diet. **(A)** A cladogram of the statistical and biological differences in the colonic microbiota among the WT VAN, WT VAD, KO VAN, and KO VAD groups, which are shown by the color of the most abundant phylotypes (n = 10). **(B)** A histogram of the LDA scores for the most abundant phylotypes (n = 10). Mean ± SEM. VAN, vitamin A normal; VAD, vitamin A deficiency. WT VAN refers to WT mice fed a VAN diet; WT VAD refers to WT mice fed a VAD diet; KO VAN refers to TLR4$^{-/-}$ mice fed a VAN diet; KO VAD refers to TLR4$^{-/-}$ mice fed a VAD diet.

release of inflammatory and cytotoxic factors from the gut for maintenance of a stable intestinal microecology (Cheng et al., 2017; Ma et al., 2017; Wu et al., 2017). Although there were no differences among the WT VAN, WT VAD, and KO VAD groups in terms of the abundance of *Ruminiclostridium_9*, we found that the *Ruminiclostridium_9* abundance in the KO VAN group was significantly higher than that in the other three groups. These results suggest that TLR4 may be involved in the regulation of intestinal microbiota by VA, but the specific mechanism of regulation and the clinical significance of this regulation need to be further explored. A cross-study analysis showed that *Alistipes* species are associated with healthy subjects rather than with individuals with gut disease (Mancabelli et al., 2017). In a murine model of DSS-induced colitis, *Alistipes finegoldii (Alistipes)* was seen to be protective against colitis (Dziarski et al., 2016). However, the *Alistipes* genus was found to be associated with colorectal cancer, and the abundance of this genus exhibited a negative correlation with the consumption of fruits and vegetables (Feng et al., 2015; Dai et al., 2018). *Rikenellaceae_RC9*, similar to *Alistipes*, belongs to the *Rikenellaceae* family. The abundance of both these genera was affected by VA and TLR4. However, the specific regulatory mechanisms remain unclear.

A large number of studies have shown that the VA nutritional state affects the abundance and composition of the intestinal microbiota (Amit-Romach et al., 2009; Hibberd et al., 2017). There is also considerable variation and discrepancy associated with identification of bacterial markers of VAD among different studies (Chiodini et al., 2015; Liu et al., 2016) and different disease models, such as models of persistent diarrhea and necrotizing enterocolitis (Lv et al., 2016; Xiao et al., 2018). On the other hand, the intestinal microflora was shown to affect the bioavailability of dietary α- and β-carotene and the activity of VA in rats (Grolier et al., 1998). These results indicate that VA levels and the intestinal microbiota are interrelated. Many studies have shown that VAD impairs GI mucosal barrier integrity by altering bacterial populations, the expression of innate immunity-related genes and the number of immune cells (Amit-Romach et al., 2009; Liu et al., 2014; Li et al., 2017). Our study further demonstrated the independent effects of VA and TLR4 on intestinal mucosa-related bacteria. Our study is the first to indicate that TLR4 is involved in regulation of the colonic mucosal microbiota by VA, providing a foundation for elucidating the relationships among VA, the intestinal microecology and intestinal innate immunity. This result further shows that VA regulates TLR4 to improve intestinal barrier function, as described in our previous study (Li et al., 2017). However, elucidation of the specific regulatory mechanism remains challenging and requires further study and the development of novel approaches.

CONCLUSION

VAD decreased the abundance of *Anaerotruncus* (*Firmicutes*), *Oscillibacter* (*Firmicutes*), *Lachnospiraceae _NK4A136 _group* (*Firmicutes*), and *Mucispirillum* (*Deferribacteres*) and increased the abundance of *Parasutterella* (*Proteobacteria*). TLR4 KO decreased the abundance of *Bacteroides* (*Bacteroidetes*) and *Alloprevotella* (*Bacteroidetes*). However, the abundance of *Allobaculum* (*Firmicutes*), *Ruminiclostridium_9* (*Firmicutes*),

Alistipes (*Bacteroidetes*), and *Rikenellaceae_RC9* (*Bacteroidetes*) impacted the interaction between VA and TLR4. Therefore, TLR4 may play a pivotal role in the regulation of the intestinal mucosa-associated microbiota and maintenance of the intestinal microecology mediated by VA.

AUTHOR CONTRIBUTIONS

LX performed the experiments and analyzed the data. BC and DF assisted in completion of the experiments. TY provided technical guidance regarding the use of HPLC. LX and JC wrote the manuscript. TL and JC designed the study. JC provided financial support for the study. All authors read and approved the final manuscript.

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REFERENCES

Amit-Romach, E., Uni, Z., Cheled, S., Berkovich, Z., and Reifen, R. (2009). Bacterial population and innate immunity-related genes in rat gastrointestinal tract are altered by vitamin A-deficient diet. *J. Nutr. Biochem.* 20, 70–77. doi: 10.1016/j.jnutbio.2008.01.002

Anitha, M., Vijay-Kumar, M., Sitaraman, S. V., Gewirtz, A. T., and Srinivasan, S. (2012). Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. *Gastroenterology* 143, 1006.e4–1016.e4. doi: 10.1053/j.gastro.2012.06.034

Berry, D., Schwab, C., Milinovich, G., Reichert, J., Ben Mahfoudh, K., Decker, T., et al. (2012). Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis. *ISME J.* 6, 2091–2106. doi: 10.1038/ismej.2012.39

Cheng, M., Liang, J., Zhang, Y., Hu, L., Gong, P., Cai, R., et al. (2017). The bacteriophage EF-P29 efficiently protects against lethal vancomycin-resistant *Enterococcus faecalis* and alleviates gut microbiota imbalance in a murine bacteremia model. *Front. Microbiol.* 8:837. doi: 10.3389/fmicb.2017.00837

Chiodini, R. J., Dowd, S. E., Chamberlin, W. M., Galandiuk, S., Davis, B., Glassing, A., et al. (2015). Microbial population differentials between mucosal and submucosal intestinal tissues in advanced Crohn's disease of the ileum. *PLoS One* 10:e0134382. doi: 10.1371/journal.pone.0134382

Clagett-Dame, M., and DeLuca, H. F. (2002). The role of vitamin A in mammalian reproduction and embryonic development. *Annu. Rev. Nutr.* 22, 347–381. doi: 10.1146/annurev.nutr.22.010402.102745E

Cox, L. M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J. M., Cho, I., et al. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* 158, 705–721. doi: 10.1016/j.cell.2014.05.052

Dai, Z., Coker, O. O., Nakatsu, G., Wu, W. K. K., Zhao, L., Chen, Z., et al. (2018). Multi-cohort analysis of colorectal cancer metagenome identified altered bacteria across populations and universal bacterial markers. *Microbiome* 6:70. doi: 10.1186/s40168-018-0451-2

Dheer, R., Santaolalla, R., Davies, J. M., Lang, J. K., Phillips, M. C., Pastorini, C., et al. (2016). Intestinal epithelial Toll-like receptor 4 signaling affects epithelial function and colonic microbiota and promotes a risk for transmissible colitis. *Infect. Immun.* 84, 798–810. doi: 10.1128/IAI.01374-15

Donohoe, D. R., Garge, N., Zhang, X., Sun, W., O'Connell, T. M., Bunger, M. K., et al. (2011). The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab.* 13, 517–526. doi: 10.1016/j.cmet.2011.02.018

Dziarski, R., Park, S. Y., Kashyap, D. R., Dowd, S. E., and Gupta, D. (2016). Pglyrp- regulated gut microflora *Prevotella falsenii*, *Parabacteroides distasonis* and *Bacteroides eggerthii* enhance and *Alistipes finegoldii* attenuates colitis in mice. *PLoS One* 11:e0146162. doi: 10.1371/journal.pone.0146162

El Aidy, S., Derrien, M., Aardema, R., Hooiveld, G., Richards, S. E., Dane, A., et al. (2014). Transient inflammatory-like state and microbial dysbiosis are pivotal in establishment of mucosal homeostasis during colonisation of germ-free mice. *Benef. Microbes* 5, 67–77. doi: 10.3920/BM2013.0018

Feng, Q., Liang, S., Jia, H., Stadlmayr, A., Tang, L., Lan, Z., et al. (2015). Gut microbiome development along the colorectal adenoma-carcinoma sequence. *Nat. Commun.* 6:6528. doi: 10.1038/ncomms7528

Frosali, S., Pagliari, D., Gambassi, G., Landolfi, R., Pandolfi, F., and Cianci, R. (2015). How the intricate interaction among Toll-like receptors, microbiota, and intestinal immunity can influence gastrointestinal pathology. *J. Immunol. Res.* 2015:489821. doi: 10.1155/2015/489821

Fukata, M., Chen, A., Klepper, A., Krishnareddy, S., Vamadevan, A. S., Thomas, L. S., et al. (2006). Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: role in proliferation and apoptosis in the intestine. *Gastroenterology* 131, 862–877. doi: 10.1053/j.gastro.2006.06.017

Fukata, M., Michelsen, K. S., Eri, R., Thomas, L. S., Hu, B., Lukasek, K., et al. (2005). Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288, G1055–G1065. doi: 10.1152/ajpgi.00328.2004

Furuta, T., Kikuchi, T., Akira, S., Watanabe, N., and Yoshikawa, Y. (2006). Roles of the small intestine for induction of toll-like receptor 4-mediated innate resistance in naturally acquired murine toxoplasmosis. *Int. Immunol.* 18, 1655–1662. doi: 10.1093/intimm/dxl099

Grolier, P., Borel, P., Duszka, C., Lory, S., Alexandre-Gouabau, M. C., Azais-Braesco, V., et al. (1998). The bioavailability of α- and β-carotene is affected by gut microflora in the rat. *Br. J. Nutr.* 80, 199–204.

Hibberd, M. C., Wu, M., Rodionov, D. A., Li, X., Cheng, J., Griffin, N. W., et al. (2017). The effects of micronutrient deficiencies on bacterial species from the human gut microbiota. *Sci. Transl. Med.* 9:eaal4069. doi: 10.1126/scitranslmed.aal4069

Hoffmann, C., Hill, D. A., Minkah, N., Kirn, T., Troy, A., Artis, D., et al. (2009). Community-wide response of the gut microbiota to enteropathogenic *Citrobacter rodentium* infection revealed by deep sequencing. *Infect. Immun.* 77, 4668–4678. doi: 10.1128/IAI.00493-09

Huang, C., Chen, J., Wang, J., Zhou, H., Lu, Y., Lou, L., et al. (2017). Dysbiosis of intestinal microbiota and decreased antimicrobial peptide level in paneth cells during hypertriglyceridemia-related acute necrotizing Pancreatitis in rats. *Front. Microbiol.* 8:776. doi: 10.3389/fmicb.2017.00776

Lau, S. K., Woo, P. C., Woo, G. K., Fung, A. M., Ngan, A. H., Song, Y., et al. (2006). Bacteraemia caused by *Anaerotruncus colihominis* and emended description of the species. *J. Clin. Pathol.* 59, 748–752. doi: 10.1136/jcp.2005.031773

Lawson, P. A., Song, Y., Liu, C., Molitoris, D. R., Vaisanen, M. L., Collins, M. D., et al. (2004). *Anaerotruncus colihominis* gen. nov., sp. nov., from human faeces. *Int. J. Syst. Evol. Microbiol.* 54(Pt 2), 413–417. doi: 10.1099/ijs.0.02653-0

Li, G., Yang, M., Zhou, K., Zhang, L., Tian, L., Lv, S., et al. (2015). Diversity of duodenal and rectal microbiota in biopsy tissues and luminal contents in healthy volunteers. *J. Microbiol. Biotechnol.* 25, 1136–1145. doi: 10.4014/jmb.1412.12047

Li, Y., Gao, Y., Cui, T., Yang, T., Liu, L., Li, T., et al. (2017). Retinoic acid facilitates Toll-like receptor 4 expression to improve intestinal barrier function through retinoic acid receptor beta. *Cell. Physiol. Biochem.* 42, 1390–1406. doi: 10.1159/000479203

Liu, H. X., Hu, Y., and Wan, Y. J. (2016). Microbiota and bile acid profiles in retinoic acid-primed mice that exhibit accelerated liver regeneration. *Oncotarget* 7, 1096–1106. doi: 10.18632/oncotarget.6665

Liu, P., Zhao, J., Guo, P., Lu, W., Geng, Z., Levesque, C. L., et al. (2017). Dietary corn bran fermented by *Bacillus subtilis* MA139 decreased gut cellulolytic

bacteria and microbiota diversity in finishing pigs. *Front. Cell. Infect. Microbiol.* 7:526. doi: 10.3389/fcimb.2017.00526

Liu, X., Li, Y., Wang, Y., Wang, Q., Li, X., Bi, Y., et al. (2014). Gestational vitamin A deficiency reduces the intestinal immune response by decreasing the number of immune cells in rat offspring. *Nutrition* 30, 350–357. doi: 10.1016/j.nut.2013.09.008

Loy, A., Pfann, C., Steinberger, M., Hanson, B., Herp, S., Brugiroux, S., et al. (2017). Lifestyle and horizontal gene transfer-mediated evolution of *Mucispirillum schaedleri*, a core member of the murine gut microbiota. *mSystems* 2:e00171-16. doi: 10.1128/mSystems.00171-16

Lv, Z., Wang, Y., Yang, T., Zhan, X., Li, Z., Hu, H., et al. (2016). Vitamin A deficiency impacts the structural segregation of gut microbiota in children with persistent diarrhea. *J. Clin. Biochem. Nutr.* 59, 113–121. doi: 10.3164/jcbn.15-148

Ma, N., Wu, Y., Xie, F., Du, K., Wang, Y., Shi, L., et al. (2017). Dimethyl fumarate reduces the risk of mycotoxins via improving intestinal barrier and microbiota. *Oncotarget* 8, 44625–44638. doi: 10.18632/oncotarget.17886

Magurran, A. E. (1988). *Ecological Diversity and Measurement*. Princeton, NJ: Princeton University Press. doi: 10.1007/978-94-015-7358-0

Man, S. M., Kaakoush, N. O., and Mitchell, H. M. (2011). The role of bacteria and pattern-recognition receptors in Crohn's disease. *Nat. Rev. Gastroenterol. Hepatol.* 8, 152–168. doi: 10.1038/nrgastro.2011.56

Mancabelli, L., Milani, C., Lugli, G. A., Turroni, F., Cocconi, D., van Sinderen, D., et al. (2017). Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. *FEMS Microbiol. Ecol.* 93:fix153. doi: 10.1093/femsec/fix153

Meehan, C. J., and Beiko, R. G. (2014). A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. *Genome Biol. Evol.* 6, 703–713. doi: 10.1093/gbe/evu050

Mondot, S., Kang, S., Furet, J. P., Aguirre de Carcer, D., McSweeney, C., Morrison, M., et al. (2011). Highlighting new phylogenetic specificities of Crohn's disease microbiota. *Inflamm. Bowel Dis.* 17, 185–192. doi: 10.1002/ibd.21436

Polansky, O., Sekelova, Z., Faldynova, M., Sebkova, A., Sisak, F., and Rychlik, I. (2015). Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Appl. Environ. Microbiol.* 82, 1569–1576. doi: 10.1128/AEM.03473-15

Quadro, L., Gamble, M. V., Vogel, S., Lima, A. A., Piantedosi, R., Moore, S. R., et al. (2000). retinol and retinol-binding protein: gut integrity and circulating immunoglobulins. *J. Infect. Dis.* 182, S97–S102. doi: 10.1086/315920

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229–241. doi: 10.1016/j.cell.2004.07.002

Ren, W., Chen, S., Zhang, L., Liu, G., Hussain, T., Hao, X., et al. (2016). Interferon Tau Affects Mouse Intestinal Microbiota and Expression of IL-17. *Mediat. Inflamm.* 2016:2839232. doi: 10.1155/2016/2839232

Robertson, B. R., O'Rourke, J. L., Neilan, B. A., Vandamme, P., On, S. L., Fox, J. G., et al. (2005). *Mucispirillum schaedleri* gen. Nov., sp. Nov., a spiral-shaped bacterium colonizing the mucuslayer of the gastrointestinal tract of laboratory rodents. *Int. J. Syst. Evol. Microbiol.* 55(Pt 3), 1199–1204. doi: 10.1099/ijs.0.63472-0

Rooks, M. G., Veiga, P., Wardwell-Scott, L. H., Tickle, T., Segata, N., Michaud, M., et al. (2014). Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. *ISME J.* 8, 1403–1417. doi: 10.1038/ismej.2014.3

Round, J. L., O'Connell, R. M., and Mazmanian, S. K. (2010). Coordination of tolerogenic immune responses by the commensal microbiota. *J. Autoimmun.* 34, J220–J225. doi: 10.1016/j.jaut.2009.11.007

Stephensen, C. B. (2001). Vitamin A, infection, and immune function. *Annu. Rev. Nutr.* 21, 167–192. doi: 10.1146/annurev.nutr.21.1.167

Swidsinski, A., Loening-Baucke, V., and Herber, A. (2009). Mucosal flora in Crohn's disease and ulcerative colitis - an overview. *J. Physiol. Pharmacol.* 60(Suppl. 6), 61–71.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll -like receptors. *Annu. Rev. Immunol.* 21, 335–376. doi: 10.1146/annurev.immunol.21.120601.141126

Tap, J., Mondot, S., Levenez, F., Pelletier, E., Caron, C., Furet, J. P., et al. (2009). Towards the human intestinal microbiota phylogenetic core. *Environ. Microbiol.* 11, 2574–2584. doi: 10.1111/j.1462-2920.2009.01982.x

Thornton, K. A., Mora-Plazas, M., Marín, C., and Villamor, E. (2014). Vitamin A deficiency is associated with gastrointestinal and respiratory morbidity in school-age children. *J. Nutr.* 144, 496–503. doi: 10.3945/jn.113.185876

Togo, A. H., Valero, R., Delerce, J., Raoult, D., and Million, M. (2016). "*Anaerotruncus massiliensis*," a new species identified from human stool from an obese patient after bariatric surgery. *New Microbes New Infect.* 14, 56–57. doi: 10.1016/j.nmni.2016.07.015

Topping, D. L., and Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* 81, 1031–1064. doi: 10.1152/physrev.2001.81.3.1031

Troy, E. B., and Kasper, D. L. (2010). Beneficial effects of *Bacteroides fragilis* polysaccharides on the immune system. *Front. Biosci.* 15, 25–34. doi: 10.2741/3603

Wang, J., Tang, H., Zhang, C., Zhao, Y., Derrien, M., Rocher, E., et al. (2015). Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice. *ISME J.* 9, 1–15. doi: 10.1038/ismej.2014.99

World Health Organization [WHO] (1995). *Global Prevalence of Vitamin A Deficiency. Micronutrient Deficiency Information System, Nutrition Unit*. Geneva: World Health Organization.

Wu, W., Lv, L., Shi, D., Ye, J., Fang, D., Guo, F., et al. (2017). Protective effect of *Akkermansia muciniphila* against immune-mediated liver injury in a mouse model. *Front. Microbiol.* 8:1804. doi: 10.3389/fmicb.2017.01804

Xiao, S., Li, Q., Hu, K., He, Y., Ai, Q., H2u, L., et al. (2018). Vitamin A and retinoic acid exhibit protective effects on necrotizing enterocolitis by regulating intestinal flora and enhancing the intestinal epithelial barrier. *Arch. Med. Res.* 49, 1–9. doi: 10.1016/j.arcmed.2018.04.003

Xu, N., Tan, G., Wang, H., and Gai, X. (2016). Effect of biochar additions to soil on nitrogen leaching, microbial biomass and bacterial community structure. *Eur. J. Soil Biol.* 74, 1–8. doi: 10.1016/j.ejsobi.2016.02.004

Zhou, Y., and Zhi, F. (2016). Lower level of *Bacteroides* in the gut microbiota is associated with inflammatory bowel disease: a meta-analysis. *Biomed Res. Int.* 2016:5828959. doi: 10.1155/2016/5828959

Zoetendal, E. G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A. D., and de Vos, W. M. (2002). Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl. Environ. Microbiol.* 68, 3401–3407. PMCID:PMC126800 doi: 10.1128/AEM.68.7.3401-3407.2002

Conflict of Interest Statement: Our team bears a patent (Patent No. ZL201010233032.8) on the formula of the vitamin A normal and vitamin A deficiency animal feed in China.

frontiers in Immunology

REVIEW
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Microbiome Dependent Regulation of T_{regs} and Th17 Cells in Mucosa

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Mammals co-exist with resident microbial ecosystem that is composed of an incredible number and diversity of bacteria, viruses and fungi. Owing to direct contact between resident microbes and mucosal surfaces, both parties are in continuous and complex interactions resulting in important functional consequences. These interactions govern immune homeostasis, host response to infection, vaccination and cancer, as well as predisposition to metabolic, inflammatory and neurological disorders. Here, we discuss recent studies on direct and indirect effects of resident microbiota on regulatory T cells (T_{regs}) and Th17 cells at the cellular and molecular level. We review mechanisms by which commensal microbes influence mucosa in the context of bioactive molecules derived from resident bacteria, immune senescence, chronic inflammation and cancer. Lastly, we discuss potential therapeutic applications of microbiota alterations and microbial derivatives, for improving resilience of mucosal immunity and combating immunopathology.

Keywords: microbiome, mucosa, T_{reg}, mucosal immunity, inflammation, Th17, antibiotics, resident microbes

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INTRODUCTION

Mammals harbor a highly diverse microbiome of at least 1000 species, and an astounding number of 10–100 trillion microbial cells, co-existing in a remarkable balance with the host immune system. Healthy human microbiome is mostly bacteria although other microbial domains such as archaea, viruses, and eukaryotes (principally fungi and protists) are also present (1). While these microbes are distributed in skin, and mucosa of ocular, nasal, oral, eye, and reproductive organs, gastrointestinal (GI) tract mucosa is the major reservoir of resident microbes in terms of abundance and species diversity (2, 3). The human colon harbors approximately 3.8×10^{13} microorganisms, followed by skin in the range of $\sim 10^{11}$ (4). Since the resurgence of microbiome research in recent years, there has been a sharp increase in understanding of how resident microbiome shapes immunity, health and disease of humans. Only a perennial holiday on a lonely island could excuse an immunologist's incognizance on intimate interrelationships between intestinal microbiota and immune balance. Direct crosstalk between resident microbes and host immune cells in mucosa emerges as a pivotal determinant of such an immune balance. Dysbiosis of resident microbes has strong association with a number of immunological disorders, including opportunistic and pathogenic infections (5–13).

Mucosal immune system has not only evolved to protect the mucosal barrier surface against external insults, it has also co-evolved with resident microbes in an interdependent harmonious relationship with them (14–21). The resulting immune balance is crucial to drive optimal immune responses without causing an over-exuberant inflammation (22–25). Past few decades have seen that an increase in hyper-hygiene mentality, mindless use of antibiotics and diet changes, have led to reduced diversity and impaired resilience in resident microbiota (26). Consequently, a disruption in aforementioned immune balance leads to rise in autoimmune and inflammatory disorders. Therefore, understanding the mechanisms of these mutualistic relationships between resident microbiota and different components of innate and adaptive immunity is vital to our understanding of immune diseases. Although gut microbiota in laboratory mice and humans differ significantly, murine models have provided a powerful tool to explore host-microbiota-pathogen interactions in mucosa (27, 28). Here we review the effects of resident microbiota on T_{regs} and Th17 cells, important players in determining immune balance, mucosal barrier integrity and host protective functions in mucosa. These cells mucosa can develop in mucosa independent of commensal microbiota. For example, there is evidence in germ free mice that T_{reg} cells can be induced by dietary antigens from solid food (29). These T_{reg} cells are of limited life span, but are distinguishable from microbiota-induced T_{reg} cells and capable of repressing inadvertent immune responses to ingested protein antigens. Similarly, in oral mucosa, mechanical damage from mastication of food induces barrier protective Th17 cells, independent of oral commensal microbiota under homeostatic conditions (30). However, dysbiosis can lead excessive Th17 cells and lead to periodontal inflammation (31). Thus, while it is known that these cells can develop independent of microbiota, resident bacterial dysbiosis is strongly associated with alterations in these cells, causing mucosal inflammation seen in many diseases including HIV immunopathogenesis (32–41). Although other cells also play important roles in mucosal tolerance and immunity, we will not review them here.

T_{REGS} AND TH17 CELLS IN MUCOSA UNDER STEADY STATE-CONDITIONS

Majority of the studies on mucosa-microbiota interactions discuss GI tract. Indeed, GI mucosa harbors by far the largest and most diverse microbiota, as well as abundant and dynamic population of T_{regs} and Th17 cells. T_{regs} are defined by the expression of CD25 and Foxp3, and are predominantly known for their immunosuppressive properties. These cells also express other molecules such as Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), PD-1, interleukin 10 (IL-10), transforming growth factor beta 1(TGF-β1), and amphiregulin. Each of the aforementioned proteins has been shown to be either important, or dispensable for different mechanisms of T_{reg}-mediated immunosuppression. Divergent conclusions derived from various T_{reg} mechanism investigations have been strikingly similar to those in the popular parable of the "Blind men and an elephant." It is now increasingly clear that suppressive and non-suppressive functions of $Foxp3^+$ cells are largely variable, depending on local tissues, disease phenotypes, responding effector cells, and cytokine milieu (42–49).

While $CD4^+$ effector T cell responses contribute to overt intestinal inflammation, T_{regs} are associated with controlling immunopathology (42, 43, 50). It is well known that T_{regs} are also pivotal for commensal tolerance (51–53). There have been contentions regarding the T_{regs} found in colon mucosa (colon T_{regs}; cT_{regs}); whether they develop in thymus (thymic $T_{regs;}$ tT_{regs}), or periphery (peripheral T_{regs}; pT_{regs}). The usage of Nrp-1 and Helios as markers of tT_{regs}, and the extent to which the TCR repertoire of cT_{reg} overlaps with that of tT_{regs} have been debated (54, 55). Nevertheless, it is well established that cT_{regs} require the presence of microbiota for their development, sustenance and function (56–58). There is also evidence that mucosal sites are the primary sites of development and maintenance of pT_{regs} (59–61). First formal proof for the requirement of microbiota for the induction and maintenance of intestinal T_{regs} was provided by studies using germ-free (GF) animal models. GF mice show a several-fold reduction in the frequency of $Helios^-$ T_{regs}, when compared with conventionally housed specific pathogen free (SPF) mice. Association of individual bacterial isolates or defined consortia in GF mice is sufficient to induce intestinal T_{regs} (56, 57). Even antibiotic treated mice, which show depletion in resident microbiota correlating with a drastic reduction in the frequency of T_{regs}, lend further credence to the positive role of microbiota in sustenance of T_{regs} (53, 55, 62). In addition to commensal tolerance, mucosal T_{regs} have been shown to regulate excessive immune responses during infections (43, 63–65). Recently, they are also shown to accumulate in other tissues and provide functions such as non-suppressive tissue repair functions in muscle (66). While T_{regs} play diverse and often opposite roles in mucosal infections (**Table 1**), effects of microbiome on T_{regs} during these infections are largely ignored in many studies.

Th17 cells are $ROR\gamma t^+$, $CCR6^+$, $IL\text{-}17A^+$, $IL\text{-}17F^+$, with some cells expressing IL-21 and IL-22, and have been implicated both in mucosal barrier functions. Th17 cells are an important subset of effector T cells that are protective during extracellular bacterial and fungal invasion (83, 88–91). However, excessive Th17 responses are also associated with a variety of pathogenic conditions, depending on the pro-inflammatory cytokines they co-produce (30, 91–95). Littman and colleagues showed for the first time that commensal microbiota play important roles in the development of intestinal Th17 cells (22, 53, 96–100). Th17 development and differentiation is controlled by cytokine and epigenetic regulation (91, 92, 101, 102), but the mechanistic details of microbiome dependent control of Th17 development during mucosal infection is largely unclear.

IMPACT OF MICROBIOME ON T_{REGS} AND TH17 CELLS DURING GI INFECTION AND INFLAMMATION

"Healthy" GI microbiota is mainly composed of the phyla Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria,

TABLE 1 | $Foxp3^+T_{reg}$ functions in mucosal infections.

Pathogen	T_{reg} manipulation	Outcome
BACTERIA		
Listeria monocytogenes	T_{regs} cause increased pathogen burden (67)	Detrimental
Salmonella enterica	Foxp3+ cell ablation accelerates bacterial clearance (68)	Detrimental
Aggregatibacter actinomycetemcomitans	T_{regs} attenuate experimental periodontitis progression (69)	Protective
Yersinia Enterocolitica	T_{regs} reduce pathogenic burden and attenuate inflammation (70)	Protective
VIRUSES		
HIV	Early interference with the T_{reg}'s suppressive function worsened infection and inflammation (71, 72)	Protective
	T_{regs} are preserved in elite controllers in humans (73)	Protective
	T_{regs} suppress anti-viral CD8 responses (74)	Detrimental
	Foxp3+ cell ablation accelerates mortality and increases viral load (197)	Protective
Herpes simplex virus 2	Foxp3+ cell ablation increases mortality (75)	Protective
West Nile virus		
PARASITES		
Toxoplasma gondii	Loss of Foxp3+ T_{reg} cells results in fatal pathology (76)	Protective
Toxoplasma gondii	Loss of Foxp3+ T_{reg} cells results in pathology (77)	Protective
Toxoplasma gondii	Loss of Foxp3+ T_{reg} cells results in pathology (78)	Protective
Heligmosomoides polygyrus	No changes in pathogen burden with T_{reg} ablation (79)	No effect
Leishmania major	T_{regs} promote increased pathogen burden (80).	Detrimental
Schistosoma mansoni	$CD4^+CD25^+$ depletion increases inflammation (81)	Protective
FUNGUS		
Candida albicans	CD4+CD25+T_{regs} regulate immunopathology in Th1 mediated gastrointestinal/disseminated Candidiasis (82)	Protective
	CD4+CD25+Foxp3+T_{regs} promote Th17 antifungal immunity and dampen immunopathology (41, 83)	Protective
	T_{regs} regulate immunopathology (84)	
	T_{regs} suppress pulmonary hyperinflammation (85)	
Aspergillus fumigatus		Protective
Pneumocystis carinii		Protective
MYCOBACTERIA		
Mycobacterium tuberculosis	Selective depletion of T_{regs} reduces pathogen burden (86).	Detrimental
	Foxp3+ cells induce resistance to TB lesions (87)	Protective

Proteobacteria, and Verrucomicrobia. Small intestine is dominated by Enterobacteriaceae and Lactobacillaceae, whereas colon contains the members of Bacteroidaceae, Lachnospiraceae, Prevotellaceae, Rikenellaceae, and Ruminococcaceae respectively (3). A number of factors including diverse environmental conditions, intake of diet and medication, as well as host genetic factors determine the dynamic composition of gut microbiota in individuals (103–107). Gut microbiota are capable of restraining the mucosal colonization by enteric pathogens, a process defined as colonization resistance (108). Thus, administration of antibiotics, and altering the resident microbiota during a mucosal infection is known to lead to post-antibiotic expansion of the pathogens. Loss of overall diversity, or even deficit in single group of bacteria can alter the susceptibility to gastrointestinal infections. For example, Clostridium difficile (C. difficile) infection, the most common cause of nosocomial diarrhea is often preceded by antibiotic usage. Colonization of C. difficile in healthy mice in fact requires a pre-exposure to a cocktail of antibiotics to alter the microbiota composition (109). However, mono-colonization of GF mice with a murine isolate from the family Lachnospiraceae could limit the colonization of C. difficile, suggesting that individual bacterial species are sufficient to confer colonization resistance to C. difficile (110). Enhanced susceptibility toward other infections after antibiotic-mediated disruption of the intestinal microbiota composition has also been reported for vancomycin-resistant Enterococcus Spp and Salmonella enterica serovar typhimurium (*S. typhimurium*) (108, 111). Mechanistically, mucosal carbohydrates such as fucose and sialic acid liberated by resident microbiota have been shown to control the growth of enteric pathogens. Antibiotics cause spikes in sugars that can worsen *S. typhimurium* and C. difficile infections (112). Microbiota alterations reduce the numbers of germinal centers in IL21-receptor knockout mice, resulting in diminished IgA^+ B cells and reduced activation-induced cytidine deaminase in Peyer's patches. These events lead to the expansion of T_{regs} and Th17 cells, and higher bacterial burdens, but dampening of Citrobacter rodentium-induced immunopathology (113). Resident microbiota at mucosal

interfaces can govern transmission and progress of parasitic protozoan infections such as Toxoplasmosis and Amoebiasis (114). In the case of Toxoplama gondii infection in mice, reduction of microbiota in the gut by prolonged antibiotic treatment leads to impaired Toll like receptor (TLR)-11 and Myeloid differentiation response 88 (MyD88) signaling and subsequent deficit in Th1 immunity, substantiating that gut commensals serve as natural molecular adjuvants during T. gondii infection (115). In a mouse model of Giardia duodenalis infection, antibiotic induced alteration of the microbiome prevents CD8 T cell activation by G. duodenalis. Conversely, GI infection can also modulate microbiota specific adaptive immunity (116). For example, a pathogenic GI infection, in parallel to specific immune reactions against the pathogen, induces immune responses to commensals and generates long-lived commensal-specific T cells. Thus an adaptive response against commensals is an integral component of mucosal immunity. However, such a commensal specific-adaptive response in a dysbiosis setting can also contribute to excessive inadvertent inflammation. In the context of HIV-1 infection, damages in GI tract and gut microbial translocation (Proteobacterial species) are associated with reduction of systemic and gut/rectal mucosal Th17 cells and T_{regs} (despite increased T_{reg}/Th17 ratio) (36, 71, 72, 117, 118). A large body of evidence suggests that increased T_{regs} in circulation correlate to reduced immune activation in HIV+ patients, underlining the anti-inflammatory protective roles of T_{regs} in patients (71–73, 118–125). While combined anti-retroviral (cART) therapy in HIV^+ patients generally ensures immune reconstitution in the peripheral blood, dysbiosis and T_{reg}/Th17 abnormalities persist in gut and other mucosae (41, 126–132). This can present residual inflammation and heightened morbidities in cART treated HIV^+ patients. However, in cART-treated HIV^+ patients with elevated levels of immune activation, it is not clear whether altered levels and function of mucosal T_{regs}/Th17 cells are associated with local microbial dysbiosis (131), and if these alterations contribute to residual inflammation in HIV disease. Collectively, these findings highlight the role of microbiota in restraining pathogens and inflammation by having significant impact on T_{regs} and Th17 cells.

Alterations in resident microbiota and host immune cells, caused by host genetic makeup also play a role in the pathogenesis of inflammatory bowel diseases (IBD). One of the adaptive arms of immunity that is impacted by such changes is T_{regs} (133). *Bacteroides fragilis* for example, has been found to invade mucosa and cause excessive activation of the host intestinal immune response in genetically susceptible patients (134), while under steady-state conditions the same bacterium can enhance T_{reg} differentiation and ensure intestinal homeostasis. Loss of autophagy protein ATG16L1 in T_{regs} results in aberrant type 2 responses and spontaneous intestinal inflammation (135). It is unclear whether microbiota directly induce the expression of ATG16L1 in T_{regs}, but it is evident that ATG16L1 and autophagic process directly promote T_{reg} survival and metabolic adaptation in the intestine. Similarly, other genetic risk variants associated with IBD such as: *NOD2, CARD9, ATG16L1, IRGM and FUT2* significantly influence the gut microbiota changes (136). For example, a decrease in *Roseburia* spp (known acetate to butyrate converters), *Clostridiaceae* family, the genera *Bifidobacterium*, *Ruminococcus* and *Faecalibacterium* has been observed in patients with IBD. Although many of these communities are strongly implicated in T_{reg} maintenance, direct mechanisms of T_{reg} regulation in the context of these genetic variants and IBD are unclear. Combined deficiency of MyD88 and JH gene, which disrupts innate interactions of immune cells with intestinal microbiota and IgA responses respectively, causes overt inflammation, highlighting the requirement of T_{reg}-IgA mediated mechanism in tolerance (51, 137). It has also been shown that microbiota-specific $Foxp3^+$ T_{reg} cells can convert to interferon-γ-producing $Foxp3^+$ T cells that have a potential to establish mucosal tolerance (138). Disruption of TLR/MyD88 signaling in Foxp3-deficient mice protect them from excessive inflammation at the environmental interfaces of skin, lungs, and intestine, showing that T_{regs} normally also restrain commensal dependent tonic MyD88-dependent pro-inflammatory signals (139). Mice lacking *CLEC7A* gene (Dectin-1), thus having dys-regulated interactions with fungal microbiome (mycobiome) show an increased susceptibility to dextran sulfate sodium (DSS) induced colitis (140). The role of Th17 cells and T_{regs} in this model is unknown. Certain proportion of intestinal T_{regs} co-expresses RORγt, the master transcription factor of the Th17 lineage, with up to 35 % in small intestine and 65 % in colon (141–143). Some of these $ROR\gamma t^+$ T_{reg} co-produce IL-17A ($T_{reg}17$), and are substantially diminished in GF or antibiotics-treated mice. Mono-association of GF mice with a panel of 22 bacterial species from the human gastrointestinal tract shows that a number of microbes, not only *Clostridiales*, are capable of induce colonic $ROR\gamma t^+$ T_{regs} (142). Segmented filamentous bacteria (SFB) were only mediocre inducers of $ROR\gamma t^+$ T_{regs} in that study (142). These studies demonstrate that intestinal $ROR\gamma t^+$ T_{regs} are highly microbiota-dependent and have functions in promoting host immunity (62). Yet, RORγt is not a perfect marker for pT_{regs}, because recent reports show the existence of $ROR\gamma t^+$ tT_{regs}, particularly developing under inflammatory conditions (143–145).

While most studies have focused on in-depth characterization of mechanisms by which microbiota engage to counter-regulate their immunostimulatory properties, the reciprocal effect of T_{regs} on the composition and function of the intestinal microbiota was largely ignored (53, 56, 99, 146, 147). Very recently, analysis of mice harboring a reduced number of TGF-β-dependent pT_{regs} demonstrated numerous underrepresented metabolic processes and a limited overall diversity of the microbiome, including a significant reduction of *Lactobacillus johnsonii* and *Mucispirillum schaedleri* (148). Mechanistically, it was confirmed that the impaired pT_{reg} generation could adversely affect the microbiota niche by elevating type 2 immune responses in the host, thereby declining the microbiota abundance during the process of community assembly. In conclusion, the presence of pT_{regs} in the intestinal immune system has a strong impact on the composition and function of the intestinal microbiota. Similarly, IL-17F deficiency induces T_{reg} cells in the colon and modifies the composition of the intestinal microbiota and mediates protection against colitis

(149). Taken together, two-way interactions between resident microbiota and host intestinal immunity confer intestinal tolerance and immunomodulation.

IMPACT OF MICROBIOTA ON T_{REGS} AND TH17 CELLS IN ORAL MUCOSA

Oral microbiome is vital to maintaining both oral and systemic immune homeostasis because oral mucosa is the primary gateway for the GI tract, the biggest component of the immune system (150). While a vast majority of microbiota studies has focused on intestinal mucosae and their interactions with gut microbiota, little is known about oral mucosal microenvironment colonized with a large array of resident microbes, which is structurally and functionally distinct from the GI tract (151–160). *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* are the major phyla accounting for ∼96–99% of the oral microbiome, while SR1, TM7, *Cyanobacteria, Spirochaetes, Synergistetes,* and *Tenericutes,* are also found (<1% distribution). It is well established that oral-resident microbiota in poly-microbial interactions and soft-tissue biofilms avert oral diseases, but direct effect of such interactions on host oral immune cells is less clear (161–166). Oral mucosa maintains subsets of dendritic cells (DC), which produce immunomodulatory cytokines such as IL-10, TGF-β1 and Prostaglandin E2, and are predominantly tolerogenic (89, 167–169). These cells may be in intimate cross-talk with oral mucosal T_{regs} (58, 62, 170, 171), albeit details of such interactions between these cells are unexplored in oral mucosa. However, alterations in T_{regs} and Th17 functions have been implicated in human oral *Candida* infections and periodontitis (36, 38, 40, 69, 172–176). We and others have shown the presence of oral mucosal $Foxp3^+$ T_{regs} with protective functions during local infection (89, 158, 169, 170). The interrelationship between these cells and oral commensals during an oral infection was also explored (58, 170). In the context of oropharyngeal candidiasis (OPC) infection, T_{reg} cells play a critical role in reducing fungal burden and establishing homeostasis during post anti-fungal response (177). T_{regs} play rather an unconventional role of enhancing the Th17 cell response and neutrophil infiltration during early acute response, but are associated with reduced TNF-α expression in CD4 T cells at resolution phase (83, 91, 178). *Candida* infection in mice by itself increases the proportion of $Foxp3^+T_{regs}$, in a TLR2/MyD88 dependent manner in oral mucosal tissues and draining cervical lymph nodes (58, 83, 91). A small proportion of those $Foxp3^+$ cells co-express RORγt and IL-17A ($T_{reg}17$). Antibiotic mediated depletion of resident bacteria significantly diminishes the frequency of $Foxp3^+T_{reg}$ $IL\text{-}17A^-$ and $T_{reg}17$ cells, as well as conventional Th17 cells not expressing Foxp3. Reduction of these cells is concomitant with an increase in tissue pathology and fungal burden in oral mucosa, demonstrating that resident bacteria are important for controlling $Foxp3^+$ cells and Th17 cells, as well as mucosal immunity (**Figure 1**). Interestingly, *Candida* can also promote Th17 and T_{reg} responses in oral mucosa (83, 179, 180). The impact of oral resident microbiome in periodontal inflammation, which is now considered a "resident microbial perturbation" rather than a disease caused by a single pathogen, is well known (181). Resident bacterium *P. gingivalis*, the keystone pathogen contributes to altering the abundance and composition of other normal microbiota. Shift and accumulation of gram-positive aerobes to gram-negative anaerobes such as *P. gingivalis, T. denticola, F. nucleatum, and Prevotella sp*. are strongly associated with damage in gingival barrier, loss of immune balance and destruction of oral tissue in periodontal disease (150). During this process, bacterial antigens from skewed microbiota can access connective tissues causing abnormal activation and expansion of inflammatory $CD4^+CD69^+CD103^-$ memory T cells and Th17 cells (182). Another recent study showed that periodontitis-associated expansion of Th17 cells required both IL-6 and IL-23, and was dependent on the local dysbiotic microbiome (31). Shift in resident microbiota can also include increase in *C. albicans*, a part of resident mycobiome in ∼50–70% of healthy humans, which can rapidly transition to a pathogen and cause infections in immune-compromised and cancer patients. *C. albicans* is also shown to heighten *P. gingivalis* accumulation, worsening the series of inflammatory events associated with periodontitis severity (183, 184). It is known that $T_{reg}17$ cells exist in periodontitis lesions and could be involved in inflammatory responses against periodontopathic bacteria (185). While there may be only small changes in oral microbiome in HIV+ individuals, underlying mechanisms causing dysbiosis and its association with HIV associated periodontitis during SIV/HIV infection are unclear (117, 186, 187). Precise events defining Th17 and T_{reg} dysfunctions in the context of underlying dysbiosis and aggravating oral inflammation in HIV disease and periodontitis remain to be seen.

MICROBIOME IN MUCOSAL IMMUNITY AND INFLAMMATION IN OTHER MUCOSAE

Lung, previously thought to be sterile, is now known to harbor a complex and dynamic microbial community of ∼500 species, with a high resemblance to oral microbiome (188, 189). Lung microbiome strongly influences the development and progression of allergic responses and asthma (190). Disrupting the normal microbiome with childhood antibiotic exposure increases the risk of childhood asthma. *Proteobacteria* abundance in lower airway secretions correlates with pro-inflammatory Th17 cell proportions in asthmatic individuals (191, 192). Similarly, in cystic fibrosis patients, alterations of some groups in the polymicrobial community significantly affect the disease progression. Also, in chronic obstructive pulmonary disease (COPD) patients, microbial dysbiosis associated with mucus hyper-secretion and reduced airway clearance results in chronic aberrant inflammation and airway damage (193). Lung microbiota alterations are also associated with differences in pneumococcal clearance (194).

Multiple genera of microbiota exist in vaginal mucosa, often dominated by species of *Lactobacillus*, and a diverse array of anaerobic microorganisms, including *Atopobium,*

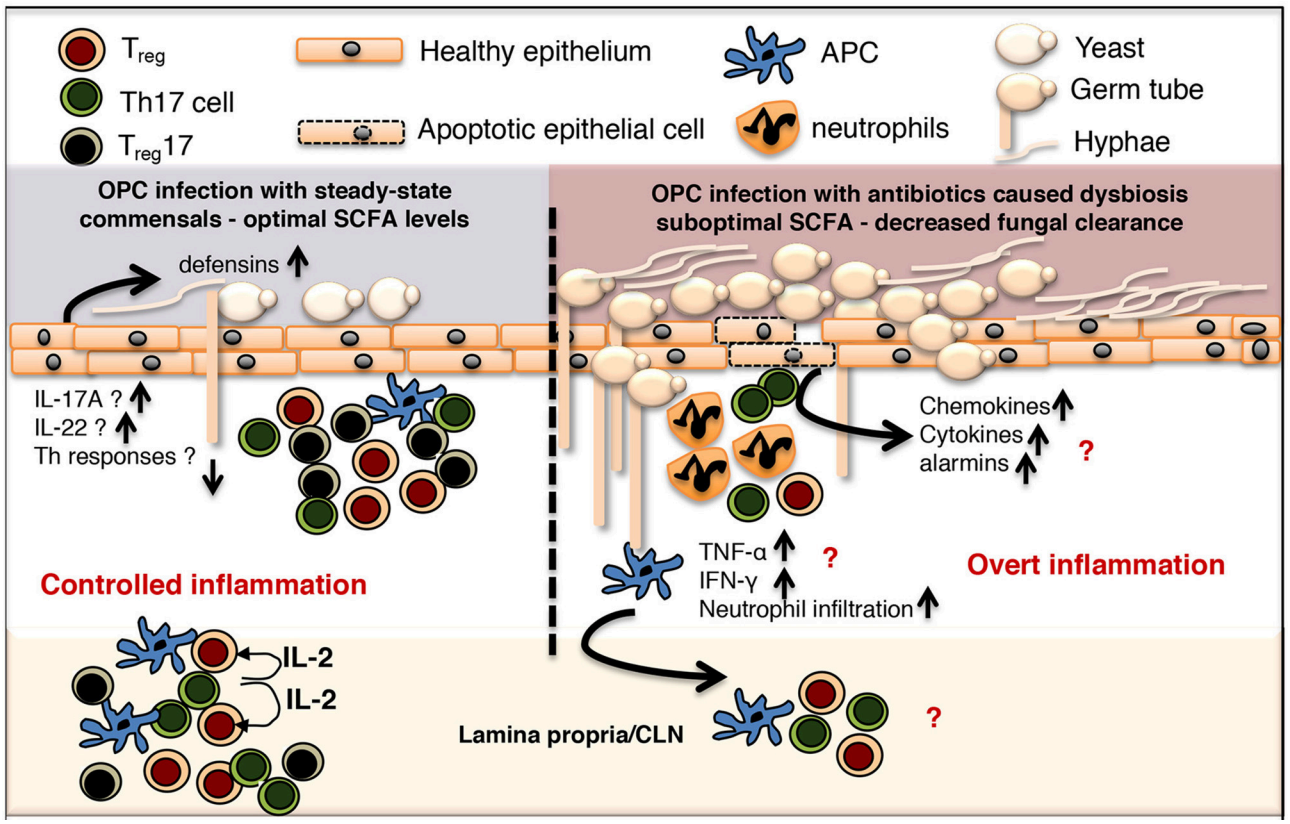

FIGURE 1 | Controlled commensal bacteria/T_{reg}/T_{reg}17/Th17 cell interplay functions as a switch between protective immunity and overt inflammation in oral mucosa. OPC, Oropharyngeal candidiasis; SCFA, short chain fatty acid; CLN, cervical lymph node; APC, antigen presenting cells.

Anaerococcus, Corynebacterium, Eggerthella, Gardnerella, Mobiluncus, Peptoniphilus, Prevotella, Sneathia, and *Finegoldia* genera (195). *Lactobacilli* largely impact the susceptibility to *T. vaginalis* infection in women. Although mechanisms are still under investigation, there is precedence that Th17 cells and T_{regs} can have protective and anti-inflammatory effects during *T. vaginalis* infection (196). During a vaginal herpes simplex virus-2 (HSV-2) infection, mice lacking T_{regs} fail to timely accumulate HSV-2-specific CD4 T cells and control the infection. This finding underscores the protective role of T_{regs} in facilitating productive mucosal immunity in vaginal mucosa (197, 198). However, mechanisms of direct control of vaginal microbiome on T_{regs} and Th17 cells and infection responses remain to be seen. In ocular mucosa, *Corynebacterium mastiditis* induces commensal specific IL-17 response γδ T cells, recruiting neutrophils and protecting the ocular mucosa from pathogenic infections (199). In nasal mucosa, on the one hand there is evidence that butyric acid-producing microorganisms associate with an impaired olfactory function (200–202). On the other, nasal microbiome is structured by IL-17 Signaling that that supports resistance to *S. pneumoniae* colonization in the nasal mucosa of mice (203). Collectively, while mcrobial dysbiosis and T_{regs}/Th17 changes are associated with many of these infections, detailed mechanisms remain to be investigated.

MOLECULAR MECHANISMS OF MICROBIOTA-ASSOCIATED ALTERATIONS OF T_{REG}/TH17 CELLS IN MUCOSAE

Resident microbes have a variety of mechanisms for conferring mucosal colonization resistance (17, 204–207). They include: (1) directly competing for shared metabolites, (2) expression of inhibitory bacteriocins, (3) induction of protective mucus layer, and (4) priming of protective immune responses (208, 209). Some of the examples include commensal dependent metabolism of secondary bile acids to deoxycholate, production of organic acids, induction of antimicrobial peptides in Paneth cells, and promoting elevated antibacterial T cell responses preventing colonization and dissemination of pathogens (210–213). Although resident bacteria are known to modulate energy metabolism producing pyruvic acid, citric acid, fumaric acid and malic acid (214), how pH changes determine the mucosal immunity and T cells warrants further investigation. Resident microbiota employ multiple mechanisms that contribute to coordination of T_{reg}/Th17 axis and safeguarding of mucosa (**Figure 2**). For example, microbiota dependent TLR signaling in host is one of the important mechanisms by which microbiota control inflammation and tolerance. TLR2/MyD88signaling is required for generation and expansion of $Nrp1^{low}$ Foxp3+

cells and $T_{reg}17$ cells in oral and gut mucosa (58). In gut mucosa the capsular polysaccharide A of the *Bacteroides fragilis* stimulates production of IL-10 by $Foxp3^+$ cells in a TLR2 dependent manner, thus facilitating mucosal tolerance (215). Recently it was found that this commensal also delivers immunomodulatory molecules to immune cells via secretion of outer membrane vesicles through a non-canonical autophagy pathway for inducing IL-10 expressing $Foxp3^+$ cells. This mechanism requires the expression of host genes *ATG16L1* and *NOD2,* whose polymorphisms are known to be associated with IBD (216). Selective deletion of *Atg16l1* in T cells in mice also results in loss of $Foxp3^+$ T_{reg} cells and spontaneous intestinal inflammation characterized by aberrant Th2 responses. These data indicate microbiota-host interactions intimately involve the processes of autophagy and T_{reg} differentiation. Moreover, loss of MyD88-STAT3 signaling in T_{regs} causes loss of mucosal T_{regs} and impaired T follicular regulatory cell interactions, resulting in poor IL-21 and anti-microbial IgA responses (217). Failure of this pathway results in over-growth of pathobionts, overt Th17 cell expansion and intestinal inflammation. However, the requirement of resident microbiome induced MyD88 signaling specifically in T_{regs}, to promote T_{reg} sustenance and intestinal tolerance is still debated (217–219). Similar to *B.fragilis*, colonic *Clostridium rhamnosus* also potently induces IL-10^+T_{regs} in a TGF-β1 dependent manner, which is correlated to increase in systemic IgE and resistance to colonic inflammation (56, 99). Similarly, microbiota and immune cell networks are known to control the production of IgA, which is central for mucosal barrier and intestinal tolerance. For example, *Mucispirillum* spp. and SFB have been directly implicated in production of intestinal IgA (137, 220, 221). T_{regs} are also known to promote IgA secretion, and maintenance of diversified and balanced microbiota, which in turn facilitates their expansion through a symbiotic regulatory loop, and prevent overt inflammation (222, 223). Moreover, $ROR\gamma t^+$ Th17 cells, as well as IL-17A from other cells also promote epithelial polymeric Ig receptor and intestinal IgA expression, further contributing to intestinal homeostasis (224, 225). SFB also control commensal tolerance and anti-microbial host responses through intestinal epithelial cell fucosyl tranferase 2 expression and fucosylation, a process that is dependent on $ROR\gamma t^+$ group 3 innate lymphoid cells (ILC3s) and IL-22 expression (226, 227). Loss of intestinal fucosylation results in increased susceptibility to infection by *Salmonella typhimurium*. ILC3s can also express major histocompatibility complex class II (MHCII) and mediate intestinal selection of $CD4^+$ T cells in order to limit commensal bacteria-specific CD4 T-cell responses (228). Although IL-6, induction of $T_{regs,}$ or Th17 cells were shown to be not required for ILC-mediated tolerance, alterations in $T_{reg}17$ and Th17 cells in the context of fucosylation remain to be studied. T_{reg}/Th17 cell differentiation and expansion are also independently controlled by specific members of anaerobic bacteria producing short chain fatty acids (SCFAs), such as acetate, propionate and butyrate (229, 230). Some of these bacteria include *Bacteroides, Bifidobacterium, Feacalibacterium* genera, *and Enterobacteriaceae family, Porphyromonas gingivalis, Fusobacterium nucleatum* (mouth), *Clostridium cochlearium, Eubacterium multiforme* (intestine), and *Anaerococcus tetradius* (vagina). These bacteria ferment indigestible oligosaccharides and cell surface fucosylated proteins by anaerobic glycolysis, resulting in SCFA production. SCFAs are present in the intestinal lumen at a total concentration of ∼100 mM at a ratio of ∼6:3:1, for acetate, propionate and butyrate respectively. Although this ratio hinges on carbohydrate availability, microbiota composition and intestinal transit time, acetate and butyrate appear to be the highest and least in abundance respectively (231). Emerging data show that SCFAs contribute to immune homeostasis in mucosa, although excessive and suboptimal levels of SCFAs are often associated with inflammation and cancer. Intestinal SCFAs have been shown to potentiate $Foxp3^+$ cell differentiation and immunomodulatory activity in the colon (53, 99, 147, 232). Mechanistically, in addition to direct histone deacetylase (HDAC) inhibition, SCFAs can induce the expression of retinal aldehyde dehydrogenase 1 family member 1a (Aldh1a) and TGF-β1 in intestinal epithelial cells and DCs (100, 221, 233, 234). Aldh1a could further convert vitamin A into its metabolite retinoic acid in G protein–coupled receptor43 (GPCR43) and Gpr109a manner, which is capable of facilitating T_{reg} induction. These tolerogenic DCs express CD103, sample antigens in the intestinal lamina propria, and migrate to the draining mesenteric lymph node (MLN) to induce immunomodulatory T cells (235–237). Whether SCFA mediated induction and or sustenance of mucosal T_{regs} require these aforementioned processes is unclear and remain to be studied. However, antibiotics precipitously decrease the oral SCFAs in saliva, showing that in the oral resident bacteria-derived-SCFA is functionally involved in controlling oral mucosal immunity and inflammation (62). Lending credence to this tenet, antibiotics treated mice show not only increased oral inflammation, but also intestinal immunopathology, when infected with oral *Candida*. Mechanistically, antibiotic treatment results in reduced T_{regs}, Th17 and $T_{reg}17$ cells in oral mucosa and tissue draining cervical and axillary lymph nodes in infected mice. Intestinal inflammation in oral *Candida* infected mice is characterized by an increase in IFN-γ producing Th1 cells and co-producers of IFN-γ and IL-17A (Th1*) cells. Although the exact mechanism of antibiotic mediated reduction of T_{regs}, Th17 cells and $T_{reg}17$ cells is unclear, administration of SCFA partially restored these populations and reduces oral immunopathology during the infection. SCFA administration however, only moderately ameliorates the intestinal inflammation. Therefore, the mechanism of Th1-mediated gut inflammation during oral *Candida* infection in the context of altered microbiota remains to be addressed. Recently, Atarashi et al. showed that oral bacterium *Klebsiella* spp. isolated from the salivary microbiota elicits a severe Th1 gut inflammation in the context of intestinal dysbiosis, in a genetically susceptible host (238). This finding underscores the role of oral resident microbes such as *Klebsiella* spp. and *C. albicans* in modulating T cells, possibly translocating to gut and causing overt inflammation in the gut in the context of resident microbial dysbiosis. Supporting this tenet, post oral gavage of *C. albicans*-infected mice pre-treated with antibiotics showed significantly altered composition of intestinal microbiota as well as $CD4^+$ T cell mediated lung inflammation, following

aerosol introduction of an allergen. However, mice without any antibiotics pre-treatment did not develop an allergic response in the airways (239, 240). Whether changes in SCFA, or T_{reg} and Th17 cells in the lung contribute to the inflammation is unknown.

Mechanistically, SCFAs also cause acetylation of p70 S6 kinase and phosphorylation rS6, promoting the mTOR activity. mTOR activity was shown to be required for generation of Th17 (T helper type 17), Th1, and IL-10^+ T cells (241). Moreover phosphoinositide 3-kinase and mTOR pathways play pivotal roles in integrating growth signals in $CD4^+$ T cell differentiation (242–249). Multiple studies support the role of mTORC1 and mTORC2 proteins in regulating Th17 and T_{reg} fate decisions (247, 250, 251). mTORC1 signaling is constitutively active in T_{reg} cells, and disruption of mTOR protein as well as unrestrained mTOR hyper-activation, both have been shown to cause autoimmunity by impairing Foxp3 expression and T_{reg} functions (252–260). Another study has also shown that mTORC1 and its downstream target hypoxia-inducible factor-1α (HIF-1α) are needed for Foxp3 induction, T_{reg} lipid and cholesterol biosynthesis from glucose, and proliferation and suppressive function *in vivo* (244, 254). Taken together, while direct role of SCFA in mediating mTOR activation and subsequent T_{reg} induction in mucosa is unclear, these studies highlight the importance of how immunologically relevant microbiome can control T_{regs} and mucosal homeostasis through multiple mechanisms.

MICROBIOTA AND T_{REG}/TH17 CELL REGULATION OF IMMUNE SENESCENCE AND CHRONIC INFLAMMATION

While resident microbes have aforementioned protective functions in mucosa, they can also trigger and sustain inflammation during aging and other chronic inflammatory conditions. Some studies demonstrate direct relationship between aging and changes in microbiota, albeit the mechanisms remain largely unstudied. Aging causes increased accumulation of gut *Enterobacteria, Streptococci, and yeasts* but declining levels of *Akkermansia muciniphila*, *Bifidobacteria* and *Bacteroides* (261–266). Reduced *Akkermansia muciniphila* is associated with reduced butyrate and impaired intestinal barrier. Consequently, aged mice display endotoxin leakage, and triggering of 4-1BB receptor signaling and insulin resistance. In oral mucosa, aging causes higher levels of $RANKL^+$ cells, and increased inflammatory Th17 cell accumulation, with concomitant loss of alveolar bone, which are dependent on the presence of commensal microbiota (30, 267, 268). In contrast, these events do not occur in in germfree mice periodontium, showing potentially pathogenic roles of commensal microbiota in aging associated dysbiosis setting. Similarly, resident microbiota have been implicated in the onset and progression of experimental autoimmune encephalomyelitis (EAE) (269). GF mice exhibit lower levels of the pro-inflammatory cytokine IFN-γ and IL-17A producing cells, and a reciprocal increase in T_{regs} in the intestine and spinal cord. These changes in GF mice correlate with a significantly attenuated EAE, compared with conventionally raised mice. Remarkably, intestinal colonization with SFB alone can promote Th17 cells in the gut and in the central nervous system (CNS), enhancing disease progression (270). Furthermore, partial elimination of intestinal microbiota ameliorates established collagen-induced arthritis by dampening Th17 responses in mice (271). Some bacteria also provide inflammatory signals resulting in chronic inflammation and tumorigenesis, likely by inducing genetic and epigenetic changes in host cells. For example, *Fusobabacteria* spp. has been implicated in increased risk of IBD and colorectal cancer (272–275). Also, in oral mucosa, the abundance of *Fusobacterium* increases, while the number of *Streptococcus, Haemophilus, Porphyromonas, and Actinomyces* decreases with cancer progression in oral squamous cell carcinoma (276). Interestingly, *Fusobabacteria,* and several other bacteria of oral mucosal origin, including genera of *Streptococcus, Staphylococcus, Peptostreptococcus* may translocate to intestine in the context of gut inflammation and carcinogenesis (277–279), similar to *Klebsiella* spp *and C. albicans* in susceptible host (62, 238). It is tempting to speculate that loss of T_{reg} functions in the context of dybiosis, excessive SCFA and oral microbial translocation may have contributed to exuberant intestinal inflammation and predisposition to carcinogenesis in these studies (**Figure 2**). However, whether the mouth- to -gut translocation is a cause, or consequence of dysbiosis and intestinal inflammation, and the underlying mechanisms still remain to be understood and warrant further investigation.

THERAPEUTIC APPLICATIONS OF MICROBIOTA ALTERATIONS AND MICROBIOTA DERIVED METABOLITES

As we discussed above, studies on patient cohorts, mechanistic studies on mice and epidemiological studies have led to a better understanding of how microbiota changes impact mucosal immunity, and *vice versa.* Mechanistic "proof-of principle" studies using disease models have opened ways to manipulate these processes, providing therapeutic approaches. Some of the widely used approaches include administration of sodium butyrate and pre- and pro-biotics, and transplantation of fecal microbiota (280–283). However, there are hurdles in pro-biotic and microbiota transplantation approaches. Existing microbiota, whether it is healthy or dysbiotic is largely stable over time in an individual. Without profound perturbation of the existing microbiota, it is challenging to introduce microbiota exogenously. The effects of exogenous bacteria introduced by probiotic and transplant approaches are greatly influenced by existing microbiota in a competitive niche, and are inconsistent. Therefore, approaches to target these niches in favor of exogenous bacteria are being studied (283, 284). Direct administration of microbial derivatives appears to be a promising venue. Butyrate has been shown to alleviate high-fat-diet induced non-alcoholic fatty liver disease. It potently down modulates peroxisome proliferator-activated receptor α-mediated activation of β oxidation, causing reduced inflammation (285). For cART

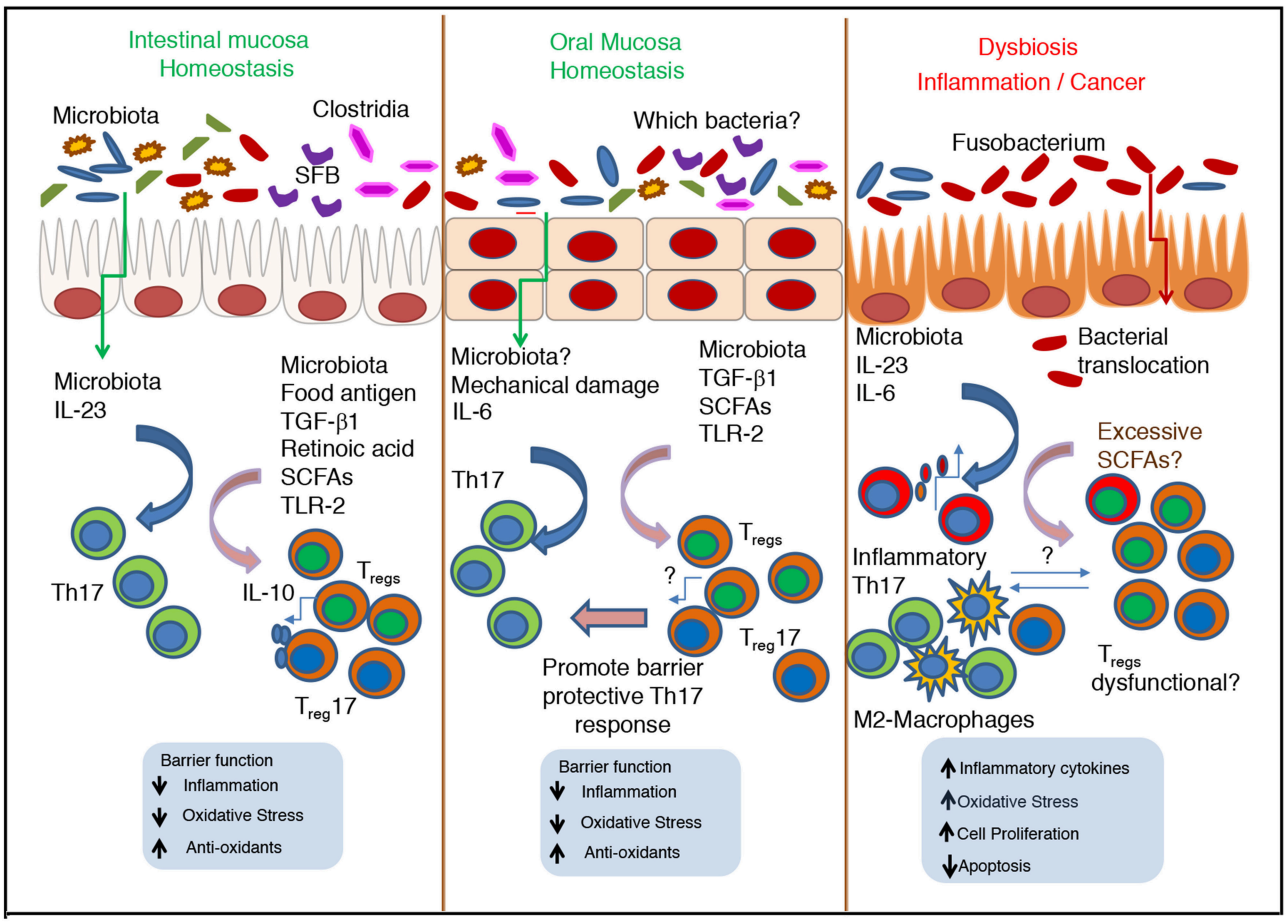

FIGURE 2 | Cross talk between microbiota and immune cells during homeostasis and dysbiosis–Role of Th17 cells and T_{regs} in oral and intestinal mucosa. During homeostatic conditions, normal microbiota promote the stimulation of epithelial cells, Th17 cells and T_{regs}, and maintain barrier function and commensal tolerance. In oral mucosa, Th17 cells are induced by mastication induced mechanical damage, independent of commensals. However, in both mucosae SCFA mediated induction of T_{regs} is key for mucosal barrier function and immunomodulation. During inflammation and cancer, excessive SCFAs can increase inflammatory Th17 cells and T_{reg} population that may be dysfunctional. The nature of their interaction with Th17 cells, tumor associated M2-type macrophages and other cells remain unclear.

treated HIV^{+} individuals, aside from cART treatment, probiotics have been studied to combat persistent systemic inflammation. This approach in the context of cART may lead to improved and holistic management of inflammatory events and higher cancer susceptibility in HIV+ patients. Application of probiotics has also shown positive effect on the course of pneumonia, acute exacerbation of bronchial asthma and COPD in mice models, but warrants further studies in humans (286). SCFA has been shown to have therapeutic potential in microbiome-targeted interventions in anti-aging medicine. Butyrate and dietary fibers have been shown to promote anti-inflammatory effects in the context of aging associated neuro-inflammation in mice (287). Adult and aged mice fed with 5% inulin (high fiber) diet for 4 weeks show an altered gut microbiome and increased butyrate, acetate, and total SCFA production, coinciding with a reduction in neuro-inflammation. High fiber supplementation in aging is a non-invasive strategy to increase butyrate levels, and these data suggest that an increase in butyrate through added soluble fiber such as inulin could counterbalance the age-related microbiota dysbiosis, potentially leading to neurological benefits (287, 288). Similarly, dietary fiber also suppresses colon carcinogenesis in polyposis mice (289). Mechanistically it has been shown to inhibit colorectal cancer cell migration through micro-RNA regulation (290). In summary, alterations of mechanisms of microbiota-host interactions are proving to hold promise for treating a variety of disorders in humans.

CONCLUSION

It is now well established that resident microbes provide enormous advantages to the host, while dysbiosis can trigger acute and chronic inflammatory conditions. One of the mechanisms by which these microbes regulate immunity id through controlling T_{regs} and Th17 cells. These cells present in various mucosal locations and share various signaling pathways for their development and sustenance, as stated above. However,

signals modulating these subsets unique to each mucosal environment in different epithelial cell contexts are unclear. Most mechanistic studies showing T_{reg}/Th17 developmental regulation were performed using the *in vitro* cultures using cells isolated from blood (human), spleen and lymph nodes (mice). While there is enough evidence to show that these cells could be regulated by overlapping signaling mechanisms, cells from these mucosae were not directly compared for similarities and differences in their development and functions. Such studies are warranted to get further insights in to homeostatic and dysbiotic conditions in different mucosae. Such studies in the context of microbial manipulation approach will offer new avenues to manipulate their interactions with the host for treating immune-mediated and metabolic disorders. While mono-association of certain genera in GF mice have proven to alter mucosal T_{regs} and Th17 cells and offer some beneficial effects in some experimental settings (98), from a therapeutic perspective, the field is still at its infancy and warrants intense mechanistic investigations. Taken together, further research in microbiota targeted approaches will enable the field to take the center stage in the management of health and disease in humans.

AUTHOR CONTRIBUTIONS

PP and JH wrote the manuscript. NB, ES, MZ, and SJ contributed to the discussion.

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REFERENCES

1. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. *Genome Med.* (2016) 8:51. doi: 10.1186/s13073-016-0307-y
2. Savage DC. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol.* (1977) 31:107–33. doi: 10.1146/annurev.mi.31.100177.000543
3. Donaldson GP, Lee SM, Mazmanian SK. Gut biogeography of the bacterial microbiota. *Nat Rev Microbiol.* (2016) 14:20–32. doi: 10.1038/nrmicro3552
4. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol.* (2016) 14:e1002533. doi: 10.1371/journal.pbio.1002533
5. Gallo RL, Nakatsuji T. Microbial symbiosis with the innate immune defense system of the skin. *J Invest Dermatol.* (2011) 131:1974–80. doi: 10.1038/jid.2011.182
6. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity*. (2012) 37:158–70. doi: 10.1016/j.immuni.2012.04.011
7. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. *Nat Rev Gastroenterol Hepatol.* (2012) 9:577–89. doi: 10.1038/nrgastro.2012.156
8. Hill DA, Siracusa MC, Abt MC, Kim BS, Kobuley D, Kubo M, et al. Commensal bacteria-derived signals regulate basophil hematopoiesis and allergic inflammation. *Nat Med.* (2012) 18:538–46. doi: 10.1038/nm.2657
9. Abt MC, Artis D. The dynamic influence of commensal bacteria on the immune response to pathogens. *Curr Opin Microbiol.* (2013) 16:4–9. doi: 10.1016/j.mib.2012.12.002
10. Hill DA, Artis D. The influence of commensal bacteria-derived signals on basophil-associated allergic inflammation. *Gut Microb.* (2013) 4:76–83. doi: 10.4161/gmic.22759
11. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol.* (2013) 14:685–90. doi: 10.1038/ni.2608
12. Kamada N, Seo SU, Chen GY, Nunez G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol.* (2013) 13:321–35. doi: 10.1038/nri3430
13. Spasova DS, Surh CD. Blowing on embers: commensal microbiota and our immune system. *Front Immunol.* (2014) 5:318. doi: 10.3389/fimmu.2014.00318
14. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol.* (2004) 4:478–85. doi: 10.1038/nri1373
15. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell.* (2006) 124:823–35. doi: 10.1016/j.cell.2006.02.016
16. Lee YK, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science*. (2010) 330:1768–73. doi: 10.1126/science.1195568
17. Belkaid Y, Naik S. Compartmentalized and systemic control of tissue immunity by commensals. *Nat Immunol.* (2013) 14:646–53. doi: 10.1038/ni.2604
18. Brito F, Zaltman C, Carvalho AT, Fischer RG, Persson R, Gustafsson A, et al. Subgingival microflora in inflammatory bowel disease patients with untreated periodontitis. *Eur J Gastroenterol Hepatol.* (2013) 25:239–45. doi: 10.1097/MEG.0b013e32835a2b70
19. Huffnagle GB, Noverr MC. The emerging world of the fungal microbiome. *Trends Microbiol.* (2013) 21:334–41. doi: 10.1016/j.tim.2013.04.002
20. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell*. (2014) 157:121–41. doi: 10.1016/j.cell.2014.03.011
21. Blander JM, Longman RS, Iliev ID, Sonnenberg GF, Artis D. Regulation of inflammation by microbiota interactions with the host. *Nat Immunol.* (2017) 18:851–60. doi: 10.1038/ni.3780
22. Bilate, A. M., Bousbaine, D., Mesin, L., Agudelo, M., Leube, J., Kratzert, A., et al. (2016). Tissue-specific emergence of regulatory and intraepithelial T cells from a clonal T cell precursor. *Sci Immunol* 1:eaaf7471. doi: 10.1126/sciimmunol.aaf7471
23. Calderon-Gomez E, Bassolas-Molina,H, Mora-Buch R, Dotti I, Planell N, Esteller M, et al. Commensal-Specific CD4(+) Cells From Patients With Crohn's Disease Have a T-Helper 17 Inflammatory Profile. *Gastroenterology*. (2016) 151:489–500 e483. doi: 10.1053/j.gastro.2016.05.050
24. Hegazy AN, West NR, Stubbington MJT, Wendt E, Suijker KIM, Datsi A, et al. Circulating and tissue-resident CD4(+) T cells with reactivity to intestinal microbiota are abundant in healthy individuals and function is altered during inflammation. *Gastroenterology*. (2017) 153:1320–37 e1316. doi: 10.1053/j.gastro.2017.07.047
25. Navabi N, Whitt J, Wu SE, Woo V, Moncivaiz J, Jordan MB, et al. Epithelial histone deacetylase 3 instructs intestinal immunity by coordinating local lymphocyte activation. *Cell Rep.* (2017) 19:1165–75. doi: 10.1016/j.celrep.2017.04.046
26. Sonnenburg ED, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS, Sonnenburg JL. Diet-induced extinctions in the gut microbiota compound over generations. *Nature*. (2016) 529:212–5. doi: 10.1038/nature16504
27. Stappenbeck TS, Virgin HW. Accounting for reciprocal host-microbiome interactions in experimental science. *Nature*. (2016) 534:191–9. doi: 10.1038/nature18285

28. Willyard C. Squeaky clean mice could be ruining research. *Nature*. (2018) 556:16–8. doi: 10.1038/d41586-018-03916-9
29. Kim KS, Hong SW, Han D, Yi J, Jung J, Yang BG, et al. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. *Science*. (2016) 351:858–63. doi: 10.1126/science.aac5560
30. Dutzan N, Abusleme L, Bridgeman H, Greenwell-Wild T, Zangerle-Murray T, Fife ME, et al. On-going mechanical damage from mastication drives homeostatic Th17 cell responses at the oral barrier. *Immunity*. (2017) 46:133–47. doi: 10.1016/j.immuni.2016.12.010
31. Dutzan N, Kajikawa T, Abusleme L, Greenwell-Wild T, Zuazo CE, Ikeuchi T, et al. A dysbiotic microbiome triggers TH17 cells to mediate oral mucosal immunopathology in mice and humans. *Sci Transl Med*. (2018) 10:eaat0797. doi: 10.1126/scitranslmed.aat0797
32. Klein RS, Harris CA, Small CB, Moll B, Lesser M, Friedland GH. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *N Engl J Med*. (1984) 311:354–8. doi: 10.1056/NEJM198408093110602
33. Patton LL. Sensitivity, specificity, and positive predictive value of oral opportunistic infections in adults with HIV/AIDS as markers of immune suppression and viral burden. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. (2000) 90:182–8. doi: 10.1067/moe.2000.108799
34. Patton LL, McKaig R, Strauss R, Rogers D, Eron JJJr. Changing prevalence of oral manifestations of human immuno-deficiency virus in the era of protease inhibitor therapy. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. (2000) 89:299–304. doi: 10.1016/S1079-2104(00)70092-8
35. Gaitan Cepeda LA, Ceballos Salobrena A, Lopez Ortega K, Arzate Mora N, Jimenez Soriano Y. Oral lesions and immune reconstitution syndrome in HIV+/AIDS patients receiving highly active antiretroviral therapy. Epidemiological evidence. *Med Oral Patol Oral Cir Bucal*. (2008) 13:E85–93.
36. Kanwar B, Favre D, McCune JM. Th17 and regulatory T cells: implications for AIDS pathogenesis. *Curr Opin HIV AIDS*. (2010) 5:151–7. doi: 10.1097/COH.0b013e328335c0c1
37. Fidel, P. L., and Jr. (2011). *Candida*-host interactions in HIV disease: implications for oropharyngeal candidiasis. *Adv Dent Res*. 23, 45–49. doi: 10.1177/0022034511399284
38. Li D, Chen J, Jia M, Hong K, Ruan Y, Liang H, et al. Loss of balance between T helper type 17 and regulatory T cells in chronic human immunodeficiency virus infection. *Clin Exp Immunol*. (2011) 165:363–71. doi: 10.1111/j.1365-2249.2011.04435.x
39. Cassone A, Cauda R. *Candida* and candidiasis in HIV-infected patients: where commensalism, opportunistic behavior and frank pathogenicity lose their borders. *AIDS*. (2012) 26:1457–72. doi: 10.1097/QAD.0b013e3283536ba8
40. Huppler AR, Bishu S, Gaffen SL. Mucocutaneous candidiasis: the IL-17 pathway and implications for targeted immunotherapy. *Arthr Res Ther*. (2012) 14:217. doi: 10.1186/ar3893
41. Pandiyan P, Younes S, Ribeiro S, Talla A, Bhaskaran N, McDonald D, et al. Mucosal regulatory T cells and T helper 17 cells in HIV associated immune activation. *Front immunol*. (2016) 7:228. doi: 10.3389/fimmu.2016.00228
42. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4(+)CD25(+)Foxp3(+) regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4(+) T cells. *Nat Immunol*. (2007) 8:1353–62. doi: 10.1038/ni1536
43. Pandiyan P, Zheng L, Lenardo MJ. The molecular mechanisms of regulatory T cell immunosuppression. *Front Immunol*. (2011) 2:60. doi: 10.3389/fimmu.2011.00060
44. Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, et al. A special population of regulatory T cells potentiates muscle repair. *Cell*. (2013) 155:1282–95. doi: 10.1016/j.cell.2013.10.054
45. Edwards JP, Fujii H, Zhou AX, Creemers J, Unutmaz D, Shevach EM. Regulation of the expression of GARP/latent TGF-beta1 complexes on mouse T cells and their role in regulatory T cell and Th17 differentiation. *J Immunol*. (2013) 190:5506–15. doi: 10.4049/jimmunol.1300199
46. Arpaia N, Green JA, Moltedo B, Arvey A, Hemmers S, Yuan S, et al. A Distinct Function of Regulatory T *Cells* in Tissue Protection. *Cell*. (2015) 162:1078–89. doi: 10.1016/j.cell.2015.08.021
47. Worthington JJ, Kelly A, Smedley C, Bauche D, Campbell S, Marie JC, et al. Integrin alphavbeta8-Mediated TGF-beta Activation by Effector Regulatory T Cells Is Essential for Suppression of T-Cell-Mediated Inflammation. *Immunity*. (2015) 42:903–15. doi: 10.1016/j.immuni.2015.04.012
48. Jin RM, Warunek J, Wohlfert EA. Therapeutic administration of IL-10 and amphiregulin alleviates chronic skeletal muscle inflammation and damage induced by infection. *Immunohorizons*. (2018) 2:142–54. doi: 10.4049/immunohorizons.1800024
49. Povoleri GAM, Nova-Lamperti E, Scotta C, Fanelli G, Chen YC, Becker PD, et al. Human retinoic acid-regulated CD161(+) regulatory T cells support wound repair in intestinal mucosa. *Nat Immunol*. (2018) 19:1403–14. doi: 10.1038/s41590-018-0230-z
50. Izcue A, Coombes JL, Powrie F. Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol*. (2009) 27:313–38. doi: 10.1146/annurev.immunol.021908.132657
51. Cong Y, Feng T, Fujihashi K, Schoeb TR, Elson CO. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci USA*. (2009) 106:19256–61. doi: 10.1073/pnas.0812681106
52. Cebula A, Seweryn M, Rempala GA, Pabla SS, McIndoe RA, Denning TL, et al. Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. *Nature*. (2013) 497:258–62. doi: 10.1038/nature12079
53. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science*. (2013) 341:569–73. doi: 10.1126/science.1241165
54. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature*. (2011) 478:250–4. doi: 10.1038/nature10434
55. Nutsch K, Chai JN, Ai TL, Russler-Germain E, Feehley T, Nagler CR, et al. Rapid and efficient generation of regulatory T cells to commensal antigens in the periphery. *Cell Rep*. (2016) 17:206–20. doi: 10.1016/j.celrep.2016.08.092
56. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*. (2011) 331:337–41. doi: 10.1126/science.1198469
57. Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity*. (2011) 34:794–806. doi: 10.1016/j.immuni.2011.03.021
58. Bhaskaran N, Cohen S, Zhang Y, Weinberg A, Pandiyan P. TLR-2 signaling promotes IL-17A production in $CD4^+CD25^+$Foxp3+ regulatory cells during oropharyngeal candidiasis. *Pathogens*. (2015) 4:90–110. doi: 10.3390/pathogens4010090
59. Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity*. (2011) 34:237–46. doi: 10.1016/j.immuni.2011.01.016
60. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med*. (2012) 209:1723–42, S1721. doi: 10.1084/jem.20120914
61. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S, et al. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets *in vivo*. *J Exp Med*. (2012) 209:1713–22, S1711-9. doi: 10.1084/jem.20120822
62. Bhaskaran N, Quigley C, Paw C, Butala S, Schneider E, Pandiyan P. Role of short chain fatty acids in controlling tregs and immunopathology during mucosal infection. *Front Microbiol*. (2018) 9:1995. doi: 10.3389/fmicb.2018.01995
63. Sakaguchi S. The origin of FOXP3-expressing $CD4^+$ regulatory T cells: thymus or periphery. *J Clin Invest*. (2003) 112:1310–2. doi: 10.1172/JCI200320274
64. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. (2008) 133:775–87. doi: 10.1016/j.cell.2008.05.009
65. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity*. (2009) 30:636–45. doi: 10.1016/j.immuni.2009.04.010
66. Panduro M, Benoist C, Mathis D. Tissue Tregs. *Annu Rev Immunol*. (2016) 34:609–33. doi: 10.1146/annurev-immunol-032712-095948
67. Rowe JH, Ertelt JM, Aguilera MN, Farrar MA, Way SS. Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host

defense against prenatal bacterial pathogens. *Cell Host Microbe*. (2011) 10:54–64. doi: 10.1016/j.chom.2011.06.005
68. Johanns TM, Ertelt JM, Rowe JH, Way SS. Regulatory T cell suppressive potency dictates the balance between bacterial proliferation and clearance during persistent Salmonella infection. *PLoS Pathog.* (2010) 6:e1001043. doi: 10.1371/journal.ppat.1001043
69. Garlet GP, Cardoso CR, Mariano FS, Claudino M, de Assis GF, Campanelli AP, et al. Regulatory T cells attenuate experimental periodontitis progression in mice. *J Clin Periodontol.* (2010) 37:591–600. doi: 10.1111/j.1600-051X.2010.01586.x
70. Zhong Y, Cantwell A, Dube PH. Transforming growth factor beta and CD25 are important for controlling systemic dissemination following *Yersinia enterocolitica* infection of the gut. *Infect Immun.* (2010) 78:3716–25. doi: 10.1128/IAI.00203-10
71. Eggena MP, Barugahare B, Jones N, Okello M, Mutalya S, Kityo C, et al. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol.* (2005) 174:4407–14.
72. Chase AJ, Sedaghat AR, German JR, Gama L, Zink MC, Clements JE, et al. Severe depletion of $CD4^+$ CD25+ regulatory T cells from the intestinal lamina propria but not peripheral blood or lymph nodes during acute simian immunodeficiency virus infection. *J Virol.* (2007) 81:12748–57. doi: 10.1128/JVI.00841-07
73. Chase AJ, Yang HC, Zhang H, Blankson JN, Siliciano RF. Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low $CD4^+$ T-cell activation. *J Virol.* (2008) 82:8307–15. doi: 10.1128/JVI.00520-08
74. Elahi S, Dinges WL, Lejarcegui N, Laing KJ, Collier AC, Koelle DM, et al. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med.* (2011) 17:989–95. doi: 10.1038/nm.2422
75. Lanteri MC, O'Brien KM, Purtha WE, Cameron MJ, Lund JM, Owen RE, et al. Tregs control the development of symptomatic West Nile virus infection in humans and mice. *J Clin Invest.* (2009) 119:3266–77. doi: 10.1172/JCI39387
76. Oldenhove G, Bouladoux N, Wohlfert EA, Hall JA, Chou D, Dos Santos L, et al. Decrease of $Foxp3^+$ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity*. (2009) 31:772–86. doi: 10.1016/j.immuni.2009.10.001
77. Morampudi V, De Craeye S, Le Moine A, Detienne S, Braun MY, D'Souza S. Partial depletion of CD4(+)CD25(+)Foxp3(+) T regulatory cells significantly increases morbidity during acute phase Toxoplasma gondii infection in resistant BALB/c mice. *Microbes Infect.* (2011) 13:394–404. doi: 10.1016/j.micinf.2011.01.006
78. Tenorio EP, Olguin JE, Fernandez J, Vieyra P, Saavedra R. Reduction of Foxp3+ cells by depletion with the PC61 mAb induces mortality in resistant BALB/c mice infected with Toxoplasma gondii. *J Biomed Biotechnol.* (2010) 2010:786078. doi: 10.1155/2010/786078
79. Rausch S, Huehn J, Loddenkemper C, Hepworth MR, Klotz C, Sparwasser T, et al. Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of Foxp3+ cells. *Eur J Immunol.* (2009) 39:3066–77. doi: 10.1002/eji.200939644
80. Mendez S, Reckling SK, Piccirillo CA, Sacks D, Belkaid Y. Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. *J Exp Med.* (2004) 200:201–10. doi: 10.1084/jem.20040298
81. Baumgart M, Tompkins F, Leng J, Hesse M. Naturally occurring $CD4^+Foxp3+$ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation. *J Immunol.* (2006) 176:5374–87. doi: 10.4049/jimmunol.176.9.5374
82. Montagnoli C, Bacci A, Bozza S, Gaziano R, Mosci P, Sharpe AH, et al. B7/CD28-dependent $CD4^+CD25+$ regulatory T cells are essential components of the memory-protective immunity to *Candida albicans*. *J Immunol.* (2002) 169:6298–308. doi: 10.4049/jimmunol.169.11.6298
83. Pandiyan P, Conti HR, Zheng L, Peterson AC, Mathern DR, Hernandez-Santos N, et al. CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse *Candida albicans* Th17 cell infection model. *Immunity*. (2011) 34:422–34. doi: 10.1016/j.immuni.2011.03.002
84. Montagnoli C, Fallarino F, Gaziano R, Bozza S, Bellocchio S, Zelante T, et al. Immunity and tolerance to Aspergillus involve functionally distinct regulatory T cells and tryptophan catabolism. *J Immunol.* (2006) 176:1712–23. doi: 10.4049/jimmunol.176.3.1712
85. Hori S, Carvalho TL, Demengeot J. CD25+$CD4^+$ regulatory T cells suppress $CD4^+$ T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice. *Eur J Immunol.* (2002) 32:1282–91. doi: 10.1002/1521-4141(200205)32:5<1282::AID-IMMU1282>3.0.CO;2-#
86. Scott-Browne JP, Shafiani S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky AY, et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med.* (2007) 204:2159–69. doi: 10.1084/jem.20062105
87. Chen CY, Huang D, Yao S, Halliday L, Zeng G, Wang RC, et al. IL-2 simultaneously expands Foxp3+ T regulatory and T effector cells and confers resistance to severe tuberculosis (TB): implicative Treg-T effector cooperation in immunity to TB. *J Immunol.* (2012) 188:4278–88. doi: 10.4049/jimmunol.1101291
88. Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med.* (2009) 206:299–311. doi: 10.1084/jem.20081463
89. Allam JP, Duan Y, Winter J, Stojanovski G, Fronhoffs F, Wenghoefer M, et al. Tolerogenic T cells, Th1/Th17 cytokines and TLR2/TLR4 expressing dendritic cells predominate the microenvironment within distinct oral mucosal sites. *Allergy*. (2011) 66:532–9. doi: 10.1111/j.1398-9995.2010.02510.x
90. Cheng SC, van de Veerdonk FL, Lenardon M, Stoffels M, Plantinga T, Smeekens S, et al. The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of *Candida albicans*. *J Leukoc Biol.* (2011) 90:357–66. doi: 10.1189/jlb.1210702
91. Bhaskaran N, Liu Z, Saravanamuthu SS, Yan C, Hu Y, Dong L, et al. Identification of Casz1 as a regulatory protein controlling T helper cell differentiation, inflammation, and immunity. *Front Immunol.* (2018) 9:184. doi: 10.3389/fimmu.2018.00184
92. Martinez GJ, Nurieva RI, Yang XO, Dong C. Regulation and function of proinflammatory TH17 cells. *Ann NY Acad Sci.* (2008) 1143:188–211. doi: 10.1196/annals.1443.021
93. Miossec P, Kolls JK. Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov.* (2012) 11:763–76. doi: 10.1038/nrd3794
94. Pandiyan P, Yang XP, Saravanamuthu SS, Zheng L, Ishihara S, O'Shea JJ, et al. The role of IL-15 in activating STAT5 and fine-tuning IL-17A production in CD4 T lymphocytes. *J Immunol.* (2012) 189:4237–46. doi: 10.4049/jimmunol.1201476
95. Duhen R, Glatigny S, Arbelaez CA, Blair TC, Oukka M, Bettelli E. Cutting edge: the pathogenicity of IFN-gamma-producing Th17 cells is independent of T-bet. *J Immunol.* (2013) 190:4478–82. doi: 10.4049/jimmunol.1203172
96. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. (2009) 139:485–98. doi: 10.1016/j.cell.2009.09.033
97. Littman DR, Pamer EG. Role of the commensal microbiota in normal and pathogenic host immune responses. *Cell Host Microbe*. (2011) 10:311–23. doi: 10.1016/j.chom.2011.10.004
98. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature*. (2013) 500:232–6. doi: 10.1038/nature12331
99. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*. (2013) 504:446–50. doi: 10.1038/nature12721
100. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity*. (2014) 40:128–39. doi: 10.1016/j.immuni.2013.12.007
101. Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol.* (2007) 19:652–7. doi: 10.1016/j.coi.2007.07.020

102. Jiang Y, Liu Y, Lu H, Sun SC, Jin W, Wang X, et al. Epigenetic activation during T helper 17 cell differentiation is mediated by Tripartite motif containing 28. *Nat Commun.* (2018) 9:1424. doi: 10.1038/s41467-018-03852-2
103. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, et al. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. *J Clin Invest.* (2010) 120:4332–41. doi: 10.1172/JCI 43918
104. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. (2011) 145:745–57. doi: 10.1016/j.cell.2011. 04.022
105. Rehman A, Sina C, Gavrilova O, Hasler R, Ott S, Baines JF, et al. Nod2 is essential for temporal development of intestinal microbial communities. *Gut*. (2011) 60:1354–62. doi: 10.1136/gut.2010.216259
106. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. (2014) 505:559–63. doi: 10.1038/nature12820
107. Zhang H, Sparks JB, Karyala SV, Settlage R, Luo XM. Host adaptive immunity alters gut microbiota. *ISME J.* (2015) 9:770–81. doi: 10.1038/ismej. 2014.165
108. Bohnhoff, M., M. C. (1962). Enhanced susceptibility to Salmonella infection in streptomycin-treated mice. *J Infect Dis* 111:11.
109. Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, et al. A mouse model of *Clostridium* difficile-associated dieases. *Gastroenterology*. (2008) 135:1984–92. doi: 10.1053/j.gastro.2008.09.002
110. Reeves AE, Koenigsknecht MJ, Bergin IL, Young VB. Suppression of *Clostridium* difficile in the gastrointestinal tracts of germfree mice inoculated with a murine isolate from the family Lachnospiraceae. *Infect Immun.* (2012) 80:3786–94. doi: 10.1128/IAI.00647-12
111. Sekirov I, Tam NM, Jogova M, Robertson ML, Li Y, Lupp C, et al. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infect Immun.* (2008) 76:4726–36. doi: 10.1128/IAI.00319-08
112. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature*. (2013) 502:96–9. doi: 10.1038/nature12503
113. Cho H, Jaime H, de Oliveira RP, Kang B, Spolski R, Vaziri T, et al. Defective IgA response to atypical intestinal commensals in IL-21 receptor deficiency reshapes immune cell homeostasis and mucosal immunity. *Mucosal Immunol.* (2018). doi: 10.1038/s41385-018-0056-x
114. Bar AK, Phukan N, Pinheiro J, Simoes-Barbosa A. The interplay of host microbiota and parasitic protozoans at mucosal interfaces: implications for the outcomes of infections and diseases. *PLoS Negl Trop Dis.* (2015) 9:e0004176. doi: 10.1371/journal.pntd.0004176
115. Benson A, Pifer R, Behrendt CL, Hooper LV, Yarovinsky F. Gut commensal bacteria direct a protective immune response against *Toxoplasma gondii*. *Cell Host Microbe.* (2009) 6:187–96. doi: 10.1016/j.chom.2009.06.005
116. Hand TW, Dos Santos LM, Bouladoux N, Molloy MJ, Pagan AJ, Pepper M, et al. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. *Science.* (2012) 337:1553–6. doi: 10.1126/science.1220961
117. Nilsson J, Boasso A, Velilla PA, Zhang R, Vaccari M, Franchini G, et al. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood*. (2006) 108:3808–17. doi: 10.1182/blood-2006-05-021576
118. Baker CA, Clark R, Ventura F, Jones NG, Guzman D, Bangsberg DR, et al. Peripheral CD4 loss of regulatory T cells is associated with persistent viraemia in chronic HIV infection. *Clin Exp Immunol.* (2007) 147:533–9. doi: 10.1111/j.1365-2249.2006.03319.x
119. Kinter AL, Hennessey M, Bell A, Kern S, Lin Y, Daucher M, et al. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses *in vitro* and are associated with favorable clinical markers of disease status. *J Exp Med.* (2004) 200:331–43. doi: 10.1084/jem.20032069
120. Kornfeld C, Ploquin MJ, Pandrea I, Faye A, Onanga R, Apetrei C, et al. Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. *J Clin Invest.* (2005) 115:1082–91. doi: 10.1172/JCI23006
121. Mozos A, Garrido M, Carreras J, Plana M, Diaz A, Alos L, et al. Redistribution of FOXP3-positive regulatory T cells from lymphoid tissues to peripheral blood in HIV-infected patients. *J Acquir Immune Defic Syndr.* (2007) 46:529–37. doi: 10.1097/QAI.0b013e31815b69ae
122. Tenorio AR, Martinson J, Pollard D, Baum L, Landay A. The relationship of T-regulatory cell subsets to disease stage, immune activation, and pathogen-specific immunity in HIV infection. *J Acquir Immune Defic Syndr.* (2008) 48:577–80. doi: 10.1097/QAI.0b013e31817bbea5
123. Jiao Y, Fu J, Xing S, Fu B, Zhang Z, Shi M, et al. The decrease of regulatory T cells correlates with excessive activation and apoptosis of $CD8^+$ T cells in HIV-1-infected typical progressors, but not in long-term non-progressors. *Immunology*. (2009) 128:e366–375. doi: 10.1111/j.1365-2567.2008.02978.x
124. Owen RE, Heitman JW, Hirschkorn DF, Lanteri MC, Biswas HH, Martin JN, et al. HIV+ elite controllers have low HIV-specific T-cell activation yet maintain strong, polyfunctional T-cell responses. *AIDS*. (2010) 24:1095–105. doi: 10.1097/QAD.0b013e3283377a1e
125. Angin M, Kwon DS, Streeck H, Wen F, King M, Rezai A, et al. Preserved function of regulatory T cells in chronic HIV-1 infection despite decreased numbers in blood and tissue. *J Infect Dis.* (2012) 205:1495–500. doi: 10.1093/infdis/jis236
126. Favre D, Mold J, Hunt PW, Kanwar B, Loke P, Seu L, et al. Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Sci Transl Med*. (2010) 2:32ra36. doi: 10.1126/scitranslmed.3000632
127. Mavigner, M., Cazabat, M., Dubois, M., L'Faqihi, F. E., Requena, M., Pasquier, C., et al. (2012). Altered $CD4^+$ T cell homing to the gut impairs mucosal immune reconstitution in treated HIV-infected individuals. *J Clin Invest.* 122, 62–69. doi: 10.1172/JCI59011
128. Brenchley JM. Mucosal immunity in human and simian immunodeficiency lentivirus infections. *Mucosal Immunol.* (2013) 6:657–65. doi: 10.1038/mi.2013.15
129. Kim CJ, McKinnon LR, Kovacs C, Kandel G, Huibner S, Chege D, et al. Mucosal Th17 cell function is altered during HIV infection and is an independent predictor of systemic immune activation. *J Immunol.* (2013) 191:2164–73. doi: 10.4049/jimmunol.1300829
130. Pallikkuth S, Micci L, Ende ZS, Iriele RI, Cervasi B, Lawson B, et al. Maintenance of intestinal Th17 cells and reduced microbial translocation in SIV-infected rhesus macaques treated with interleukin (IL)-21. *PLoS Pathog.* (2013) 9:e1003471. doi: 10.1371/journal.ppat.1003471
131. Klase Z, Ortiz A, Deleage C, Mudd JC, Quinones M, Schwartzman E, et al. Dysbiotic bacteria translocate in progressive SIV infection. *Mucosal Immunol.* (2015) 8:1009–20. doi: 10.1038/mi.2014.128
132. Hensley-McBain T, Berard AR, Manuzak JA, Miller CJ, Zevin AS, Polacino P, et al. Intestinal damage precedes mucosal immune dysfunction in SIV infection. *Mucosal Immunol.* (2018) 11:1429–40. doi: 10.1038/s41385-018-0032-5
133. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*. (2011) 474:298–306. doi: 10.1038/nature10208
134. Zhang M, Sun K, Wu Y, Yang Y, Tso P, Wu Z. Interactions between intestinal microbiota and host immune response in inflammatory bowel disease. *Front Immunol.* (2017) 8:942. doi: 10.3389/fimmu.2017.00942
135. Kabat AM, Pott J, Maloy KJ. The mucosal immune system and its regulation by autophagy. *Front Immunol.* (2016) 7:240. doi: 10.3389/fimmu.2016.00240
136. Imhann F, Vich Vila A, Bonder MJ, Fu J, Gevers D, Visschedijk MC, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut*. (2018) 67:108–19. doi: 10.1136/gutjnl-2016-312135
137. Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, et al. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science*. (2009) 325:617–20. doi: 10.1126/science.1172747
138. Feng T, Cao AT, Weaver CT, Elson CO, Cong Y. Interleukin-12 converts Foxp3+ regulatory T cells to interferon-gamma-producing Foxp3+ T cells that inhibit colitis. *Gastroenterology*. (2011) 140:2031–43. doi: 10.1053/j.gastro.2011.03.009

139. Rivas MN, Koh YT, Chen A, Nguyen A, Lee YH, Lawson G, et al. MyD88 is critically involved in immune tolerance breakdown at environmental interfaces of Foxp3-deficient mice. *J Clin Invest*. (2012) 122:1933–47. doi: 10.1172/JCI40591
140. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science*. (2012) 336:1314–7. doi: 10.1126/science.1221789
141. Ohnmacht C, Park JH, Cording S, Wing JB, Atarashi K, Obata Y, et al. MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORgammat+ T cells. *Science*. (2015) 349:989–93. doi: 10.1126/science.aac4263
142. Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, et al. MUCOSAL IMMUNOLOGY. *Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. Science*. (2015) 349:993–7. doi: 10.1126/science.aaa9420
143. Yang BH, Hagemann S, Mamareli P, Lauer U, Hoffmann U, Beckstette M, et al. Foxp3+ T cells expressing RORgammat represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol.* (2016) 9:444–57. doi: 10.1038/mi.2015.74
144. Kim BS, Lu H, Ichiyama K, Chen X, Zhang YB, Mistry NA, et al. Generation of RORgammat(+) Antigen-Specific T Regulatory 17 Cells from Foxp3(+) Precursors in Autoimmunity. *Cell Rep*. (2017) 21:195–207. doi: 10.1016/j.celrep.2017.09.021
145. Yang J, Zou M, Pezoldt J, Zhou X, Huehn J. Thymus-derived Foxp3(+) regulatory T cells upregulate RORgammat expression under inflammatory conditions. *J Mol Med.* (2018) 96:1387–94. doi: 10.1007/s00109-018-1706-x
146. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature*. (2008) 453:620–5. doi: 10.1038/nature07008
147. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*. (2013) 504:451–5. doi: 10.1038/nature12726
148. Campbell C, Dikiy S, Bhattarai SK, Chinen T, Matheis F, Calafiore M, et al. Extrathymically generated regulatory T cells establish a niche for intestinal border-dwelling bacteria and affect physiologic metabolite balance. *Immunity*. (2018) 48:1245-1257 e1249. doi: 10.1016/j.immuni.2018.04.013
149. Tang C, Kakuta S, Shimizu K, Kadoki M, Kamiya T, Shimazu T, et al. Suppression of IL-17F, but not of IL-17A, provides protection against colitis by inducing Treg cells through modification of the intestinal microbiota. *Nat Immunol.* (2018) 19:755–65. doi: 10.1038/s41590-018-0134-y
150. Sultan AS, Kong EF, Rizk AM, Jabra-Rizk MA. The oral microbiome: a Lesson in coexistence. *PLoS Pathog.* (2018) 14:e1006719. doi: 10.1371/journal.ppat.1006719
151. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol.* (2005) 43:5721–32. doi: 10.1128/JCM.43.11.5721-5732.2005
152. Avila M, Ojcius DM, Yilmaz O. The oral microbiota: living with a permanent guest. *DNA Cell Biol.* (2009) 28:405–11. doi: 10.1089/dna.2009.0874
153. Zaura E, Keijser BJ, Huse SM, Crielaard W. Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiol.* (2009) 9:259. doi: 10.1186/1471-2180-9-259
154. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog.* (2010) 6:e1000713. doi: 10.1371/journal.ppat.1000713
155. Ahn J, Yang L, Paster BJ, Ganly I, Morris L, Pei Z, et al. Oral microbiome profiles: 16S rRNA pyrosequencing and microarray assay comparison. *PLoS ONE*. (2011) 6:e22788. doi: 10.1371/journal.pone.0022788
156. Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simon-Soro A, Pignatelli M, et al. The oral metagenome in health and disease. *ISME J.* (2012) 6:46–56. doi: 10.1038/ismej.2011.85
157. Dang AT, Cotton S, Sankaran-Walters S, Li CS, Lee CY, Dandekar S, et al. Evidence of an increased pathogenic footprint in the lingual microbiome of untreated HIV infected patients. *BMC Microbiol.* (2012) 12:153. doi: 10.1186/1471-2180-12-153
158. Yamazaki S, Maruyama A, Okada K, Matsumoto M, Morita A, Seya T. Dendritic cells from oral cavity induce Foxp3(+) regulatory T cells upon antigen stimulation. *PLoS ONE*. (2012) 7:e51665. doi: 10.1371/journal.pone.0051665
159. Bashan A, Gibson TE, Friedman J, Carey VJ, Weiss ST, Hohmann EL, et al. Universality of human microbial dynamics. *Nature*. (2016) 534:259–62. doi: 10.1038/nature18301
160. Teng F, Darveekaran Nair SS, Zhu P, Li S, Huang S, Li X, et al. Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. *Sci Rep*. (2018) 8:16321. doi: 10.1038/s41598-018-34294-x
161. Shirtliff ME, Peters BM, Jabra-Rizk MA. Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol Lett.* (2009) 299:1–8. doi: 10.1111/j.1574-6968.2009.01668.x
162. Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin Microbiol Rev.* (2012) 25:193–213. doi: 10.1128/CMR.00013-11
163. Wright CJ, Burns LH, Jack AA, Back CR, Dutton LC, Nobbs AH, et al. Microbial interactions in building of communities. *Mol Oral Microbiol.* (2013) 28:83–101. doi: 10.1111/omi.12012
164. Guo L, He X, Shi W. Intercellular communications in multispecies oral microbial communities. *Front Microbiol.* (2014) 5:328. doi: 10.3389/fmicb.2014.00328
165. Murray JL, Connell JL, Stacy A, Turner KH, Whiteley M. Mechanisms of synergy in polymicrobial infections. *J Microbiol.* (2014) 52:188–99. doi: 10.1007/s12275-014-4067-3
166. Xu H, Sobue T, Thompson A, Xie Z, Poon K, Ricker A, et al. Streptococcal co-infection augments *Candida* pathogenicity by amplifying the mucosal inflammatory response. *Cell Microbiol.* (2014) 16:214–31. doi: 10.1111/cmi.12216
167. Cutler CW, Jotwani R. Dendritic cells at the oral mucosal interface. *J Dent Res.* (2006) 85:678–89. doi: 10.1177/154405910608500801
168. Novak N, Haberstok J, Bieber T, Allam JP. The immune privilege of the oral mucosa. *Trends Mol Med.* (2008) 14:191–8. doi: 10.1016/j.molmed.2008.03.001
169. Hovav AH. Dendritic cells of the oral mucosa. *Mucosal Immunol.* (2013) 7:27–37. doi: 10.1038/mi.2013.42
170. Pandiyan P, Bhaskaran N, Zhang Y, Weinberg A. Isolation of T cells from mouse oral tissues. *Biol Proced Online.* (2014) 16:4. doi: 10.1186/1480-9222-16-4
171. Park JY, Chung H, DiPalma DT, Tai X, Park JH. Immune quiescence in the oral mucosa is maintained by a uniquely large population of highly activated Foxp3(+) regulatory T cells. *Mucosal Immunol.* (2018). doi: 10.1038/s41385-018-0027-2
172. Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature*. (2008) 452:773–6. doi: 10.1038/nature06764
173. Blaschitz C, Raffatellu M. Th17 cytokines and the gut mucosal barrier. *J Clin Immunol.* (2010) 30:196–203. doi: 10.1007/s10875-010-9368-7
174. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol.* (2010) 8:481–90. doi: 10.1038/nrmicro2337
175. Pion M, Jaramillo-Ruiz D, Martinez A, Munoz-Fernandez MA, Correa-Rocha R. HIV infection of human regulatory T cells downregulates Foxp3 expression by increasing DNMT3b levels and DNA methylation in the FOXP3 gene. *AIDS.* (2013) 27:2019–29. doi: 10.1097/QAD.0b013e32836253fd
176. Cheng WC, Hughes FJ, Taams LS. The presence, function and regulation of IL-17 and Th17 cells in periodontitis. *J Clin Periodontol.* (2014) 41:541–9. doi: 10.1111/jcpe.12238
177. Bhaskaran N, Quigley C, Weinberg A, Huang A, Popkin D, Pandiyan P. Transforming growth factor-beta1 sustains the survival of Foxp3 regulatory cells during late phase of oropharyngeal candidiasis infection. *Mucosal Immunol.* (2016) 9:1015–26. doi: 10.1038/mi.2015.115
178. Bhaskaran N, Weinberg A, Pandiyan P. Th17 inflammation model of oropharyngeal candidiasis in immunodeficient mice. *J Vis Exp*. (2015). doi: 10.3791/52538
179. Hernandez-Santos N, Huppler AR, Peterson AC, Khader SA, McKenna KC, Gaffen SL. Th17 cells confer long-term adaptive immunity to oral

mucosal *Candida albicans* infections. *Mucosal Immunol.* (2013) 6:900–10. doi: 10.1038/mi.2012.128
180. Whibley N, Gaffen SL. Brothers in arms: Th17 and Treg responses in *Candida albicans* immunity. *PLoS Pathog.* (2014) 10:e1004456. doi: 10.1371/journal.ppat.1004456
181. Khan SA, Kong EF, Meiller TF, Jabra-Rizk MA. Periodontal diseases: bug induced, host promoted. *PLoS Pathog.* (2015) 11:e1004952. doi: 10.1371/journal.ppat.1004952
182. Mahanonda R, Champaiboon C, Subbalekha K, Sa-Ard-Iam N, Yongyuth A, Isaraphithakkul B, et al. Memory T cell subsets in healthy gingiva and periodontitis tissues. *J Periodontol.* (2018) 89:1121–30. doi: 10.1002/JPER.17-0674
183. Tamai R, Sugamata M, Kiyoura Y. *Candida albicans* enhances invasion of human gingival epithelial cells and gingival fibroblasts by *Porphyromonas gingivalis*. *Microb Pathog.* (2011) 51:250–4. doi: 10.1016/j.micpath.2011.06.009
184. Canabarro A, Valle C, Farias MR, Santos FB, Lazera M, Wanke B. Association of subgingival colonization of *Candida albicans* and other yeasts with severity of chronic periodontitis. *J Periodontal Res.* (2013) 48:428–32. doi: 10.1111/jre.12022
185. Okui T, Aoki Y, Ito H, Honda T, Yamazaki K. The presence of IL-17+/FOXP3+ double-positive cells in periodontitis. *J Dent Res.* (2012) 91:574–9. doi: 10.1177/0022034512446341
186. Andersson J, Fehniger TE, Patterson BK, Pottage J, Agnoli M, Jones P, et al. Early reduction of immune activation in lymphoid tissue following highly active HIV therapy. *AIDS*. (1998) 12:F123–129.
187. Starr JR, Huang Y, Lee KH, Murphy CM, Moscicki AB, Shiboski CH, et al. Oral microbiota in youth with perinatally acquired HIV infection. *Microbiome*. (2018) 6:100. doi: 10.1186/s40168-018-0484-6
188. Bassis CM, Erb-Downward JR, Dickson RP, Freeman CM, Schmidt TM, Young VB, et al. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *MBio*. (2015) 6:e00037. doi: 10.1128/mBio.00037-15
189. Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Beck JM, Huffnagle GB, et al. Spatial variation in the healthy human lung microbiome and the adapted island model of lung biogeography. *Ann Am Thorac Soc.* (2015) 12:821–30. doi: 10.1513/AnnalsATS.201501-029OC
190. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe*. (2015) 17:704–15. doi: 10.1016/j.chom.2015.03.008
191. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. *PLoS ONE*. (2010) 5:e8578. doi: 10.1371/journal.pone.0008578
192. Huang YJ, Nariya S, Harris JM, Lynch SV, Choy DF, Arron JR, et al. The airway microbiome in patients with severe asthma: associations with disease features and severity. *J Allergy Clin Immunol.* (2015) 136:874–84. doi: 10.1016/j.jaci.2015.05.044
193. Madan JC, Koestler DC, Stanton BA, Davidson L, Moulton LA, Housman ML, et al. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: interaction between intestinal and respiratory tracts and impact of nutritional exposures. *MBio*. (2012) 3:e00251-12. doi: 10.1128/mBio.00251-12
194. Krone CL, Biesbroek G, Trzcinski K, Sanders EA, Bogaert D. Respiratory microbiota dynamics following *Streptococcus pneumoniae* acquisition in young and elderly mice. *Infect Immun.* (2014) 82:1725–31. doi: 10.1128/IAI.01290-13
195. Ma B, Forney LJ, Ravel J. Vaginal microbiome: rethinking health and disease. *Annu Rev Microbiol.* (2012) 66:371–89. doi: 10.1146/annurev-micro-092611-150157
196. Nemati M, Malla N, Yadav M, Khorramdelazad H, Jafarzadeh A. Humoral and T cell-mediated immune response against trichomoniasis. *Parasite Immunol.* (2018) 40:e12510. doi: 10.1111/pim.12510
197. Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. *Science*. (2008) 320:1220–4. doi: 10.1126/science.1155209
198. Soerens AG, Da Costa A, Lund JM. Regulatory T cells are essential to promote proper CD4 T-cell priming upon mucosal infection. *Mucosal Immunol.* (2016) 9:1395–406. doi: 10.1038/mi.2016.19
199. St Leger AJ, Desai JV, Drummond RA, Kugadas A, Almaghrabi F, Silver P, et al. An Ocular Commensal Protects against Corneal Infection by Driving an Interleukin-17 Response from Mucosal gammadelta T Cells. *Immunity*. (2017) 47:148–58 e145. doi: 10.1016/j.immuni.2017.06.014
200. Mahdavinia M, Keshavarzian A, Tobin MC, Landay AL, Schleimer RP. A comprehensive review of the nasal microbiome in chronic rhinosinusitis (CRS). *Clin Exp Aller*. (2016) 46:21–41. doi: 10.1111/cea.12666
201. Koskinen K, Reichert JL, Hoier S, Schachenreiter J, Duller S, Moissl-Eichinger C, et al. The nasal microbiome mirrors and potentially shapes olfactory function. *Sci Rep.* (2018) 8:1296. doi: 10.1038/s41598-018-19438-3
202. Yang HJ, LoSavio PS, Engen PA, Naqib A, Mehta A, Kota R, et al. Association of nasal microbiome and asthma control in patients with chronic rhinosinusitis. *Clin Exp Aller*. (2018) 48:1744–7. doi: 10.1111/cea.13255
203. Ritchie ND, Ijaz UZ, Evans TJ. IL-17 signalling restructures the nasal microbiome and drives dynamic changes following Streptococcus pneumoniae colonization. *BMC Genomics*. (2017) 18:807. doi: 10.1186/s12864-017-4215-3
204. Oliveira MR, Tafuri WL, Afonso LC, Oliveira MA, Nicoli JR, Vieira EC, et al. Germ-free mice produce high levels of interferon-gamma in response to infection with Leishmania major but fail to heal lesions. *Parasitology*. (2005) 131(Pt 4), 477–488. doi: 10.1017/S0031182005008073
205. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*. (2006) 124:837–48. doi: 10.1016/j.cell.2006.02.017
206. Kelly D, King T, Aminov R. Importance of microbial colonization of the gut in early life to the development of immunity. *Mutat Res.* (2007) 622:58–69. doi: 10.1016/j.mrfmmm.2007.03.011
207. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science*. (2012) 336:489–93. doi: 10.1126/science.1219328
208. Caballero S, Pamer EG. Microbiota-mediated inflammation and antimicrobial defense in the intestine. *Annu Rev Immunol.* (2015) 33:227–56. doi: 10.1146/annurev-immunol-032713-120238
209. Thiemann S, Smit N, Strowig T. Antibiotics and the intestinal microbiome: individual responses, resilience of the ecosystem, and the susceptibility to infections. *Curr Topics Microbiol Immunol.* (2016) 398:124–38. doi: 10.1007/82_2016_504
210. Sorg JA, Sonenshein AL. Bile salts and glycine as cogerminants for *Clostridium* difficile spores. *J Bacteriol.* (2008) 190:2505–12. doi: 10.1128/JB.01765-07
211. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci USA*. (2008) 105:20858–63. doi: 10.1073/pnas.0808723105
212. Sun Y, O'Riordan MX. Regulation of bacterial pathogenesis by intestinal short-chain Fatty acids. *Adv Appl Microbiol.* (2013) 85:93–118. doi: 10.1016/B978-0-12-407672-3.00003-4
213. Thiemann S, Smit N, Roy U, Lesker TR, Galvez EJC, Helmecke J, et al. Enhancement of IFNgamma production by distinct commensals ameliorates salmonella-induced disease. *Cell Host Microbe*. (2017) 21:682–94 e685. doi: 10.1016/j.chom.2017.05.005
214. Shukla SD, Budden KF, Neal R, Hansbro PM. Microbiome effects on immunity, health and disease in the lung. *Clin Transl Immunol.* (2017) 6:e133. doi: 10.1038/cti.2017.6
215. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science*. (2011) 332:974–7. doi: 10.1126/science.1206095
216. Kabat AM, Harrison OJ, Riffelmacher T, Moghaddam AE, Pearson CF, Laing A, et al. The autophagy gene Atg16l1 differentially regulates Treg and TH2 cells to control intestinal inflammation. *Elife*. (2016) 5:e12444. doi: 10.7554/eLife.12444
217. Wang S, Charbonnier LM, Noval Rivas M, Georgiev P, Li N, Gerber G, et al. MyD88 Adaptor-dependent microbial sensing by regulatory t cells promotes mucosal tolerance and enforces commensalism. *Immunity*. (2015) 43:289–303. doi: 10.1016/j.immuni.2015.06.014

218. Schenten D, Nish SA, Yu S, Yan X, Lee HK, Brodsky I, et al. Signaling through the adaptor molecule MyD88 in $CD4^+$ T cells is required to overcome suppression by regulatory T cells. *Immunity*. (2014) 40:78–90. doi: 10.1016/j.immuni.2013.10.023
219. Cording S, Fleissner D, Heimesaat MM, Bereswill S, Loddenkemper C, Uematsu S, et al. Commensal microbiota drive proliferation of conventional and Foxp3(+) regulatory CD4(+) T cells in mesenteric lymph nodes and Peyer's patches. *Eur J Microbiol Immunol.* (2013) 3:1–10. doi: 10.1556/EuJMI.3.2013.1.1
220. Moor K, Diard M, Sellin ME, Felmy B, Wotzka SY, Toska A, et al. High-avidity IgA protects the intestine by enchaining growing bacteria. *Nature*. (2017) 544:498–502. doi: 10.1038/nature22058
221. Wu W, Sun M, Chen F, Cao AT, Liu H, Zhao Y, et al. Microbiota metabolite short-chain fatty acid acetate promotes intestinal IgA response to microbiota which is mediated by GPR43. *Mucosal Immunol.* (2017) 10:946–56. doi: 10.1038/mi.2016.114
222. Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science.* (2009) 323:1488–92. doi: 10.1126/science.1169152
223. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, et al. Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity*. (2014) 41:152–65. doi: 10.1016/j.immuni.2014.05.016
224. Cao AT, Yao S, Gong B, Elson CO, Cong Y. Th17 cells upregulate polymeric Ig receptor and intestinal IgA and contribute to intestinal homeostasis. *J Immunol.* (2012) 189:4666–73. doi: 10.4049/jimmunol.1200955
225. Hirota K, Turner JE, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol.* (2013) 14:372–9. doi: 10.1038/ni.2552
226. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. *Science*. (2014) 345:1254009. doi: 10.1126/science.1254009
227. Goto Y, Uematsu S, Kiyono H. Epithelial glycosylation in gut homeostasis and inflammation. *Nat Immunol.* (2016) 17:1244–51. doi: 10.1038/ni.3587
228. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate $CD4^+$ T-cell responses to intestinal commensal bacteria. *Nature*. (2013) 498:113–7. doi: 10.1038/nature12240
229. Victoriano AF, Imai K, Okamoto T. Interaction between endogenous bacterial flora and latent HIV infection. *Clin Vaccine Immunol.* (2013) 20:773–9. doi: 10.1128/CVI.00766-12
230. Yu X, Shahir AM, Sha J, Feng Z, Eapen B, Nithianantham S, et al. Short-chain fatty acids from periodontal pathogens suppress histone deacetylases, EZH2, and SUV39H1 to promote Kaposi's sarcoma-associated herpesvirus replication. *J Virol.* (2014) 88:4466–79. doi: 10.1128/JVI.03326-13
231. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. *Proc Nutr Soc.* (2003) 62:67–72. doi: 10.1079/PNS2002207
232. Zeng H, Chi H. Metabolic control of regulatory T cell development and function. *Trends Immunol.* (2015) 36:3–12. doi: 10.1016/j.it.2014.08.003
233. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* (2007) 204:1757–64. doi: 10.1084/jem.20070590
234. Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol.* (2009) 2:340–50. doi: 10.1038/mi.2009.13
235. Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol.* (2007) 8:1086–94. doi: 10.1038/ni1511
236. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, et al. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. *J Immunol.* (2011) 186:1934–42. doi: 10.4049/jimmunol.1001672
237. Goverse G, Molenaar R, Macia L, Tan J, Erkelens MN, Konijn T, et al. Diet-derived short chain fatty acids stimulate intestinal epithelial cells to induce mucosal tolerogenic dendritic cells. *J Immunol.* (2017) 198:2172–81. doi: 10.4049/jimmunol.1600165
238. Atarashi K, Suda W, Luo C, Kawaguchi T, Motoo I, Narushima S, et al. Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science*. (2017) 358:359–65. doi: 10.1126/science.aan4526
239. Noverr MC, Noggle RM, Toews GB, Huffnagle GB. Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. *Infect Immun.* (2004) 72:4996–5003. doi: 10.1128/IAI.72.9.4996-5003.2004
240. Samuelson DR, Welsh DA, Shellito JE. Regulation of lung immunity and host defense by the intestinal microbiota. *Front Microbiol.* (2015) 6:1085. doi: 10.3389/fmicb.2015.01085
241. Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, et al. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. *Mucosal Immunol.* (2015) 8:80–93. doi: 10.1038/mi.2014.44
242. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev.* (1999) 13:2905–27.
243. Pandiyan P, Gartner D, Soezeri O, Radbruch A, Schulze-Osthoff K, Brunner-Weinzierl MC. CD152 (CTLA-4) determines the unequal resistance of Th1 and Th2 cells against activation-induced cell death by a mechanism requiring PI3 kinase function. *J Exp Med.* (2004) 199:831–42. doi: 10.1084/jem.20031058
244. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity*. (2009) 30:832–44. doi: 10.1016/j.immuni.2009.04.014
245. Esposito M, Ruffini F, Bellone M, Gagliani N, Battaglia M, Martino G, et al. Rapamycin inhibits relapsing experimental autoimmune encephalomyelitis by both effector and regulatory T cells modulation. *J Neuroimmunol.* (2010) 220:52–63. doi: 10.1016/j.jneuroim.2010.01.001
246. Procaccini C, De Rosa V, Galgani M, Abanni L, Cali G, Porcellini A, et al. An oscillatory switch in mTOR kinase activity sets regulatory T cell responsiveness. *Immunity*. (2010) 33:929–41. doi: 10.1016/j.immuni.2010.11.024
247. Delgoffe GM, Pollizzi KN, Waickman AT, Heikamp E, Meyers DJ, Horton MR, et al. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol.* (2011) 12:295–303. doi: 10.1038/ni.2005
248. So T, Croft M. Regulation of PI-3-Kinase and Akt Signaling in T lymphocytes and other cells by TNFR family molecules. *Front Immunol.* (2013) 4:139. doi: 10.3389/fimmu.2013.00139
249. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. PI3Kgamma is a molecular switch that controls immune suppression. *Nature*. (2016) 539:437–42. doi: 10.1038/nature19834
250. Chi H. Regulation and function of mTOR signalling in T cell fate decisions. *Nat Rev Immunol.* (2012) 12:325–38. doi: 10.1038/nri3198
251. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. *Annu Rev Immunol.* (2012) 30:39–68. doi: 10.1146/annurev-immunol-020711-075024
252. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci USA*. (2008) 105:7797–802. doi: 10.1073/pnas.0800928105
253. Clambey ET, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, et al. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. *Proc Natl Acad Sci USA*. (2012) 109:E2784–2793. doi: 10.1073/pnas.1202366109
254. Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature*. (2013) 499:485–90. doi: 10.1038/nature12297
255. Carbone F, De Rosa V, Carrieri PB, Montella S, Bruzzese D, Porcellini A, et al. Regulatory T cell proliferative potential is impaired in human autoimmune disease. *Nat Med.* (2014) 20:69–74. doi: 10.1038/nm.3411
256. Hurez V, Dao V, Liu A, Pandeswara S, Gelfond J, Sun L, et al. Chronic mTOR inhibition in mice with rapamycin alters T, B, myeloid, and innate lymphoid

cells and gut flora and prolongs life of immune-deficient mice. *Aging Cell*. (2015) 14:945–56. doi: 10.1111/acel.12380
257. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat Immunol.* (2015) 16:178–87. doi: 10.1038/ni.3076
258. Apostolidis SA, Rodriguez-Rodriguez N, Suarez-Fueyo A, Dioufa N, Ozcan E, Crispin JC, et al. Phosphatase PP2A is requisite for the function of regulatory T cells. *Nat Immunol.* (2016) 17:556–64. doi: 10.1038/ni.3390
259. Gabriel SS, Kallies A. Sugars and fat - A healthy way to generate functional regulatory T cells. *Eur J Immunol.* (2016) 46:2705–9. doi: 10.1002/eji.201646663
260. Kasper IR, Apostolidis SA, Sharabi A, Tsokos GC. Empowering Regulatory T Cells in Autoimmunity. *Trends Mol Med.* (2016) 22:784–97. doi: 10.1016/j.molmed.2016.07.003
261. Merchant HA, Liu F, Orlu Gul M, Basit AW. Age-mediated changes in the gastrointestinal tract. *Int J Pharm.* (2016) 512:382–95. doi: 10.1016/j.ijpharm.2016.04.024
262. Shibagaki N, Suda W, Clavaud C, Bastien P, Takayasu L, Iioka E, et al. Aging-related changes in the diversity of women's skin microbiomes associated with oral bacteria. *Sci Rep*. (2017) 7:10567. doi: 10.1038/s41598-017-10834-9
263. Zhang L, Wang Y, Xiayu X, Shi C, Chen W, Song N, et al. Altered Gut Microbiota in a Mouse Model of Alzheimer's Disease. *J Alzheimers Dis.* (2017) 60:1241–57. doi: 10.3233/JAD-170020
264. Zhang T, Xiang J, Cui B, He Z, Li P, Chen H, et al. Cost-effectiveness analysis of fecal microbiota transplantation for inflammatory bowel disease. *Oncotarget.* (2017) 8:88894–903. doi: 10.18632/oncotarget.21491
265. Zhang W, Zhu YH, Zhou D, Wu Q, Song D, Dicksved J, et al. Oral administration of a select mixture of bacillus probiotics affects the gut microbiota and goblet cell function following *Escherichia coli* challenge in newly weaned pigs of genotype MUC4 that are supposed to be enterotoxigenic *E. coli* F4ab/ac receptor negative. *Appl Environ Microbiol.* (2017) 83: e02747–16. doi: 10.1128/AEM.02747-16
266. Bodogai M, O'Connell J, Kim K, Kim Y, Moritoh K, Chen C, et al. Commensal bacteria contribute to insulin resistance in aging by activating innate B1a cells. *Sci Transl Med.* (2018) 10:eaat4271. doi: 10.1126/scitranslmed.aat4271
267. Irie K, Novince CM, Darveau RP. Impact of the oral commensal flora on alveolar bone homeostasis. *J Dent Res.* (2014) 93:801–6. doi: 10.1177/0022034514540173
268. Irie K, Tomofuji T, Ekuni D, Fukuhara D, Uchida Y, Kataoka K, et al. Age-related changes of CD4(+) T cell migration and cytokine expression in germ-free and SPF mice periodontium. *Arch Oral Biol.* (2018) 87:72–8. doi: 10.1016/j.archoralbio.2017.12.007
269. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA*. (2011) 108(Suppl. 1):4615–22. doi: 10.1073/pnas.1000082107
270. Wu HJ, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity*. (2010) 32:815–27. doi: 10.1016/j.immuni.2010.06.001
271. Rogier R, Evans-Marin H, Manasson J, van der Kraan PM, Walgreen B, Helsen MM, et al. Alteration of the intestinal microbiome characterizes preclinical inflammatory arthritis in mice and its modulation attenuates established arthritis. *Sci Rep*. (2017) 7:15613. doi: 10.1038/s41598-017-15802-x
272. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. *Cell Host Microbe*. (2013) 14:195–206. doi: 10.1016/j.chom.2013.07.012
273. Mima K, Sukawa Y, Nishihara R, Qian ZR, Yamauchi M, Inamura K, et al. *Fusobacterium nucleatum* and T Cells in Colorectal Carcinoma. *JAMA Oncol.* (2015) 1:653–61. doi: 10.1001/jamaoncol.2015.1377
274. Hamada T, Zhang X, Mima K, Bullman S, Sukawa Y, Nowak JA, et al. *Fusobacterium nucleatum* in colorectal cancer relates to immune response differentially by tumor microsatellite instability status. *Cancer Immunol Res.* (2018) 6:1327–36. doi: 10.1158/2326-6066.CIR-18-0174
275. Liu L, Tabung FK, Zhang X, Nowak JA, Qian ZR, Hamada T, et al. Diets that promote colon inflammation associate with risk of colorectal carcinomas that contain *Fusobacterium nucleatum*. *Clin Gastroenterol Hepatol.* (2018) 16:1622-1631 e1623. doi: 10.1016/j.cgh.2018.04.030
276. Yang CY, Yeh YM, Yu HY, Chin CY, Hsu CW, Liu H, et al. Oral microbiota community dynamics associated with oral squamous cell carcinoma staging. *Front Microbiol.* (2018) 9:862. doi: 10.3389/fmicb.2018.00862
277. Brennan CA, Garrett WS. *Fusobacterium nucleatum* - symbiont, opportunist and oncobacterium. *Nat Rev Microbiol.* (2019) 17:156–166. doi: 10.1038/s41579-018-0129-6
278. Dinakaran V, Mandape SN, Shuba K, Pratap S, Sakhare SS, Tabatabai MA, et al. Identification of specific oral and gut pathogens in full thickness colon of colitis patients: implications for colon motility. *Front Microbiol.* (2018) 9:3220. doi: 10.3389/fmicb.2018.03220
279. Sears CL. The who, where and how of fusobacteria and colon cancer. *Elife.* (2018) 7:e28434. doi: 10.7554/eLife.28434
280. Lee SM, Donaldson GP, Mikulski Z, Boyajian S, Ley K, Mazmanian SK. Bacterial colonization factors control specificity and stability of the gut microbiota. *Nature*. (2013) 501:426–9. doi: 10.1038/nature12447
281. D'Souza B, Slack T, Wong SW, Lam F, Muhlmann M, Koestenbauer J, et al. Randomized controlled trial of probiotics after colonoscopy. *ANZ J Surg.* (2017) 87:E65–E69. doi: 10.1111/ans.13225
282. Bagga D, Reichert JL, Koschutnig K, Aigner CS, Holzer P, Koskinen K, et al. Probiotics drive gut microbiome triggering emotional brain signatures. *Gut Microbes*. (2018) 9:486–96. doi: 10.1080/19490976.2018.1460015
283. Shepherd ES, DeLoache WC, Pruss KM, Whitaker WR, Sonnenburg JL. An exclusive metabolic niche enables strain engraftment in the gut microbiota. *Nature*. (2018) 557:434–8. doi: 10.1038/s41586-018-0092-4
284. Hoffmann D, Palumbo F, Ravel J, Roghmann MC, Rowthorn V, von Rosenvinge E. Improving regulation of microbiota transplants. *Science*. (2017) 358:1390–1. doi: 10.1126/science.aaq0034
285. Sun B, Jia Y, Hong J, Sun Q, Gao S, Hu Y, et al. Sodium butyrate ameliorates high-fat-diet-induced non-alcoholic fatty liver disease through peroxisome proliferator-activated receptor alpha-mediated activation of beta oxidation and suppression of inflammation. *J Agric Food Chem.* (2018) 66:7633–42. doi: 10.1021/acs.jafc.8b01189
286. Mortaz E, Adcock IM, Folkerts G, Barnes PJ, Paul Vos A, Garssen J. Probiotics in the management of lung diseases. *Mediat Inflamm.* (2013) 2013:751068. doi: 10.1155/2013/751068
287. Matt SM, Allen JM, Lawson MA, Mailing LJ, Woods JA, Johnson RW. Butyrate and dietary soluble fiber improve neuroinflammation associated with aging in mice. *Front Immunol.* (2018) 9:1832. doi: 10.3389/fimmu.2018.01832
288. Lu M, Wang Z. Microbiota and Aging. *Adv Exp Med Biol.* (2018) 1086:141–56. doi: 10.1007/978-981-13-1117-8_9
289. Bishehsari F, Engen PA, Preite NZ, Tuncil YE, Naqib A, Shaikh M, et al. Dietary fiber treatment corrects the composition of gut microbiota, promotes SCFA production, and suppresses colon carcinogenesis. *Genes*. (2018) 9:E102. doi: 10.3390/genes9020102
290. Xu Z, Tao J, Chen P, Chen L, Sharma S, Wang G, et al. Sodium butyrate inhibits colorectal cancer cell migration by downregulating Bmi-1 through enhanced miR-200c expression. *Mol Nutr Food Res.* (2018) 62:e1700844. doi: 10.1002/mnfr.201700844

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Mitochondrial DNA Leakage Caused by *Streptococcus pneumoniae* Hydrogen Peroxide Promotes Type I IFN Expression in Lung Cells

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Streptococcus pneumoniae (S. pn), the bacterial pathogen responsible for invasive pneumococcal diseases, is capable of producing substantial amounts of hydrogen peroxide. However, the impact of *S. pn*-secreted hydrogen peroxide (H_2O_2) on the host immune processes is not completely understood. Here, we demonstrated that *S. pn*-secreted H_2O_2 caused mitochondrial damage and severe histopathological damage in mouse lung tissue. Additionally, *S. pn*-secreted H_2O_2 caused not only oxidative damage to mitochondrial deoxyribonucleic acid (mtDNA), but also a reduction in the mtDNA content in alveolar epithelia cells. This resulted in the release of mtDNA into the cytoplasm, which subsequently induced type I interferons (IFN-I) expression. We also determined that stimulator of interferon genes (STING) signaling was probably involved in *S. pn* H_2O_2-inducing IFN-I expression in response to mtDNA damaged by *S. pn*-secreted H_2O_2. In conclusion, our study demonstrated that H_2O_2 produced by *S. pn* resulted in mtDNA leakage from damaged mitochondria and IFN-I production in alveolar epithelia cells, and STING may be required in this process, and this is a novel mitochondrial damage mechanism by which *S. pn* potentiates the IFN-I cascade in *S. pn* infection.

Keywords: *Streptococcus pneumoniae*, hydrogen peroxide, mitochondrial damage, mtDNA, IFNβ, STING

INTRODUCTION

Streptococcus pneumoniae (S. pn), a member of the human nasopharyngeal microbiota, can cause pneumonia and other invasive pneumococcal diseases, such as otitis media, meningitis and bacteremia, especially in children and the elderly (Weiser et al., 2018). The virulence of *S. pn* is known to be dependent upon several factors, including its polysaccharide capsule, surface proteins, enzymes, and the cholesterol-dependent cytolysin, pneumolysin (Ply) (Mitchell and Mitchell, 2010). These virulence factors play an important role in the invasion of *S. pn* into the host. Interestingly, *S. pn* can secrete a substantial amount of hydrogen peroxide (H_2O_2) via an enzymatic reaction catalyzed by pyruvate oxidase, SpxB, during the aerobic metabolism. The *spxB* gene is not only a virulence determinant in *S. pn*, but it is also essential for resistance to the toxic by-product

produced by itself (Li-Korotky et al., 2009). It can influence the synthesis of acetyl-phosphate, a potential source of ATP under the aerobic conditions in *S. pn* (Pericone et al., 2003). Meanwhile, it can affect the sugar utilization pattern and capsule biosynthesis (Carvalho et al., 2013). Research has shown that strains of *S. pn* that lack *spxB* during growth produce significantly reduced levels of H_2O_2 (Yesilkaya et al., 2013; Echlin et al., 2016). And compared with the wide type *S. pn*, the *spxB* mutant strain showed reduced virulence in animal models for nasopharyngeal colonization and pneumonia (Spellerberg et al., 1996). Through its ability to produce H_2O_2, *S. pn* is able to not only induce autolysis (Regev-Yochay et al., 2007), but also inhibit a variety of competing organisms, such as *Haemophilus influenzae* (Pericone et al., 2000) and *Staphylococcus aureus* (Regev-Yochay et al., 2006) in the aerobic environment of the respiratory tract.

Previous research has shown that *S. pn*-secreted H_2O_2 influences the host physiology and immune defense. During pneumococcal meningitis, both *S. pn*-secreted H_2O_2 and Ply are sufficient to induce mitochondrial damage, trigger the release of apoptosis-inducing factor (AIF) from mitochondria, and ultimately mediate apoptosis (Braun et al., 2002). *S. pn*-secreted H_2O_2 has been shown to induce endoplasmic reticulum (ER) stress, activate the mitogen-associated protein kinase (MAPK) signaling pathways, and regulate target genes (Loose et al., 2015). Furthermore, H_2O_2 secreted by *S. pn* is required for the induction of cardiomyocyte cell death, which is involved in the pathogenesis of *S. pn* infection in the heart (Brissac et al., 2017). However, the mechanism of how *S. pn*-secreted H_2O_2 activates the immune system are not fully understood in acute pneumonia.

Mitochondrial deoxyribonucleic acid (mtDNA) is an important damage-associated molecular pattern (DAMP), which contains a large number of unmethylated CpG sequences (Nakayama and Otsu, 2018). It is thought that mtDNA is more susceptible to damage, owing to an inefficient DNA repair mechanism and the lack of protective histone packaging. Damaged mtDNA released to into the cytoplasm or circulation has been shown to induce the transcription of pro-inflammatory cytokines, including MMP-8, TNFα, IL-6, and IL-1β (Fang et al., 2016). Moreover, mtDNA is involved in the induction of endothelial inflammation (Mao et al., 2017) and cardiomyocyte ischemia/reperfusion-injury (Hu et al., 2018). Previous research has confirmed that *S. pn*-secreted H_2O_2 leads to cytotoxic DNA damage in lung cells (Rai et al., 2015). However, the source of this oxidative DNA damage and whether it is involved in the host immune response remains unclear.

The ability to sense aberrant nucleic acids is a cornerstone of the innate immune system against pathogens. Stimulator of interferon genes (STING), a key innate immune signaling adaptor, responds to various forms of DNA species, including self-DNA from the nucleus of damaged cells. Self-DNA may cause various autoimmune diseases such as systemic lupus erythematosus (SLE) (Barber, 2015). Certain bacteria, such as *Listeria monocytogenes*, secrete cyclic dinucleotides (CDNs) that induce STING signaling within the host (Sauer et al., 2011). Similarly, *S. pn* DNA stimulates type I interferons (IFN-I) (IFNα, IFNβ) production in a STING-dependent manner (Parker et al., 2011; Koppe et al., 2012). In addition, mtDNA, which exists as a closed circular doubles-stranded DNA species, is able to activate STING, resulting in the upregulation of IFN-I and other interferon-stimulated genes (ISGs), under cellular damage and stress (Fang et al., 2016). However, there is little data to indicate mtDNA damaged by *S. pn*-secreted H_2O_2 induces IFN-I expression.

In this study, we demonstrated that *S. pn*-secreted H_2O_2 is capable of causing mitochondrial damage and mtDNA leakage into the cytosol of human alveolar epithelial cells, which further trigger the expression of IFNβ mediated by STING signaling probably. Thus, this study revealed a new strategy by which *S. pn* activates the host immune response.

MATERIALS AND METHODS

Ethics Statement

All animal experiments in this study were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chongqing Medical University and were authorized by the Animal Ethics Committee of Chongqing Medical University.

Bacterial Strains and Culture Conditions

The *S. pn* standard strain D39 (NCTC 7466, serotype 2) was purchased from the National Collection of Type Cultures (London, United Kingdom). The *S. pn* spxB knockout mutant strain (D39ΔspxB) was constructed by long flanking homology-polymerase chain reaction (LFH-PCR), as described before (Wu et al., 2014). Briefly, the *spxB* gene was substituted with an erythromycin resistant sequence. The positive clones were selected on blood agar plates containing 0.25 μg/ml erythromycin and identified by PCR. All *S. pn* strains were grown in C plus Y medium at 37°C in 5% CO_2 until the optical density at 600 nm equaled 0.5 (OD600 = 0.5).

Mouse Model of Acute Pneumonia

Female C57BL/6 mice (6–8 weeks old) were purchased from Chongqing Medical University (Chongqing, China) and were maintained under specific-pathogen-free conditions in a temperature-controlled room of the animal facility at Chongqing Medical University. All animal experiments were approved by the respective ethics committees of Chongqing Medical University. Mice were inoculated intranasally with 1×10^8 CFU of D39 (NCTC 7466, serotype 2) or D39Δ*spxB* in 30 μL of sterile PBS ($n = 5$ mice/group). Catalase was given intravenously (at 6, 12, 18, 22, 23, and 24 h) in the other five mice inoculated with 1×10^8 CFU of D39. Mice were sacrificed 24 h post-infection, and blood and lung homogenate supernatants were collected.

Cell Culture

The human lung alveolar carcinoma (type II pneumocyte) A549 cell lines were cultured in DMEM (Hyclone, United States) supplemented with 10% fetal bovine serum (FBS) (BI, United States) and 1% penicillin-streptomycin (Hyclone,

United States) at 37°C in 5% CO_2. Wild-type (WT) and STING knockout mouse embryonic fibroblast (MEF sting−/−) cells were also cultured in DMEM supplemented with 10% FBS (Gibco, United States) and 1% penicillin-streptomycin at 37°C with 5% CO_2. MEF sting−/− cells were kindly provided by Professor Chen Wang (School of Life Sciences and Technology, China Pharmaceutical University, Nanjing, China) (Lu et al., 2018). In order to construct a mtDNA-depleted cell line, A549 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and ethidium bromide (EtBr) (300 ng/ml) for 5 days at 37°C in 5% CO_2.

Mitochondrial Transmembrane Potential (ΔΨm) Assay

Mitochondrial transmembrane potential was assessed using a JC-1 kit (Solarbio, China). JC-1 is a fluorescent probe that indicates mitochondrial membrane potential loss. In normal cells, JC-1 aggregates in intact mitochondria (red fluorescence) but becomes a monomer (green fluorescence) in cells with disrupted mitochondrial membrane. After incubation with specific stimulators, including D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200) at 2 h. A549 cells were incubated in DMEM containing 10 μM JC-1 at 37°C and protected from light for 20 min. The cells were then washed with ice-cold 1× JC-1 staining buffer twice and then imaged with a fluorescent microscope (Nikon ECLIPSE 80i, Japan).

Lung Histology and Immunohistochemistry

Lung tissue was removed and fixed in 4% paraformaldehyde. The tissues were then embedded in paraffin, and then 5-μm sections were cut. The sections were stained with hematoxylin and eosin (Sigma-Aldrich, United States) and analyzed using a light microscope (Nikon ECLIPSE 80i, Japan). The degree of peribronchial inflammation was semi-quantitatively graded as described methods (Blanquiceth et al., 2016). The tissues were scored as follows: 0, normal; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells 5–6 cells deep; and 5, a ring of cells of > 6 cells deep.

For immunohistochemistry of lung tissue sections, citrate buffer was used for antigen retrieval. Lung sections were then incubated with an anti-PINK1 antibody (Novus Biologicals, United States), according to standard protocols. The mean integrated optical density (IODs) of PINK1 expression was measured using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, United States).

Transmission Electron Microscopy

A549 cells were infected with D39 or D39Δ*spxB* for 2 h. The cells were then harvested, washed with sterile PBS twice, and fixed with ice-cold 4% glutaraldehyde. Fixed cells were sectioned according to the Electron Microscopy Research Service of Chongqing Medical University and observed with a Hitachi H-7500 transmission electron microscope (Hitachi, Japan).

Extraction of Cytoplasmic DNA and Transfection

Cytoplasmic DNA was extracted as previously described (Holden and Horton, 2009). Briefly, 2×10^6 cells were stimulated as indicated. The cells were then harvested, washed with phosphate-buffered saline (PBS), and then mixed on a rotator in 500 μl of digitonin solution (25 μg/ml) containing 150 mM NaCl and 50 mM HEPES (pH 7.4) for 30 min at room temperature. The lysate was then centrifuged at 1000 × *g* for 5 min thrice in order to remove the nuclei and intact cells. The supernatant was transferred to a new tube and then centrifuged at 17,000 × *g* for 10 min in order to pellet the remaining cellular debris. Total cytosolic DNA were extracted using a DNA Blood Mini Kit (Qiagen, Germany), precipitated with 100 μl absolute ethanol, and stored at −20°C. A549 cells were transfected with the cytosolic DNA for 6 h using Lipofectamine 2000 (Lipo 2000) (Invitrogen, United States) according to the manufacturer's instructions.

mtDNA Copy Number and Transcription Level

In order to quantify mtDNA copy number, total DNA was extracted from the lungs of mice and A549 cells using a DNeasy Blood & Tissue Kit (Qiagen, Germany). For the quantification of mtDNA transcription level, total RNA was extracted from the lungs of mice and A549 cells using RNAiso plus reagent (Takara Bio, China) following the manufacturer's instructions. Cytochrome B (*Cytb*) and cytochrome c oxidase subunit 3 (*CoxIII*) were amplified in order to analyze the copy number and transcription level of mtDNA. G*apdh* was used as the internal control (Hu et al., 2018). The primers used in this study are listed in **Table 1**.

Immunofluorescence and Confocal Imaging

Treated A549 cells were incubated with MitoTracker® Red CMXRos (Yeasen, China) (mitochondrial red fluorescent probe) at 37°C for 45 min. Cells were fixed, permeabilized with 0.1% Triton X-100, blocked with 10% donkey serum, and then incubated with an anti-8-hydroxyguanine (8-OHdG) antibody (Santa Cruz Biotechnology, United States) at 4°C overnight. Then the cells were incubated with fluorescent-labeled secondary antibodies (Bioss, China). Nuclei were stained with DAPI (Beyotime, China) at room temperature for 15 min. Cells were observed with a Nikon ECLIPSE Ti confocal microscope (Nikon, Japan). The images were captured and analyzed by using NIS-Elements Viewer (Nikon, Japan).

Western Blot Analysis

A549 cells were lysed with RIPA buffer (Beyotime, China) supplemented with PMSF and phosphatase inhibitor (100:1:1) (BioTools, United States). Protein samples were separated onto sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred onto PVDF membranes (Millipore, United States). The membranes were incubated with a primary antibody

TABLE 1 | The sequences of PCR primers.

Gene	Orientation	Sequence
Human-*GAPDH*	Sense	5′-GAAGGGCTCATGACCACAGT-3′
	Anti-sense	5′-GGATGCAGGGATGATGTTCT-3′
Human-*IFN*β	Sense	5′-AGATCAACCTCACCTCAGG-3′
	Anti-sense	5′-TCAGAAACACTGTCTGCTGG-3′
Human-*IFNa2*	Sense	5′-CCTGATGAAGGAGGACTCCATT-3′
	Anti-sense	5′-AAAAAGGTGAGCTGGCATACG-3
Human-*IFNa5*	Sense	5′-TCCTCTGATGAATGTGGACTCT-3′
	Anti-sense	5′-GTACTAGTCAATGAGAATCATTTCG-3′
Human-*COXIII*	Sense	5′-CTCTGGACCCTACCGACTT-3′
	Anti-sense	5′-CAGCCAGGGCAGTAA-3′
Human-*ISG15*	Sense	5′-GAGAGGCAGCGAACTCATCT-3′
	Anti-sense	5′-CTTCAGCTCTGACACCGACA-3′
Human-*OASL-1*	Sense	5′-CCATCACGGTCACCATTGTG-3′
	Anti-sense	5′-ACCGCAGGCCTTGATCAG-3′
Human-*RNF185*	Sense	5′-AGGACCCCAGAGAGAAGACC-3′
	Anti-sense	5′-CAATTCCAAAAGACATCTGG-3′
Mouse-*Gapdh*	Sense	5′-CGGAGTCAACGGATTTGGTC-3′
	Anti-sense	5′-GACAAGCTTCCCGTTCTCAG-3′
Mouse-*Ifn*β	Sense	5′-ATTGCCTCAAGGACAGGATG-3′
	Anti-sense	5′-GGCCTTCAGGTAATGCAGAA-3′
Mouse-*Pgc1-*α	Sense	5′-TATGGAGTGACATAGAGTGTGCT-3′
	Anti-sense	5′-CCACTTCAATCCACCCAGAAAG-3′
Mouse-*Cxcl10*	Sense	5′-CCTGCCCACGTGTTGAGAT-3′
	Anti-sense	5′-TGATGGTCTTAGATTCCGGATTC-3′
Mouse-*Sting*	Sense	5′-GAGAGCCACCAGAGCACAC-3′
	Anti-sense	5′-CGCACAGTCCTCCAGTAGC-3′
Mouse-*Cytb*	Sense	5′-CTCACAGGACTGGCGAGAC-3′
	Anti-sense	5′-ACAGCCCCAATGACCCTCA-3′
Mouse-*CoxIII*	Sense	5′-TGCTGACCTCCAACAGGAAT-3′
	Anti-sense	5′-GTCCATGGAATCCAGTAGCCA-3′

overnight at 4°C, including anti-human STING and anti-human GAPDH (Cell Signaling Technology, United States). The membranes were then incubated with an HRP-conjugated secondary antibody for 1 h at 37°C. Bands were visualized by using Image Lab (Bio-Rad Laboratories, Hercules, CA, United States). GAPDH was used as a loading control.

Real-Time PCR

Total RNA was extracted from the lungs of mice and cells using RNAiso plus reagent (Takara Bio, China), according to the manufacturer's protocol. The mRNA was then reverse transcribed into cDNA using the PrimeScript^{TM} RT reagent kit (Takara Bio, China). All real-time PCR reactions were performed using TB Green Premix Ex TaqTM II on a Bio-Rad CFX-96 cycler (Bio-Rad Laboratories, United States). The expression of mRNA was normalized against GAPDH. The data shown are representative of three separate experiments. The primers used in this study are listed in **Table 1**.

ELISA

Cytokine levels in the blood, lung homogenate supernatants, and cell culture supernatants were measured by using ELISA assays, according to the manufacturer's instructions. The following ELISA kits were used in this study: LEGEND MAX^{TM} Mouse IFNβ (Biolegend, United States) and Human IFNβ (Cloud-clone, China).

Statistical Analysis

All experiments were performed at least three times, and the data are presented as the mean ± SD. Student's *t*-test was used for statistical analysis, and GraphPad Prism 5 software was used to perform statistical analysis for all experiments. $P < 0.05$ was considered statistically significant.

RESULTS

H_2O_2 Secreted by *S. pn* Induces IFN-I Expression *in vivo* and *in vitro*

In order to assess whether H_2O_2 secreted by *S. pn* induces the production of IFN-I *in vivo*, we used a mouse model of acute pneumonia. C57BL/6 mice were intranasally infected with *S. pn* D39 or D39Δ*spxB* for 24 h, catalase was given intravenously in the other five mice inoculated with 1×10^8 CFU of D39. ELISA assays demonstrated significant upregulation of IFNβ in the blood and lung homogenate supernatants of D39-infected mice compared to D39Δ*spxB*-infected mice and catalase treatment of D39-infected mice (**Figure 1A**). Similarly, *Ifn*β mRNA levels were increased in the lung tissue of D39-infected mice, but not in the lung tissues of D39Δ*spxB*-infected mice and catalase treatment of D39-infected mice (**Figure 1B**). We also infected the human alveolar epithelial cell line A549 with D39 or D39Δ*spxB* and analyzed the expression of IFN-I at 1 and 5 h post-infection. D39 infection was capable of inducing expression of *IFN*β and *IFNa2* (subtype of IFN-I) at both 1 and 5 h compared to D39Δ*spxB* infection. Pre-treatment of A549 cells with catalase prior to D39 infection resulted in diminished *IFN*β and *IFNa2* transcript levels, in which 5-fold reduction was observed in *IFN*β and *IFNa2* transcript levels at 5 h post-infection (**Figures 1C,D**, left panel). We also found that the production of IFNβ was reduced by 50% in the supernatant of A549 cells infected with D39Δ*spxB* as compared to D39-infected cells (**Figure 1C**, right panel). We further determined that D39 infection increased the expression of *IFNa5* (subtype of IFN-I) at 5 h, but not 1 h post-infection (**Figure 1D**, right panel).

Together, these data suggest that H_2O_2 secreted by *S. pn* is capable of inducing IFN-I expression in lung cells.

S. pn-Secreted H_2O_2 Is Sufficient to Induce Mitochondrial Dysfunction

Given that IFNβ production is triggered by mtDNA following mitochondrial stress (Fang et al., 2016), we theorized that H_2O_2 generated by *S. pn* induces mitochondrial damage in mouse lung tissue. The mRNA level of peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (*Pgc1-*α), which is the master regulator of mitochondrial biogenesis and functions as a transcriptional co-regulator (Dorn et al., 2015), was markedly decreased by 75% in D39-infected mice, but not

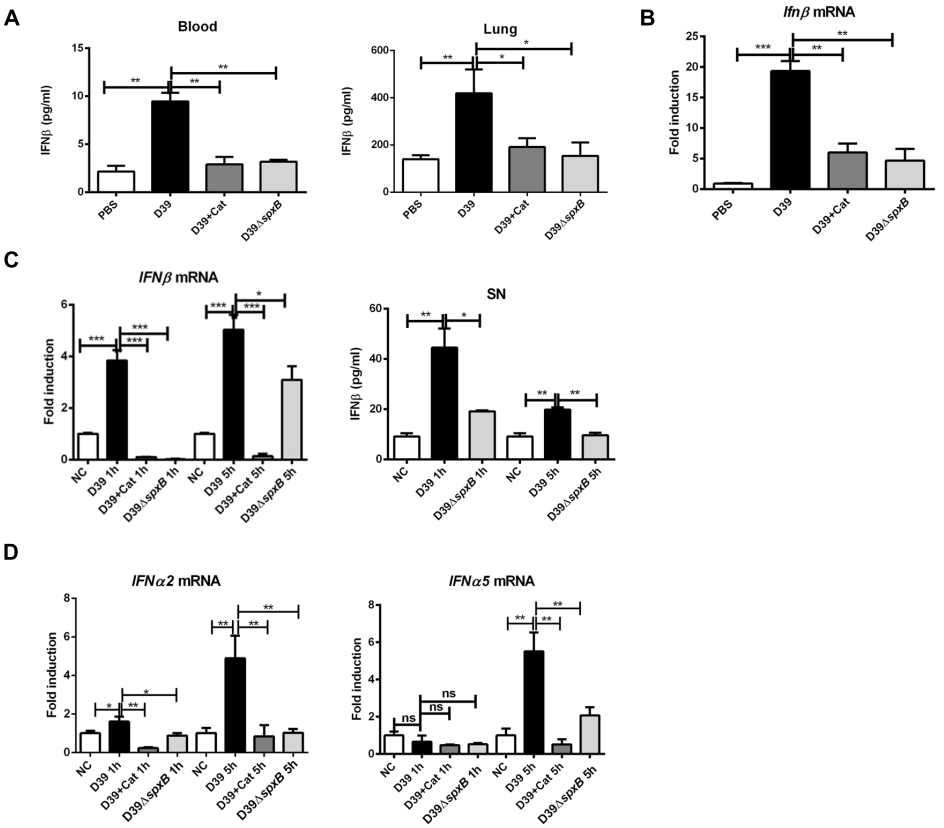

FIGURE 1 | *S. pn*-secreted H_2O_2 could augment the expression of IFN-I *in vivo and in vitro*. Female C57BL/6 mice were intranasally infected with D39 and D39Δ*spxB* (1×10^8 CFU) for 24 h, catalase was given intravenously (at 6, 12, 18, 22, 23, and 24 h) in the other five mice inoculated with 1×10^8 CFU of D39. **(A)** The production of IFNβ in blood and lung homogenates were measured by ELISA. **(B)** The expression of *Ifn*β in lungs were analyzed by real-time PCR. **(C,D)** A549 cells were infected with D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200) at 1 and 5 h, *IFN*β, (**C**, left panel) *IFNa2* and *IFNa5* **(D)** mRNA levels were determined by real-time PCR. **(C)** A549 cells were infected with D39 and D39Δ*spxB* (MOI = 200) at 1 and 5 h, IFNβ in supernatants (SN) was quantified by ELISA (right panel). NC, negative control. All data were presented as means ± SD from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ was considered statistically significant and highly statistically significant differences, respectively; ns, not significant.

in D39Δ*spxB*-infected mice and catalase treatment of D39-infected mice (**Figure 2A**). PTEN-induced putative kinase 1 (PINK1) is a regulator of mitophagy, which is rapidly degraded when mitochondria are healthy, but accumulates on the surface of damaged mitochondria (Jin et al., 2010). We observed by immunohistochemistry that infection with D39 significantly induced the accumulation of PINK1 on the mitochondria in the lung tissue of mice, while both infection with D39Δ*spxB* and catalase treatment of D39 infection did not increase PINK1-mitochondria interactions (**Figure 2B**). In addition, morphological and histopathological analyses of murine lung tissue revealed that severe pulmonary injuries were found in the D39-infected group, with obvious hemorrhage and massive inflammatory cell infiltration in the peribronchial, as compared to the D39Δ*spxB*-infected group and these pulmonary injuries were not markedly observed in the lung tissue of mice infected with D39 pre-treatment with catalase (**Figure 2C**). These data suggest that *S. pn*-secreted H_2O_2 causes mitochondrial damage in the lungs of mice.

To further understand the extent of mitochondrial damage induced by the H_2O_2 generated by *S. pn*, we assessed mitochondrial function in A549 cells infected with *S. pn* by measuring the $\Delta\Psi$m. We used an oxidative phosphorylation uncoupler, carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), as a positive control, which causes depolarization of mitochondria and mitochondrial damage (Park et al., 2018). Our results showed an increase in green fluorescence in A549 cells after infection with D39 for 2 h, indicating that the $\Delta\Psi$m values significantly decreased. Moreover, the addition of catalase significantly attenuated the decrease in the $\Delta\Psi$m values induced by D39 infection (**Figure 2D**). Similarly, we used transmission electron microscopy to monitor changes in mitochondria morphology. We observed abnormal mitochondrial morphology in A549 cells infected with D39, including mitochondrial swelling, condensation, and abnormal cristae shape. However, these changes were not observed in D39Δ*spxB*-infected cells (**Figure 2E**). These results suggest that *S. pn*-secreted H_2O_2 induces mitochondrial damage in alveolar epithelial cells.

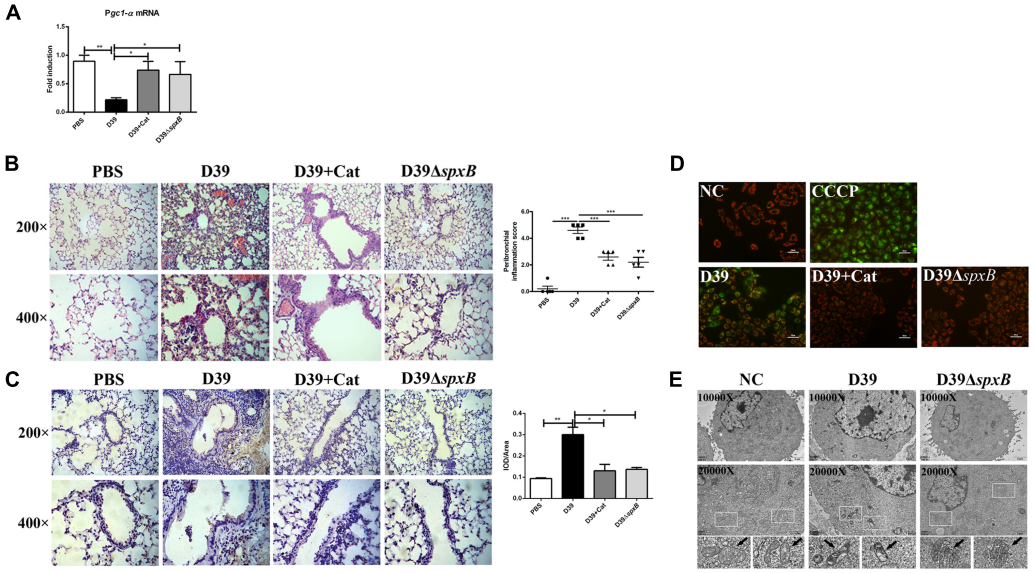

FIGURE 2 | *S. pn*-secreted H_2O_2 led to the mitochondrial malfunction in lung cells. Female C57BL/6 mice were intranasally infected with D39 and D39Δ*spxB* (1×10^8 CFU) for 24 h, catalase was given intravenously (at 6, 12, 18, 22, 23, and 24 h) in the other five mice inoculated with 1×10^8 CFU of D39. **(A)** The expression of *Pgc1*-α in lungs were analyzed by real-time PCR. **(B)** The expression of PINK1 in lung section of mouse were analyzed by immunohistochemistry (left panel). Score of PINK1 production was measured using the scale described in Section "Materials and Methods" (right panel). **(C)** Pathological analyses were done by hematoxylin and eosin staining, with lung sections examined under light microscopy at 200× (scale bar = 100 μm) and 400× (scale bar = 50 μm) magnification (left panel). Score of peribronchial inflammation was measured using the scale described in Section "Materials and Methods" (right panel). **(D)** A549 cells were infected with D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200) for 2 h, ΔΨm was measured using JC-1 probe. CCCP were applied as a positive control. Scale bar = 50 μm. **(E)** Changes of ultrastructure of A549 cells exposed to D39 and D39Δ*spxB* (MOI = 200) for 2 h were monitored by transmission electron microscopy. NC, negative control. All data were presented as means ± SD from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ was considered statistically significant and highly statistically significant differences, respectively; ns, not significant.

Taken together, our results demonstrate that H_2O_2 produced by *S. pn* causes mitochondrial dysfunction in lung cells both *in vivo* and *in vitro*.

S. pn-Secreted H_2O_2 Mediates Oxidative Damage of Mitochondrial DNA

The expression of 8-hydroxyguanine (8-OHdG) is known to be reflective of oxidative DNA damage. To further clarify if mtDNA is damaged by *S. pn*-secreted H_2O_2, we evaluated the level of 8-OHdG in A549 cells infected with D39 by immunofluorescence analysis. The number of 8-OHdG-positive A549 cells significantly increased following D39 infection, but not D39Δ*spxB* infection (**Figure 3A**). Furthermore, the addition of catalase markedly reduced the number of 8-OHdG-positive A549 cells infected with D39. These results suggest that *S. pn*-secreted H_2O_2 causes oxidative damage to the mtDNA in A549 cells, and catalase pre-treatment may prevent this phenomenon. We also explored the mtDNA copy number in A549 cells infected with D39 at 2 h post-infection. Real-time PCR analysis demonstrated that the mtDNA copy number significantly reduced by 50% over time in A549 cells after D39 infection (**Figure 3B**). Furthermore, the reduction in mtDNA copy number and mtDNA transcript level in D39-infected A549 cells was largely prevented by catalase pre-treatment, which is consistent with our data showing that catalase pre-treatment also decreased 8-OHdG levels in mtDNA after D39 infection. In D39Δ*spxB*-infected cells, the mtDNA transcript level and copy number were partially restored as compared with D39 infection. As expected, when A549 cells were exposed to 1 mM H_2O_2, we observed a 40% reduction in mtDNA copy number and a 60% reduction mtDNA transcript level by real-time PCR (**Figure 3C**). Likewise, copy number and transcript level of mtDNA were significantly decreased in D39-infected mouse lung tissue as compared with D39Δ*spxB*-infected mice and catalase treatment of D39 infected mice (**Figure 3D**).

In short, these results provide evidence that H_2O_2 secreted by *S. pn* induces significant oxidative damage in the mtDNA of lung cells.

S. pn-Secreted H_2O_2 Promotes Mitochondria DNA Leakage and the Induction of IFN-I

To address whether *S. pn*-secreted H_2O_2 could lead to the leakage of mtDNA into the cytoplasm, we assessed the cytosolic mtDNA levels in D39-infected A549 cells. We show that D39 infection significantly elevated the level of mtDNA in the cytoplasm within the first 3 h post-infection. A549 cells infected with D39Δ*spxB* did not exhibit this increase in mtDNA levels in the cytoplasm (**Figure 4A**). As expected, catalase pre-treatment inhibited D39-induced mtDNA leakage into the cytoplasm (**Figure 4B**). These findings suggest that H_2O_2 generated by *S. pn* triggers mtDNA leakage into the cytoplasm of A549 cells.

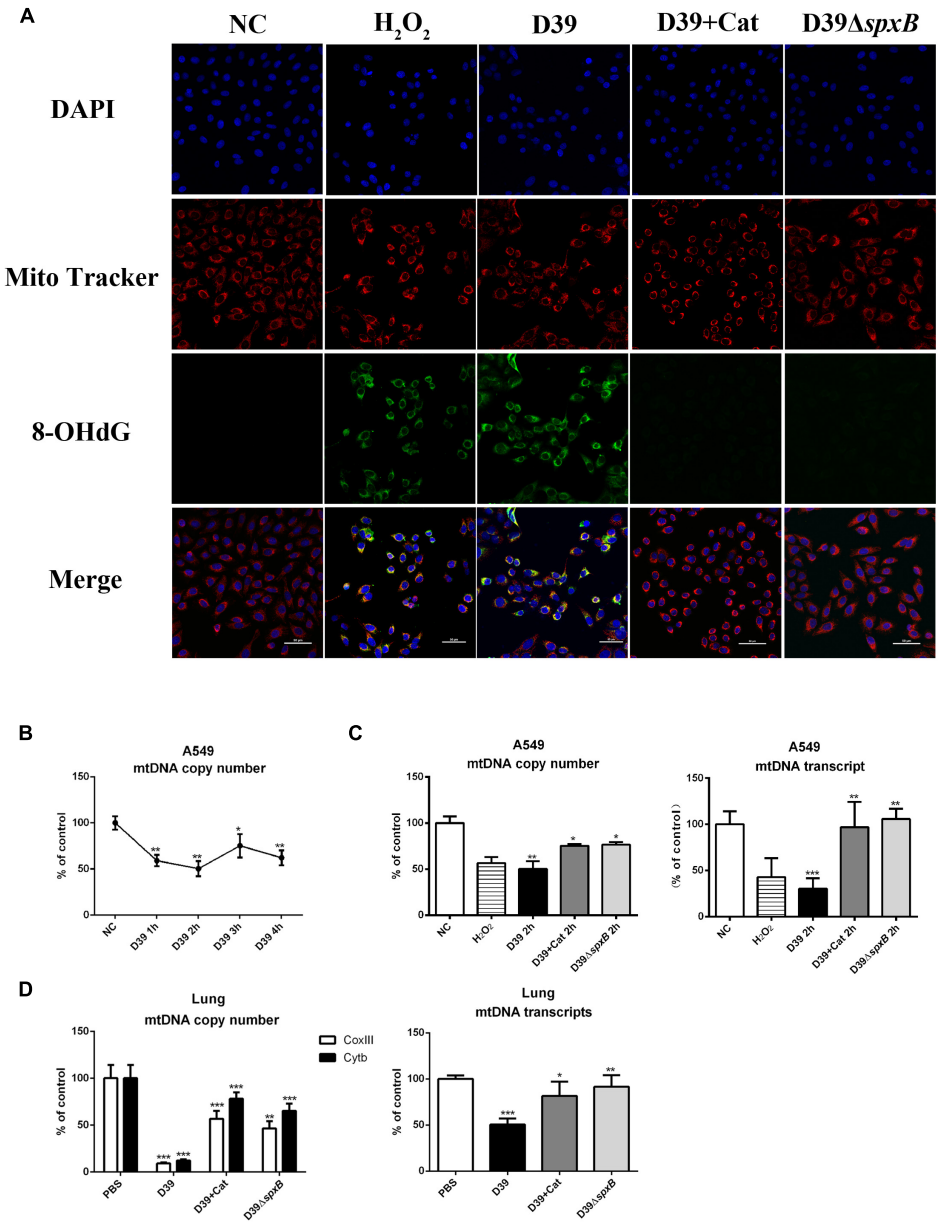

FIGURE 3 | *S. pn*-secreted H_2O_2 caused oxidative damage of mitochondrial DNA. **(A)** A549 cells were stimulated with D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200), as well as 1 mM H_2O_2 for 2 h, representative images of MitoTracker red (red), 8-OHdG immunostaining (green), and DAPI (blue) in A549 cells. The orange in the merged images of green and red fluorescence indicates 8-OHdG-positive cells. **(B)** A549 cells were infected with D39 (MOI = 200) at indicated time points, mtDNA copy number was analyzed by real-time PCR. **(C)** A549 cells were stimulated with D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200), as well as 1 mM H_2O_2 for 2 h, mtDNA copy number (left panel) and mtDNA transcript level (right panel) was analyzed by real-time PCR. **(D)** Female C57BL/6 mice were intranasally infected with D39 and D39Δ*spxB* (1×10^8 CFU) for 24 h, catalase was given intravenously (at 6, 12, 18, 22, 23, and 24 h) in the other five mice inoculated with 1×10^8 CFU of D39. mtDNA copy number (left panel) and mtDNA transcript level (right panel) were analyzed by real-time PCR. mtDNA level was normalized to the internal control GAPDH. Mitochondrial genes *CoxIII* and *Cytb* were chosen to indicate mtDNA transcription. NC, negative control. All data were presented as means ± SD from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ was considered statistically significant and highly statistically significant differences, respectively; ns, not significant.

We next sought to determine if IFN-I expression is induced by mtDNA damage caused specifically by *S. pn*-secreted H_2O_2 to mtDNA. Thus, we isolated mtDNA from the cytoplasm of A549 cells subjected to various stimulations, including D39 with or without catalase, D39Δ*spxB* and H_2O_2. We then exposed untreated A549 cells with the isolated mtDNA. Poly (dA:dT), a synthetic double-stranded DNA sequence, was used as a positive control. The cytosolic mtDNA isolated from D39-infected cells caused an upregulation in the transcription level of *IFN*β, and a similar result was obtained with mtDNA from H_2O_2-stimulated

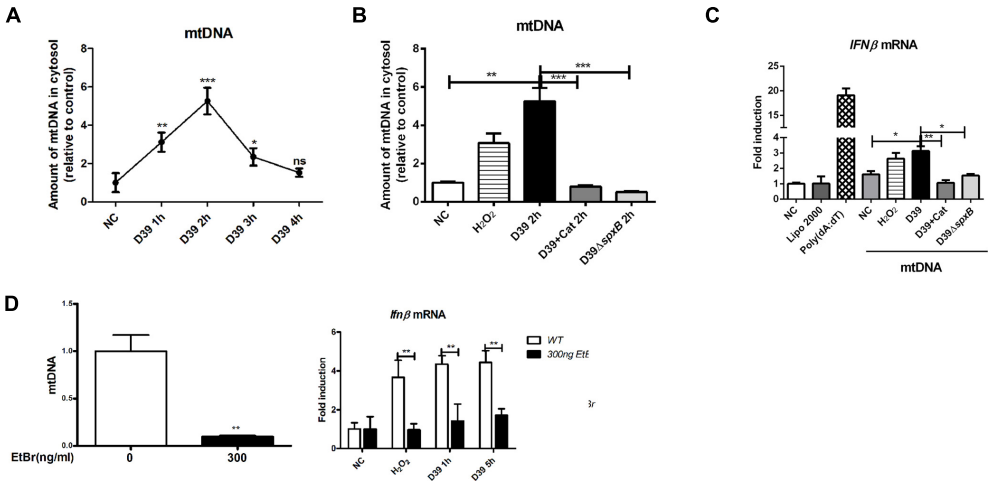

FIGURE 4 | mtDNA leakage caused by *S. pn*-secreted H_2O_2 was involved in IFN-I induction. **(A)** A549 cells were infected with D39 (MOI = 200) at indicated time points, DNA in the cytosolic fraction was isolated, and the copy number of mtDNA (mtDNA sequences as primers) was measured and normalized with the copy number of nuclear DNA (nuclear DNA sequences as primers). **(B)** A549 cells were stimulated with D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200), as well as 1 mM H_2O_2 for 2 h, DNA in the cytosolic fraction was isolated, and the copy number of mtDNA was measured and normalized with GAPDH. **(C)** A549 cells were transfected with cytosolic DNA isolated from different stimulations, including D39 with or without catalase (Cat) and D39Δ*spxB*, as well as H_2O_2, *IFN*β mRNA level were determined by real-time PCR. POLY (dA: dT) (2 μg/ml) was applied as positive control. **(D)** mtDNA in A549 cells were evaluated by real-time PCR after being treated with EtBr (300 ng/ml) for 5 days (left panel). *IFN*β mRNA level in A549 cells treated with 1 mM H_2O_2 or D39 (MOI = 200) were determined by real-time PCR (right panel). NC, negative control. All data were presented as means ± SD from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ was considered statistically significant and highly statistically significant differences, respectively; ns, not significant.

cells. However, there was no increase in the expression of *IFN*β in A549 cells treated with mtDNA from D39-infected A549 cells pretreated with catalase or D39Δ*spxB*-infected A549 cells (**Figure 4C**).

In order to ascertain the importance of mitochondria in the induction of *IFN*β expression in D39-infected A549 cells, we constructed mtDNA-depleted A549 cells. A549 cells were exposed to low concentrations of ethidium bromide over time to effectively reduce the mtDNA content within these cells. Real-time PCR results confirmed the successful construction of mtDNA-deficient cells (**Figure 4D**, left panel). The expression of *IFN*β in mtDNA-deficient cells was reduced by about 60% than that in WT cells following treatment with D39 or H_2O_2 (**Figure 4D**, right panel), which suggests that mtDNA plays a critical role in *S. pn* H_2O_2-induced production of IFNβ.

STING Signaling Is Probably Involved in the Activation of IFN-I by S. *pn*-Secreted H_2O_2

IFN-I production has been shown to be triggered by mtDNA through STING signaling (Fang et al., 2016). We examined the protein level of STING in A549 cells following different stimulations, including D39 with or without catalase, D39Δ*spxB* and H_2O_2. The results showed that both D39 infection and H_2O_2 stimulation up-regulated the expression of STING in A549 cells (**Figure 5A**), and the induction of STING was reduced by 20% by catalase treatment, a 40% reduction was observed after infection with D39Δ*spxB*. To further confirm that STING is responsible for inducing IFN-I expression in response to detecting mtDNA oxidized by S. *pn*-secreted H_2O_2, WT MEFs and STING knockout MEFs (MEFs sting−/−) were stimulated with D39, D39Δ*spxB*, and H_2O_2. Real-time PCR results demonstrated upregulation of *Ifn*β and *Ifna4* in WT MEF cells, but not in MEF sting−/− cells (**Figure 5B**). Curiously, there was no change in the transcriptional level of the IFN-responsive gene, C-X-C motif chemokine 10 (*Cxcl10*), in either WT MEFs or MEF sting−/− cells upon stimulation with D39, D39Δ*spxB* or H_2O_2. These results demonstrated that STING is indispensable in *S. pn* H_2O_2-induced production of IFN-I in MEF cells.

Additionally, we also determined the expression of IFN-I-stimulated genes *ISG15* and *OASl-1*, as well as *RNF185* (which has been reported to positively regulate the STING signaling pathway) in A549 cells infected with D39 (**Figure 5C**) (Wang Q. et al., 2017). We found that *ISG15*, *OASl-1*, and *RNF185* were induced in A549 cells infected with D39 at 1 h, but not 5 h post-infection. Furthermore, the addition of catalase or infection with D39Δ*spxB* diminished the expression of these genes at 1 h. Taken together, these findings indicate that STING signaling is probably involved in inducing the expression of IFN-I in response to mtDNA damaged by *S. pn*-secreted H_2O_2.

DISCUSSION

In this study, we ascertained that *S. pn*-secreted H_2O_2 promoted IFNβ production in lung cells, which was mediated by mtDNA leakage from mitochondria damaged by H_2O_2. Neutralizing

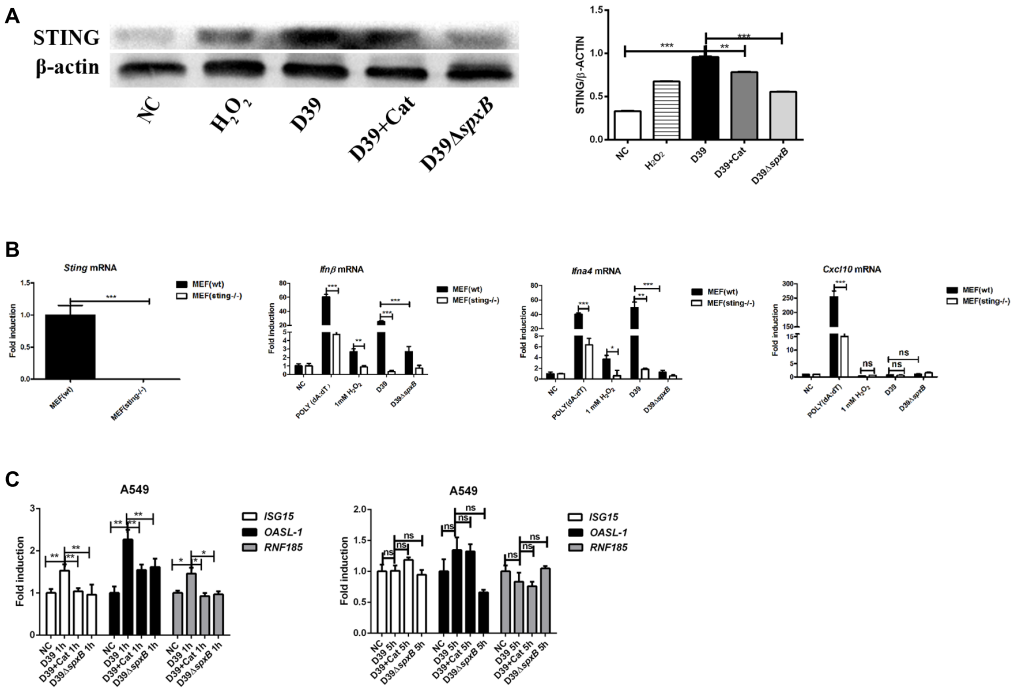

FIGURE 5 | STING signaling probably participated in IFN-I activation induced by *S. pn*-secreted H_2O_2. **(A)** Western blot analysis of STING in A549 cells stimulated with D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200), as well as 1 mM H_2O_2 for 1 h. **(B)** STING in MEF (wt) and MEF (sting–/–) were determined by real-time PCR. Transcription level of *Ifn*β, *Ifna4* and *Cxcl10* in MEF (wt) and MEF (sting–/–) were measured by real-time PCR after being treated with D39, D39Δ*spxB* (MOI = 200) and 1 mM H_2O_2 for 1 h. POLY (dA: dT) (2 μg/ml) was applied as positive control. **(C)** A549 cells were infected with D39 with or without catalase (Cat) and D39Δ*spxB* (MOI = 200) at 1 and 5 h, *ISG15*, *OASL-1*, *RNF185* mRNA levels were determined by real-time PCR. NC, negative control. All data were presented as means ± SD from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ was considered statistically significant and highly statistically significant differences, respectively; ns, not significant.

the H_2O_2 produced by *S. pn* H_2O_2 with catalase markedly attenuated mitochondrial malfunction and IFNβ expression, suggesting that targeting H_2O_2 during *S. pn* infection may offer therapeutic strategies.

There are several virulence factors of *S. pn* that are involved in the disease process. Specifically, *S. pn* is able to secrete substantial amounts of H_2O_2—up to a concentration of approximately 2 mM under aerobic conditions (Duane et al., 1993; Echlin et al., 2016; Lisher et al., 2017). Other *Streptococcus* species, such as *Streptococcus sanguis* (Sumioka et al., 2017), *Oral streptococci* (Matsushima et al., 2017), which have been reported to secrete H_2O_2. Several studies indicate that H_2O_2 secretion by most *Streptococcus* species is universal and indispensable. The production of H_2O_2 is dependent on the pyruvate oxidase gene *spxB*, which confers a selective advantage in co-colonization (Pesakhov et al., 2007; Regev-Yochay et al., 2007). Deletion of *spxB* results in a significant reduction in H_2O_2 to approximately 20% of level produced by WT *S. pn* (Echlin et al., 2016). However, *spxB* may also play other roles in the virulence of *S. pn* as there are several different serotypes. It has been shown that the virulence of a *spxB* knockout mutant of strain D39 (serotype 2) is attenuated in a murine model of nasopharyngeal colonization (Spellerberg et al., 1996), while a *spxB* mutant of *S. pn* serotype 1 is hypervirulent (Syk et al., 2014).

Research have reported that H_2O_2 inhibited cell migration in a dose-dependent manner, and this would impair airway epithelial cell repair (Hamada et al., 2016). And A549 cells exposed to H_2O_2 caused powerful LDH release and a necrotic phenotype rather than programmed cell death (Schmeck et al., 2004). These suggested that H_2O_2 is able to damage lung tissue. Some studies have supported that the toxic H_2O_2 secreted by *S. pn* could cause cellular oxidative stress and participate in cellular immune responses through different signaling pathways. A previous study reported that two pneumococcal toxins, Ply and H_2O_2, led to mitochondrial damage and consequently caused apoptosis of brain cells (Braun et al., 2002). Recent studies have shown that *S. pn*-secreted H_2O_2 induced DNA damage and apoptosis in lung cells, and contributed to the genotoxicity and virulence of *S. pn* (Rai et al., 2015). Moreover, there are some reports showing that pneumococcal H_2O_2-induced stress signaling regulated the expression of inflammatory genes (Loose et al., 2015). However, others have reported that the pneumococci-induced oxidative stress was independent of *S. pn*-secreted H_2O_2 and Ply but depended on the pneumococcal autolysin LytA (Zahlten et al., 2015). Here, we showed that *S. pn*- secreted H_2O_2 alone was able to induce mitochondrial oxidative damage, impairing mtDNA replication and decreasing mtDNA content in lung cells. Moreover, H_2O_2 is a type of reactive oxygen species (ROS), and as such, is an important

signaling molecule that mediates oxidative stress and cellular damage (Wible and Bratton, 2018). Previous studies on intestinal health have revealed that H_2O_2 upregulated intracellular and mitochondrial ROS expression (Jiang et al., 2017). However, whether H_2O_2 and the production of ROS induces mtDNA damage still needs to be further clarified.

The unique aspect of mitochondria is that it is the only source of DNA in cells that does not reside in the nucleus. mtDNA-mediated signaling is the basis of the host immune defense in several diseases. In atherosclerosis, oxidative damage and replication errors are the sources of mtDNA defects, which lead to mitochondrial dysfunction and directly promote atherosclerosis (Yu and Bennett, 2014). In intestinal ischemia reperfusion (I/R), mtDNA contributed to the early phase of I/R injury and amplified the inflammatory response (Yue et al., 2015; Hu et al., 2018). During the process of metabolic stress–induced endothelial inflammation and insulin resistance, palmitic acid caused mtDNA leakage into the cytoplasm, and activated STING signaling to mediate the intercellular adhesion molecule (ICAM)-1 expression and endothelial inflammation (Mao et al., 2017). Interestingly, mtDNA could activate several innate immune pathways including TLR9, NLRP3 and STING signaling pathways in the mammalian immune responses (Fang et al., 2016).

IFN-I are pleiotropic cytokines produced in response to viruses, bacteria, and parasites. In bacterial infection, lipopolysaccharide (LPS) or bacterial nucleic acids are recognized by innate immune receptors, triggering IFN-I production (Boxx and Cheng, 2016). IFN-I induce differential effects on the immune response of the host. *Listeria monocytogenes* was reported to induce IFNβ expression, suppress the production of IFNγ and TNFα, thereby promoting infection (Auerbuch et al., 2004; Rayamajhi et al., 2010). In contrast, *S. pn* DNA initiated an IFN-I cascade that contributed to pneumococcal clearance, and this process played an important part in the host defense against pneumococci by inhibiting bacterial transmigration (Parker et al., 2011; LeMessurier et al., 2013). Interestingly, our data showed that exposure of alveolar epithelial cells to H_2O_2 produced by *S. pn* was sufficient to induce mtDNA leakage into the cytoplasm and induce IFNβ production.

Growing evidence has indicated that STING signaling can be triggered by DNA from pathogens or damaged self-DNA in the cytoplasm (Barber, 2015). Hartlova et al. (2015) demonstrated that unrepaired DNA lesions promoted the production of IFN-I via the STING signaling pathway, which strengthened anti-microbial immunity. In acute pancreatitis, STING sensed self-DNA from dying acinar cells and promoted inflammation (Zhao et al., 2018). Our results confirmed that *S. pn*- secreted H_2O_2 resulted in both the production of IFN-I and the activation of IFN-I-stimulated genes, *ISG15* and *OASl-1*, and *RNF185* in A549 cells. We verified that deletion of STING notably impaired the expression IFN-I in MEF cells. These data suggested that STING signaling may play an indispensable role in the production of IFN-I induced by *S. pn* H_2O_2.

A previous study showed that IFNβ induced ROS production in human myotubes, which contributed to mitochondrial dysfunction and resulted in muscle impairment and continued inflammation in dermatomyositis (Meyer et al., 2017). Likewise, another study reported that caspases controlled antiviral immunity through cGAS cleavage during inflammasome activation, resulting in reduced IFN-I expression, revealing a negative feedback that regulates the output of DNA-sensing pathways (Wang Y. et al., 2017). However, further research is needed in order to elucidate the end result of IFN-I production by *S. pn*-secreted H_2O_2. Specifically, studies need to determine if this signaling pathway ultimately favors bacterial clearance or aggravates host cell apoptosis.

Overall, our findings demonstrated that *S. pn*-secreted H_2O_2 induced mtDNA leakage into the cytoplasm, which resulted in the activation of the IFN-I, and this process may be mediated via STING signaling. We also confirmed that *S. pn* H_2O_2 was sufficient to mediate mitochondrial oxidative stress, which underscores the importance of mitochondrial homeostasis during the host immune defense. In summary, we have identified a novel signaling mechanism that may serve as a potential target for controlling *S. pn* infection.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

YG, WX, and XZ conceived and designed the experiments. YG, XD, SY, and HW (fourth author) performed the experiments. YG, XZ, and HL analyzed the data. YG, XH, and HW (corresponding author) wrote the manuscript. WX and HW (corresponding author) reviewed and edited the manuscript.

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REFERENCES

Auerbuch, V., Brockstedt, D. G., Meyer-Morse, N., O'Riordan, M., and Portnoy, D. A. (2004). Mice lacking the type I interferon receptor are resistant to *Listeria monocytogenes*. *J. Exp. Med.* 200, 527–533. doi: 10.1084/jem.20040976

Barber, G. N. (2015). STING: infection, inflammation and cancer. *Nat. Rev. Immunol.* 15, 760–770. doi: 10.1038/nri3921

Blanquiceth, Y., Rodriguez-Perea, A. L., Tabares Guevara, J. H., Correa, L. A., Sanchez, M. D., Ramirez-Pineda, J. R., et al. (2016). Increase of frequency and modulation of phenotype of regulatory T Cells by atorvastatin is associated with decreased lung inflammatory cell infiltration in a murine model of acute allergic asthma. *Front. Immunol.* 7:620. doi: 10.3389/fimmu.2016.00620

Boxx, G. M., and Cheng, G. (2016). The roles of type I interferon in bacterial infection. *Cell Host Microbe* 19, 760–769. doi: 10.1016/j.chom.2016.05.016

Braun, J. S., Sublett, J. E., Freyer, D., Mitchell, T. J., Cleveland, J. L., Tuomanen, E. I., et al. (2002). Pneumococcal pneumolysin and H2O2 mediate brain cell apoptosis during meningitis. *J. Clin. Invest.* 109, 19–27. doi: 10.1172/jci12035

Brissac, T., Shenoy, A. T., Patterson, L. A., and Orihuela, C. J. (2017). Cell invasion and pyruvate oxidase derived H2O2 are critical for *Streptococcus pneumoniae* mediated cardiomyocyte killing. *Infect. Immun.* doi: 10.1128/iai.00569-17 [Epub ahead of print].

Carvalho, S. M., Farshchi Andisi, V., Gradstedt, H., Neef, J., Kuipers, O. P., Neves, A. R., et al. (2013). Pyruvate oxidase influences the sugar utilization pattern and capsule production in *Streptococcus pneumoniae*. *PLoS One* 8:e68277. doi: 10.1371/journal.pone.0068277

Dorn, G. W. II, Vega, R. B., and Kelly, D. P. (2015). Mitochondrial biogenesis and dynamics in the developing and diseased heart. *Genes Dev.* 29, 1981–1991. doi: 10.1101/gad.269894.115

Duane, P. G., Rubins, J. B., Weisel, H. R., and Janoff, E. N. (1993). Identification of hydrogen peroxide as a *Streptococcus pneumoniae* toxin for rat alveolar epithelial cells. *Infect. Immun.* 61, 4392–4397.

Echlin, H., Frank, M. W., Iverson, A., Chang, T. C., Johnson, M. D., Rock, C. O., et al. (2016). Pyruvate oxidase as a critical link between metabolism and capsule biosynthesis in *Streptococcus pneumoniae*. *PLoS Pathog.* 12:e1005951. doi: 10.1371/journal.ppat.1005951

Fang, C., Wei, X., and Wei, Y. (2016). Mitochondrial DNA in the regulation of innate immune responses. *Protein Cell* 7, 11–16. doi: 10.1007/s13238-015-0222-9

Hamada, S., Sato, A., Hara-Chikuma, M., Satooka, H., Hasegawa, K., Tanimura, K., et al. (2016). Role of mitochondrial hydrogen peroxide induced by intermittent hypoxia in airway epithelial wound repair in vitro. *Exp. Cell Res.* 344, 143–151. doi: 10.1016/j.yexcr.2016.04.006

Hartlova, A., Erttmann, S. F., Raffi, F. A., Schmalz, A. M., Resch, U., Anugula, S., et al. (2015). DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity. *Immunity* 42, 332–343. doi: 10.1016/j.immuni.2015.01.012

Holden, P., and Horton, W. A. (2009). Crude subcellular fractionation of cultured mammalian cell lines. *BMC Res. Notes* 2:243. doi: 10.1186/1756-0500-2-243

Hu, Q., Ren, J., Li, G., Wu, J., Wu, X., Wang, G., et al. (2018). The mitochondrially targeted antioxidant MitoQ protects the intestinal barrier by ameliorating mitochondrial DNA damage via the Nrf2/ARE signaling pathway. *Cell Death Dis.* 9:403. doi: 10.1038/s41419-018-0436-x

Jiang, Q., Liu, G., Wang, X., Hou, Y., Duan, Y., Wu, G., et al. (2017). Mitochondrial pathway is involved in the protective effects of alpha-ketoglutarate on hydrogen peroxide induced damage to intestinal cells. *Oncotarget* 8, 74820–74835. doi: 10.18632/oncotarget.20426

Jin, S. M., Lazarou, M., Wang, C., Kane, L. A., Narendra, D. P., and Youle, R. J. (2010). Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J. Cell Biol.* 191, 933–942. doi: 10.1083/jcb.201008084

Koppe, U., Hogner, K., Doehn, J. M., Muller, H. C., Witzenrath, M., Gutbier, B., et al. (2012). Streptococcus pneumoniae stimulates a STING- and IFN regulatory factor 3-dependent type I IFN production in macrophages, which regulates RANTES production in macrophages, cocultured alveolar epithelial cells, and mouse lungs. *J. Immunol.* 188, 811–817. doi: 10.4049/jimmunol.1004143

LeMessurier, K. S., Hacker, H., Chi, L., Tuomanen, E., and Redecke, V. (2013). Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung. *PLoS Pathog.* 9:e1003727. doi: 10.1371/journal.ppat.1003727

Li-Korotky, H. S., Lo, C. Y., Zeng, F. R., Lo, D., and Banks, J. M. (2009). Interaction of phase variation, host and pressure/gas composition: pneumococcal gene expression of PsaA, SpxB, Ply and LytA in simulated middle ear environments. *Int. J. Pediatr. Otorhinolaryngol.* 73, 1417–1422. doi: 10.1016/j.ijporl.2009.07.007

Lisher, J. P., Tsui, H. T., Ramos-Montanez, S., Hentchel, K. L., Martin, J. E., Trinidad, J. C., et al. (2017). Biological and chemical adaptation to endogenous hydrogen peroxide production in *Streptococcus pneumoniae* D39. *mSphere* 2:e291-16. doi: 10.1128/mSphere.00291-16

Loose, M., Hudel, M., Zimmer, K. P., Garcia, E., Hammerschmidt, S., Lucas, R., et al. (2015). Pneumococcal hydrogen peroxide-induced stress signaling regulates inflammatory genes. *J. Infect. Dis.* 211, 306–316. doi: 10.1093/infdis/jiu428

Lu, C., Zhang, X., Ma, C., Xu, W., Gan, L., Cui, J., et al. (2018). *Nontypeable Haemophilus influenzae* DNA stimulates type I interferon expression via STING signaling pathway. *Biochim. Biophys. Acta Mol. Cell Res.* 1865, 665–673. doi: 10.1016/j.bbamcr.2018.01.011

Mao, Y., Luo, W., Zhang, L., Wu, W., Yuan, L., Xu, H., et al. (2017). STING-IRF3 triggers endothelial inflammation in response to free fatty acid-induced mitochondrial damage in diet-induced obesity. *Arterioscler Thromb. Vasc. Biol.* 37, 920–929. doi: 10.1161/ATVBAHA.117.309017

Matsushima, H., Kumagai, Y., Vandenbon, A., Kataoka, H., Kadena, M., Fukamachi, H., et al. (2017). Microarray analysis of macrophage response to infection with *Streptococcus oralis* reveals the immunosuppressive effect of hydrogen peroxide. *Biochem. Biophys. Res. Commun.* 485, 461–467. doi: 10.1016/j.bbrc.2017.02.048

Meyer, A., Laverny, G., Allenbach, Y., Grelet, E., Ueberschlag, V., Echaniz-Laguna, A., et al. (2017). IFN-beta-induced reactive oxygen species and mitochondrial damage contribute to muscle impairment and inflammation maintenance in dermatomyositis. *Acta Neuropathol.* 134, 655–666. doi: 10.1007/s00401-017-1731-9

Mitchell, A. M., and Mitchell, T. J. (2010). *Streptococcus pneumoniae*: virulence factors and variation. *Clin. Microbiol. Infect.* 16, 411–418. doi: 10.1111/j.1469-0691.2010.03183.x

Nakayama, H., and Otsu, K. (2018). Mitochondrial DNA as an inflammatory mediator in cardiovascular diseases. *Biochem. J.* 475, 839–852. doi: 10.1042/BCJ20170714

Park, Y. S., Choi, S. E., and Koh, H. C. (2018). PGAM5 regulates PINK1/Parkin-mediated mitophagy via DRP1 in CCCP-induced mitochondrial dysfunction. *Toxicol. Lett.* 284, 120–128. doi: 10.1016/j.toxlet.2017.12.004

Parker, D., Martin, F. J., Soong, G., Harfenist, B. S., Aguilar, J. L., Ratner, A. J., et al. (2011). *Streptococcus pneumoniae* DNA initiates type I interferon signaling in the respiratory tract. *MBio* 2:e16-11. doi: 10.1128/mBio.00016-11

Pericone, C. D., Overweg, K., Hermans, P. W., and Weiser, J. N. (2000). Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. *Infect. Immun.* 68, 3990–3997. doi: 10.1128/IAI.68.7.3990-3997.2000

Pericone, C. D., Park, S., Imlay, J. A., and Weiser, J. N. (2003). Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. *J. Bacteriol.* 185, 6815–6825. doi: 10.1128/jb.185.23.6815-6825.2003

Pesakhov, S., Benisty, R., Sikron, N., Cohen, Z., Gomelsky, P., Khozin-Goldberg, I., et al. (2007). Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of *Streptococcus pneumoniae*. *Biochim. Biophys. Acta* 1768, 590–597. doi: 10.1016/j.bbamem.2006.12.016

Rai, P., Parrish, M., Tay, I. J., Li, N., Ackerman, S., He, F., et al. (2015). *Streptococcus pneumoniae* secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells. *Proc. Natl. Acad. Sci. U.S.A.* 112, E3421–E3430. doi: 10.1073/pnas.1424144112

Rayamajhi, M., Humann, J., Penheiter, K., Andreasen, K., and Lenz, L. L. (2010). Induction of IFN-alphabeta enables *Listeria monocytogenes* to suppress macrophage activation by IFN-gamma. *J. Exp. Med.* 207, 327–337. doi: 10.1084/jem.20091746

Regev-Yochay, G., Trzcinski, K., Thompson, C. M., Lipsitch, M., and Malley, R. (2007). *SpxB* is a suicide gene of *Streptococcus pneumoniae* and confers a selective advantage in an in vivo competitive colonization model. *J. Bacteriol.* 189, 6532–6539. doi: 10.1128/JB.00813-07

Regev-Yochay, G., Trzcinski, K., Thompson, C. M., Malley, R., and Lipsitch, M. (2006). Interference between *Streptococcus pneumoniae* and *Staphylococcus aureus*: in vitro hydrogen peroxide-mediated killing by *Streptococcus pneumoniae*. *J. Bacteriol.* 188, 4996–5001. doi: 10.1128/JB.00317-06

Sauer, J. D., Sotelo-Troha, K., von Moltke, J., Monroe, K. M., Rae, C. S., Brubaker, S. W., et al. (2011). The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to *Listeria monocytogenes* and cyclic dinucleotides. *Infect. Immun.* 79, 688–694. doi: 10.1128/IAI.00999-10

Schmeck, B., Gross, R., N'Guessan, P. D., Hocke, A. C., Hammerschmidt, S., Mitchell, T. J., et al. (2004). *Streptococcus pneumoniae*-induced caspase 6-dependent apoptosis in lung epithelium. *Infect. Immun.* 72, 4940–4947. doi: 10.1128/iai.72.9.4940-4947.2004

Spellerberg, B., Cundell, D. R., Sandros, J., Pearce, B. J., Idanpaan-Heikkila, I., Rosenow, C., et al. (1996). Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. *Mol. Microbiol.* 19, 803–813. doi: 10.1046/j.1365-2958.1996.425954.x

Sumioka, R., Nakata, M., Okahashi, N., Li, Y., Wada, S., Yamaguchi, M., et al. (2017). *Streptococcus sanguinis* induces neutrophil cell death by production of hydrogen peroxide. *PLoS One* 12:e0172223. doi: 10.1371/journal.pone.0172223

Syk, A., Norman, M., Fernebro, J., Gallotta, M., Farmand, S., Sandgren, A., et al. (2014). Emergence of hypervirulent mutants resistant to early clearance during systemic serotype 1 pneumococcal infection in mice and humans. *J. Infect. Dis.* 210, 4–13. doi: 10.1093/infdis/jiu038

Wang, Q., Huang, L., Hong, Z., Lv, Z., Mao, Z., Tang, Y., et al. (2017). The E3 ubiquitin ligase RNF185 facilitates the cGAS-mediated innate immune response. *PLoS Pathog.* 13:e1006264. doi: 10.1371/journal.ppat.1006264

Wang, Y., Ning, X., Gao, P., Wu, S., Sha, M., Lv, M., et al. (2017). Inflammasome activation triggers caspase-1-mediated cleavage of cGAS to regulate responses to DNA virus infection. *Immunity* 46, 393–404. doi: 10.1016/j.immuni.2017.02.011

Weiser, J. N., Ferreira, D. M., and Paton, J. C. (2018). *Streptococcus pneumoniae*: transmission, colonization and invasion. *Nat. Rev. Microbiol.* 16, 355–367. doi: 10.1038/s41579-018-0001-8

Wible, D. J., and Bratton, S. B. (2018). Reciprocity in ROS and autophagic signaling. *Curr. Opin. Toxicol.* 7, 28–36. doi: 10.1016/j.cotox.2017.10.006

Wu, K., Huang, J., Zhang, Y., Xu, W., Xu, H., Wang, L., et al. (2014). A novel protein, RafX, is important for common cell wall polysaccharide biosynthesis in *Streptococcus pneumoniae*: implications for bacterial virulence. *J. Bacteriol.* 196, 3324–3334. doi: 10.1128/jb.01696-14

Yesilkaya, H., Andisi, V. F., Andrew, P. W., and Bijlsma, J. J. (2013). *Streptococcus pneumoniae* and reactive oxygen species: an unusual approach to living with radicals. *Trends Microbiol.* 21, 187–195. doi: 10.1016/j.tim.2013.01.004

Yu, E. P., and Bennett, M. R. (2014). Mitochondrial DNA damage and atherosclerosis. *Trends Endocrinol. Metab.* 25, 481–487. doi: 10.1016/j.tem.2014.06.008

Yue, R., Xia, X., Jiang, J., Yang, D., Han, Y., Chen, X., et al. (2015). Mitochondrial DNA oxidative damage contributes to cardiomyocyte ischemia/reperfusion-injury in rats: cardioprotective role of lycopene. *J. Cell Physiol.* 230, 2128–2141. doi: 10.1002/jcp.24941

Zahlten, J., Kim, Y. J., Doehn, J. M., Pribyl, T., Hocke, A. C., Garcia, P., et al. (2015). *Streptococcus pneumoniae*-induced oxidative stress in lung epithelial cells depends on pneumococcal autolysis and is reversible by resveratrol. *J. Infect. Dis.* 211, 1822–1830. doi: 10.1093/infdis/jiu806

Zhao, Q., Wei, Y., Pandol, S. J., Li, L., and Habtezion, A. (2018). STING signaling promotes inflammation in experimental acute pancreatitis. *Gastroenterology* 154:1822–1835.e2. doi: 10.1053/j.gastro.2018.01.065

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REVIEW
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Interactions Between the Gut Microbiota and the Host Innate Immune Response Against Pathogens

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The mammalian intestine is colonized by over a trillion microbes that comprise the "gut microbiota," a microbial community which has co-evolved with the host to form a mutually beneficial relationship. Accumulating evidence indicates that the gut microbiota participates in immune system maturation and also plays a central role in host defense against pathogens. Here we review some of the mechanisms employed by the gut microbiota to boost the innate immune response against pathogens present on epithelial mucosal surfaces. Antimicrobial peptide secretion, inflammasome activation and induction of host IL-22, IL-17, and IL-10 production are the most commonly observed strategies employed by the gut microbiota for host anti-pathogen defense. Taken together, the body of evidence suggests that the host gut microbiota can elicit innate immunity against pathogens.

Keywords: gut microbiota, host innate immunity, antimicrobial peptides, inflammasome, IL-22, IL-17, IL-10

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INTRODUCTION

The mammalian intestine is home to a complex and dynamic population of microorganisms, termed the "gut microbiota" (1, 2). These microorganisms, which co-evolved with the host as part of a mutually beneficial relationship (3), include bacteria, fungi and viruses (4, 5). Accumulating evidence indicates that the gut microbiota can participate in the maturation and function of the innate immune system, while also playing many complex roles in the host defense against pathogens (6). On the one hand, the gut microbiota can help repair intestinal mucosal barrier damage (7, 8); on the other hand, gut microbiota mediates host anti-pathogen defenses (9).

In the past decade, studies of germ-free (GF) mice have provided clues to elucidate the complexity of the intestinal microbiota (10, 11) and its importance to host health (12, 13). Mounting research shows that at least a thousand different gut microbiota species, such as *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and others, contribute to host defense against harmful microorganisms (14, 15).

Recently, several studies have begun to elucidate the molecular mechanisms underlying how the gut microbiota regulates host innate immunity against pathogens (16, 17), including a role in helping the host resist pathogen colonization. In this review, we summarize the main mechanisms by which commensal bacteria, including certain probiotic species, actively prevent pathogen colonization of the host.

GUT MICROBIOTA AND ANTIMICROBIAL PEPTIDES

Defensins

The α-defensins, microbicidal peptides mainly produced by Paneth cells, are key components of innate immunity. They control pathogen growth within the intestine (18–20) and their production can be directly elicited by both Gram-negative and Gram-positive bacteria, as well as by bacterial metabolites (e.g., lipopolysaccharide, lipoteichoic acid, lipid A, and muramyl dipeptide) (21–23). By contrast, live fungi and protozoa do not appear to stimulate Paneth cells and thus fail to elicit Paneth cell degranulation (21). Nevertheless, recent research has found that the gut microbiota plays an important role in induction of α-defensins expression against pathogens (24). In one *in vitro* study, live *E. coli* or *S. aureus*, live or dead *S. typhimurium*, lipopolysaccharide (LPS), lipid A, lipoteichoic acid (LTA), or liposomes could stimulate isolated intact intestinal crypts, demonstrating that intestinal Paneth cells may contribute to α-defensins secretion by sensing the presence of exogenous bacteria and bacterial antigens (21). To investigate whether gut microbiota possess the same or similar functions, Shipra Vaishnava and colleagues used a CR2-MyD88 Tg mouse model, whereby Paneth cells were the sole cell lineage expressing MyD88, to demonstrate that Paneth cells may directly sense enteric bacteria to trigger the MyD88-dependent antimicrobial program. Furthermore, increased numbers of *Salmonella* were observed to be internalized by mesenteric lymph node (MLN) cells of MyD88$^{-/-}$ and germ-free mice as compared to corresponding numbers observed for wild-type mice (25). Similarly, transcriptional profiles have shown that α-defensin gene (*Defa)* transcripts were less abundant in intestinal microbiota-free mice and TLRs-deficient or MyD88-deficient mice, but could be recovered after stimulation with toll-like receptor (TLR) agonists, specifically agonists of TLR2 or TLR4 (26). Thus, commensal microbiota appears to protect the host against pathogen invasion by triggering enteric Paneth cell TLR-MyD88 signaling. Notably, this mechanism is distinct from the NOD2-dependent antimicrobial response (25, 27, 28), since the former mechanism entails triggering of expression of multiple antimicrobial factors (25). However, several human-based studies have demonstrated that mutations in the NOD2 peptidoglycan sensor actually did reduce secretion of α-defensins (29–33). Therefore, these contradictory human and mouse study results warrant further research. Notably, another study has demonstrated that Cd1d$^{-/-}$ mice exhibited a defect in Paneth cell granule ultrastructure that specifically resulted in an inability to degranulate after bacterial colonization, with an increased load of segmented filamentous bacteria (SFB) also noted (34). Thus, no clear evidence demonstrates that CD1d mediates regulation of gut microbiota via α-defensins expression.

Meanwhile, more recent research has begun to examine the mechanism of how the gut microbiota influences α-defensins secretion. Studies using the Caco-2 IEC line have demonstrated that lactic acid strongly suppresses transcription of the α-defensin gene, while cecal content may include as yet unidentified factors which enhance concomitant α-defensin 5 expression (35). However, contrary to the aforementioned results, Menendez et al. found that *Defa* expression was partially restored *in vivo* by *lactobacillus* administration to antibiotic-treated mice (26). Notably, an emerging role of vitamin D, a *lactobacillus* metabolite, has been recently discovered that exerts an effect opposite on α-defensins expression to that exerted by lactate (36, 37). To reconcile these results, Su et al. used a mouse model and certain feed formulations to demonstrate that VDD- and HFD ± VDD-fed mice exhibited reduced levels of expression of α-defensin and MMP7 (a metalloproteinase that can proteolytically convert pro-α-defensins to their mature and active forms) within ileal crypts as compared to results for control and HFD groups. Moreover, their results demonstrated a critical role of vitamin D signaling in maintaining steady-state expression of α-defensins and MMP7 under physiological conditions. Subsequently, Su et al. have demonstrated that dietary vitamin D deficiency resulted in loss of Paneth cell-specific α-defensins, which may lead to intestinal dysbiosis and endotoxemia (38). Of note, oral administration of α-defensin suppressed *Helicobacter hepaticus* growth *in vivo* (38). Meanwhile, using complementary mouse models of defensin deficiency (MMP7$^{-/-}$) and surplus (HD5$^{+/+}$), Salzman noted defensin-dependent reciprocal shifts in proportions of dominant bacterial species within the small intestine with no changes in total bacterial numbers observed (**Table 1**). Upon further research, this group observed that mice overexpressing HD5 exhibited a significant loss of segmented filamentous bacteria (SFB), resulting in reduced numbers of Th17 cells within the lamina propria (48). However, direct evidence implicating the involvement of SFB in α-defensin production is still lacking and studies on α-defensin regulation by specific commensal microorganisms are still rare, warranting further research. Nevertheless, in view of existing research results, we believe that the discovery of specific microorganisms through research focusing on specific metabolic pathways may be a more fruitful approach.

With regard to β-defensins, which directly kill or inhibit the growth of microorganisms (49), these agents have been shown to exert antimicrobial activity against some species of enteric pathogenic Gram-positive *S. aureus* and *S. pyogenes*, as well as against Gram-negative *P. aeruginosa*, *E. coli* and the yeast *C. albicans* (50). In fact, accumulating evidence has shown that, similarly to α-defensins, β-defensins secretion is also regulated by the gut microbiota. For example, using *in vitro* studies of HT-29 and Caco-2 human colon epithelial cell lines, human fetal intestinal xenografts have been observed to constitutively express hBD-1 but not hBD-2, with upregulation of only the latter in xenografts intraluminally infected with *Salmonella* (51). Meanwhile, it has been independently shown that preincubation of Caco-2 cells with live *E. faecium* significantly reduced *S. typhimurium* internalization by 45.8%, while heat-killed *E. faecium* pretreatment had no effect on pathogen internalization (49). This result aligns with the latest research, which has shown that only live gut microbiota, as modeled using *Lactobacillus acidophilus* PZ 1129 and PZ 1130, *Lactobacillus paracasei, Lactobacillus plantarum*, *E. coli* K-12, and *E. coli Nissle 1917*, can strongly induce expression of hBD-2 in Caco-2 intestinal epithelial cells in a time- and dose-dependent manner (39–42)

TABLE 1 | Gut microbiota protects the host against pathogen infections and the relevant mechanisms.

Pathogens	Gut microbiota	Mechanisms	References
Helicobacter hepaticus	*Lactobacillus*	Inducing α-defensin production from Paneth cells	(38)
S. aureus *S. pyogenes* *P. aeruginosa* *E. coli* *C. albicans*	*Lactobacillus acidophilus PZ 1129* *Lactobacillus acidophilus PZ 1130* *Lactobacillus paracasei* *Lactobacillus plantarum* *E. coli K-12* *E. coli Nissle 1917*	Inducing β-defensin production	(39–42)
Klebsiella pneumoniae *Citrobacter rodentium* *Enterococcus* *Plasmodium chabaudi*	*L. reuteri* *Allobaculum spp* *Clostridium spp* *Bacteroides spp*	Inducing IL-22 production	(43–46)
Salmonella typhimurium	*Bacteroides*	Inducing IL-17 production	(47)

(**Table 1**). Notably, the *E. coli* strain *Nissle 1917*, a non-pathogenic Gram-negative strain isolated in 1917 by Alfred Nissle, elicited the most marked expression of induced β-defensin expression *in vitro* (39–42). Interestingly, Schlee et al. constructed several *E. coli Nissle 1917* deletion mutants and pinpointed flagellin as the major stimulatory factor for triggering of β-defensin secretion in the presence of that strain (40). Meanwhile, Wehkamp et al. and others have found that *E. coli Nissle 1917*-induced β-defensin expression in cell culture was mediated by NF-κB- and MAPK/AP-1-dependent pathways (39–42). Nevertheless, *in vivo* studies are still needed to confirm if gut microbiota can induce β-defensins expression to reduce pathogen colonization and control gut homeostasis (**Table 1**). Recently, to further clarify the relationship between gut microbiota and β-defensin secretion, Miani et al. used a mouse model and antibiotic treatment experiments to study the participation of dysbiotic microbiota and a low-affinity aryl hydrocarbon receptor (AHR) allele in the defective pancreatic expression of mBD14 observed in NOD mice. By utilizing 16S rDNA gene sequencing and AHR ligand activity measurements, they demonstrated that gut microbiota-derived molecules, including AHR ligands and butyrate, promoted IL-22 secretion by pancreatic ILCs that subsequently induced mBD14 expression by endocrine cells. Therefore, dysbiotic microbiota and a low-affinity AHR allele appear to explain defective pancreatic mBD14 expression of mBD14 in NOD mice (24). Because only live gut microbiota can stimulate secretion of β-defensins, we believe that specific gut microbiota that possess special metabolic pathway functionality, including pathways for secretion of AHR ligands, may possess the ability to regulate secretion of β-defensins.

C-Type Lectins

The C-type lectins, also key components of innate immunity that control growth of enteric pathogens (52–54), are expressed by multiple small intestinal epithelial lineages (55, 56). REG3γ and REG3β, two C-type lectins, provide protection against infection by specific bacterial pathogens, including *Enterococcus faecalis* (57–59), *Yersinia pseudotuberculosis* (60, 61), and *Listeria monocytogenes* (57). Notably, additional evidence suggests that C-type lectins actually mediate syncytium endosymbiont defenses through prevention of pathogen colonization. To further demonstrate how these lectins control bacterial colonization of the intestinal epithelial surface, Vil-Myd88^{Tg} mice (mice with IEC-restricted Myd88 expression) were used to determine whether surface Myd88 present on epithelial cells was sufficient to restrain bacterial colonization (55). The results showed that secretion of C-type lectins required both activation of the MyD88 pathway (62) and recognition of syncytium endosymbionts by TLRs (63). Furthermore, Earle et al. used a pipeline method to assess intestinal microbiota localization within immunofluorescence images of fixed gut cross-sections. The results indicated that elimination of dietary microbiota-accessible carbohydrates (MACs) resulted in thinning of mucus within the distal colon that increased microbial proximity to the epithelium and heightened inflammatory marker REG3β expression (64). These results align with those from an earlier study of transcriptional profiles of duodenum, jejunum, ileum and colon samples, which demonstrated that MyD88 was essential for syncytium endosymbiont-induced colonic epithelial expression of antimicrobial genes Reg3β and Reg3γ, with Myd88 deficiency associated with both a shift in bacterial diversity and a greater proportion of SFB in the small intestine (65). In fact, other research found that conventionally raised Myd88$^{-/-}$ mice exhibited increased expression of antiviral genes in the colon, which correlated with norovirus infection of the colonic epithelium (65). Therefore, it can be concluded that both the activation of the MyD88 pathway and recognition of syncytium endosymbionts by TLRs are indispensable for triggering C-type lectins secretion (**Figure 1**). Recently, Ju et al. used antibiotic-treated mice to study differences between metronidazole-treated and control groups, and observed reduced abundance of *Turicibacteraceae*, overgrowth of *E. coli* and higher levels of *Reg3β* and *Reg3γ* mRNA for the metronidazole-treated group (66). These results provide a basis for the study of the effects of specific gut syncytium endosymbiont organisms on C-type lectins secretion.

Other accumulating evidence has shown that the mammalian gut contains a rich fungal community that interacts with the immune system through the C-type lectin receptor Dectin-1. To demonstrate whether symbiotic fungi influence C-type lectins secretion that prevents pathogen colonization, Iliev et al. studied mice lacking Dectin-1 and observed increased susceptibility

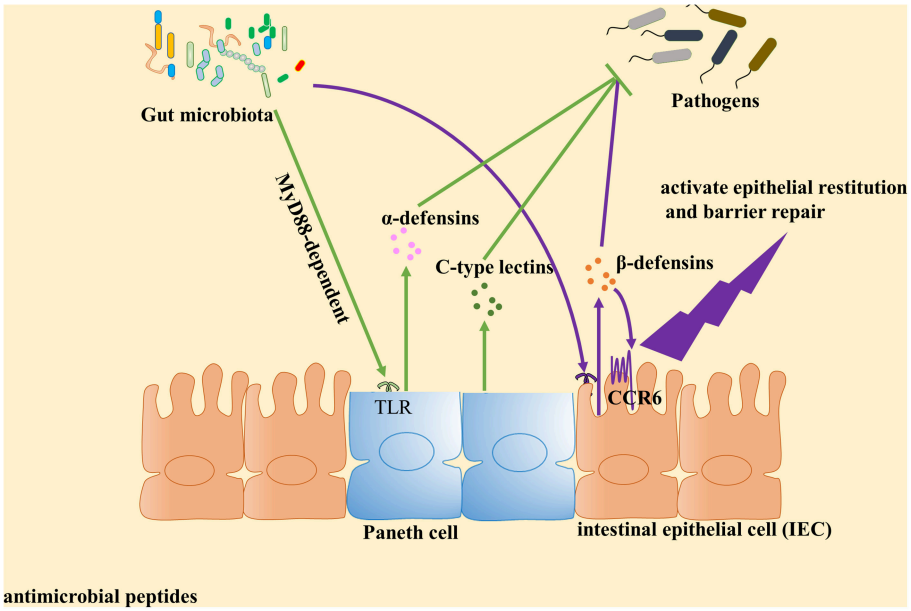

FIGURE 1 | Gut microbiota plays an important role in the induction of antimicrobial peptides expression against pathogens. Antimicrobial peptides are key components of innate immunity to control pathogen growth within the intestine. Accumulated evidence identified that gut microbiota can contribute to the expression of antimicrobial peptides and play a central role in host defense against pathogens. Paneth cells could directly sense gut microbiota through cell-autonomous myeloid differentiation primary response 88 (MyD88)-dependent toll-like receptor (TLR) activation, triggering expression of α-defensins and C-type lectins. With regard to β-defensin, gut microbiota induce β-defensin expression in cell culture mediated by NF-κB- and MAPK/AP-1-dependent pathways *in vitro*. Then β-defensin interacts with intestinal epithelial cell (IEC) through CCR6 to activate epithelial restitution and barrier repair.

to chemically induced colitis due to altered responses to indigenous fungi. Moreover, in humans they identified a gene polymorphism for Dectin-1 (CLEC7A) that is strongly linked to a severe form of ulcerative colitis (67). Independently, Eriksson et al. found that CLR-specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3 (SIGNR3) is the closest murine homolog to the human dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor. Both receptors recognize similar carbohydrate ligands, such as terminal fucose or high-mannose glycans. Notably, using the dextran sulfate sodium-induced colitis model, IGNR3 has been observed to recognize fungal members of the commensal microbiota, with SIGNR3$^{-/-}$ mice exhibiting a higher level of TNF-α in colon (68). Therefore, symbiotic fungi appear to communicate with the host via the C-type lectin receptor to maintain intestinal homeostasis. However, as yet no direct evidence has been found to determine whether symbiotic fungi can regulate selectin secretion, warranting further research.

GUT MICROBIOTA ELICITS INFLAMMASOME ACTIVATION AGAINST PATHOGENS

Inflammasome activation is an important innate immune pathway that prevents pathogen invasion via secretion of proinflammatory cytokines IL-1β and IL-18, as well as through induction of pyroptosis (69–74). It is well-documented that inflammasomes come from two main sources, namely myeloid- and epithelial-derived inflammasomes. While they share several common features, it should be noted that inflammasomes of distinct origins may exhibit different features and effector functions. For example, from a mechanistic of view, macrophage- and epithelial cell-derived inflammasomes are activated with different intermediate processes. While IL-18 processing is dependent on caspase-11 in IECs, caspase-1 is responsible for the processing of IL-18 in myeloid cells (75). In addition, compared with myeloid cells, IECs constitutively express IL-18, while produce little IL-1β (76–78). Moreover, unlike myeloid inflammasomes, IEC inflammasomes is capable of producing considerable amounts of prostaglandin upon activation (79). Intriguingly, the signaling circuitry between epithelial and myeloid inflammasomes are also different. For example, in homeostasis conditions, both *NLRP3* and *PYCARD* genes have been shown to be highly expressed in murine primary macrophages, while mouse airway epithelial cells can only express a low level of *PYCARD* and cannot express *NLRP3* (80).

Accumulating evidence suggests that gut microbiota can activate NLRC4 and NLRP3 inflammasome pathways against pathogens (81–83). *Enterobacteriaceae* and the pathobiont *Proteus mirabilis*, which are members of the normal flora of the human gastrointestinal tract (84, 85), were shown to induce robust IL-1β production through NLRP3 activation mediated by intestinal Ly6C^{high} monocytes (86, 87). Indeed, recruited Ly6C^{high} monocytes have been shown to express a variety of inflammasome components, such as NAIPs (71, 88, 89), NLRC4

(89), NLRP1 (90, 91), NLRP6 (92, 93), AIM2 (94), caspase-1 (95), caspase-4 (96) (in humans), ASC (93), and IL-18 (87, 97, 98). Meanwhile, Seo et al. have also demonstrated that *Proteus mirabilis* (a Proteobacteria phylum member) induced NLRP3 activation and IL-1β production (86). Interestingly, bacterial components from other *Proteobacteria*, such as LPS produced by *Pseudomonas* spp., have even been shown to induce host mental depression symptoms via NLRP3 inflammasome activation (99). Other interesting lines of research have shown that in addition to gut commensal bacteria, the mammalian gut contains a rich fungal community which also appears to activate the inflammasome pathway. This community includes the human commensal fungus *Candida albicans* (*C. albicans*), which colonizes gastrointestinal and vaginal tract mucosal surfaces and appears to promote inflammasome activation during AOM-DSS-induced colitis (100). In further support of this finding, direct peptide administration experiments had previously demonstrated that candidalysin, a peptide derived from the hypha-specific *ECE1* gene, acted as a fungal trigger for NLRP3 inflammasome-mediated maturation that was sufficient for inducing IL-1β secretion mature macrophages in an NLRP3 inflammasome-dependent manner (101).

In recent studies, numerous other gut microbiota metabolites have also been demonstrated to elicit inflammasome pathways against pathogens. For example, gut microbiota-derived adenosine triphosphate (ATP) has been shown to co-operate with NLRP3 (also known as CIAS1) (102) via the macrophage P2X7 receptor (103) to induce assembly of a cytosolic protein complex containing ASK and caspase-1 (70, 104–106) that eventually leads to inflammasome activation (106). Another important gut microbiota metabolite, short-chain fatty acids (SCFAs), end products of fermentation of dietary fibers by anaerobic intestinal microbiota, have also been implicated in inflammasome activation (107). SCFAs binding to GPR43 on colonic epithelial cells to stimulate K^+ efflux and hyperpolarization has been shown to lead to NLRP3 inflammasome activation, with subsequent acceleration of cell maturation and secretion of IL-1β (108) and IL-18 (77, 109).

GUT MICROBIOTA CAN ENHANCE INTERLEUKIN EXPRESSION TO CLEAR INVADING PATHOGENS

IL-22

IL-22 is important in maintaining mucosal barrier integrity and is produced by many different types of innate immune cells (110–113). This cytokine has been shown to play a host-protective role during infection by a wide range of pathogens, including *Klebsiella pneumoniae* (114), *Citrobacter rodentium* (115, 116), vancomycin-resistant *Enterococcus* (117, 118) and *Plasmodium chabaudi* (119). One IL-22-dependent mechanism involved in pathogen clearance involves the increased presence of antimicrobial proteins within the mucosa (120) that include the following: calprotectin and lipocalin-2, the latter of which binds to the siderophore enterochelin, with both acting to limit iron availability in the gut (120); C-type lectins, which regenerate islet-derivative proteins Reg3β and Reg3γ that control some components of the microbiota (58, 120, 121); and S100A8 and S100A9, two antimicrobial peptides that heterodimerize to form calprotectin, an antimicrobial protein that sequesters zinc and manganese to prevent microbial access to these nutrients (122). Although epithelial antimicrobial defenses also exist, many pathogens can still colonize mucosal surfaces to establish infections (120, 123). Nevertheless, accumulated evidence has shown that IL-22 is rapidly induced in response to pathogen invasion through activation of host AhR via specific gut microbiota-derived molecules (**Figure 2**) (124, 125). For example, *Lactobacillus species* (specifically, *L. reuteri*) can activate IL-22 production by gut type 3 innate lymphoid cells (ILC3) (126–128), while other studies have shown that supplementation with three commensal *Lactobacillus strains* with high tryptophan-metabolizing activities was sufficient to restore intestinal IL-22 production (43, 129). Indeed, additional work has shown that *Lactobacillus species* could utilize tryptophan as an energy source and produce a metabolite, indole-3-aldehyde (IAld), which could then activate AhRs present on ILCs (126, 130). In addition to *Lactobacillus strains*, other recent studies have shown that *Allobaculum* spp. (43), *Escherichia coli* (44), *Clostridium* spp. (45), and *Bacteroides* spp. (46) can also utilize tryptophan to produce IAld and elicit IL-22 production (**Table 1**). Meanwhile, other studies have shown that activated ILCs secrete IL-22 to protect the host against opportunistic pathogens by reducing pathogen colonization (120, 131). In fact, other innate immune cells, such as NKT cells, γδ T cells and macrophages, have very recently been shown to secrete IL-22 under regulation by gut microbiota via the AhR pathway (132). Therefore, gut microbiota may prevent pathogen infection by collectively enhancing IL-22 expression via the AhR pathway.

IL-17

IL-17 is a well-established crucial cytokine that is involved in limiting invasion and dissemination of pathogens, including *Salmonella typhimurium* (133), by both recruitment of neutrophils and by the induction of production of antimicrobial peptides (131, 134). Recent studies have demonstrated that both the abundance and activation status of IL-17-producing intraepithelial lymphocytes (IELs) are modulated by commensal bacteria, with enrichment of the γδT cell population of IELs representing an important source of innate IL-17 production (135, 136). Notably, a comparative study of GF mice and SPF mice has shown that the number of TCRγδ IELs is decreased in GF mice (133). Moreover, in addition to the regulation of IELs numbers, the gut microbiota may also regulate activation of TCRγδ IELs, as reflected by a report showing that production of IL-17 by TCRγδ IELs is decreased in GF mice (137). Meanwhile, antibiotic-treatment and monocolonization of mice have been used to demonstrate that the great majority of γ/δ T cells within peritonea of SPF mice are $CD62L^-$ γδT cells, which are activated γδT cells, with GF mice possessing far fewer $CD62L^-$ γ/δ T cells than SPF mice (47). Notably, additional research suggests that specific commensal bacteria, excluding metronidazole-sensitive anaerobes, such as *Bacteroides* species, are required for maintaining IL-1R1$^{\pm}$ γδT cells (47), a result

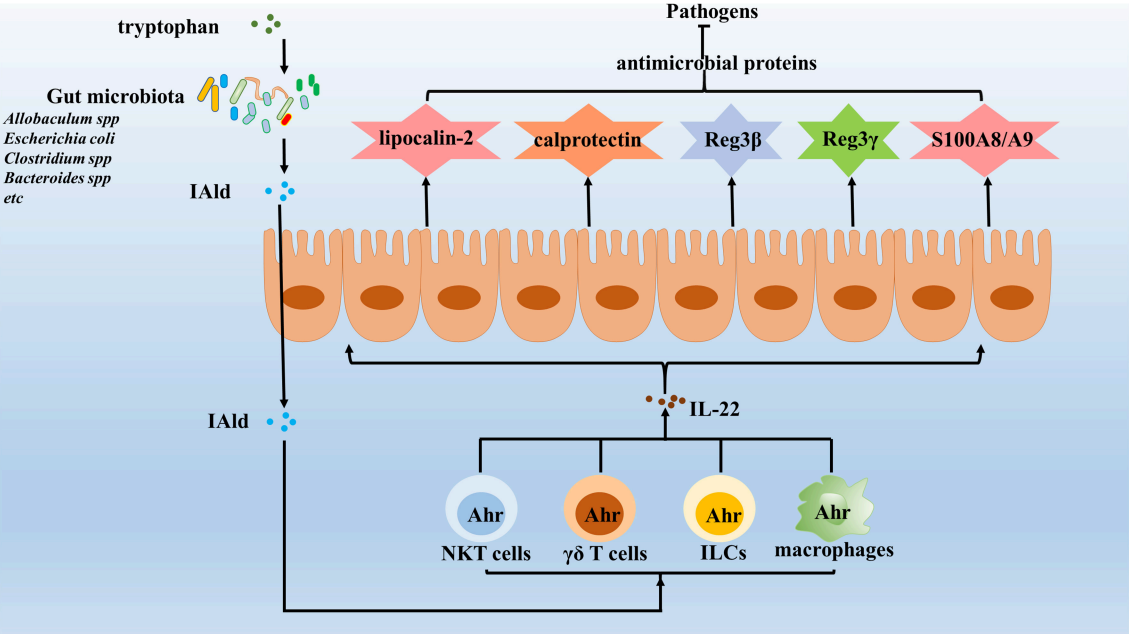

FIGURE 2 | Gut microbiota enhance the expression of IL-22 against invading pathogens. IL-22 is important in maintaining the integrity of mucosal barriers and can be produced by many different types innate immune cells, which induce the expression of various antimicrobial proteins, including lipocalin-2, calprotectin, C-type lectins, S100A8, S100A9, and so on to clear pathogens. Increasing evidence identified that gut microbiota enhances the expression of IL-22 to protect the host against pathogens. The results show that gut microbiota utilize tryptophan as an energy source and produce a metabolite, indole-3-aldehyde (IAld), which in turns activated Ahr on innate immune cells. Once activated, innate immune cells will secrete IL-22, which protects the host against opportunistic pathogens by reducing their colonization.

that aligns with previous research results by another research group (138) (**Table 1**). In conclusion, gut microbiota influences the abundance and activation status of IL-17-producing TCRγδ IELs to protect the host from pathogen infection and to maintain intestinal homeostasis. In addition, lymphoid tissue inducer (LTi) cells and NCR^{-} ILC3 cells also appear to function as important sources of innate IL-17 production (127). However, few studies have investigated how gut microbiota regulate these cell types, warranting further research in this area.

IL-10

IL-10 is an anti-inflammatory cytokine that plays a central role in regulating the host immune response to pathogens, thereby preventing host damage and maintaining normal tissue homeostasis (139–141). Accumulating evidence suggests that macrophages are an important source of innate IL-10 and that the gut microbiota plays a vital role in mucosal innate IL-10 generation under homeostatic conditions (142–144). For example, studies in GF mice and SPF mice have shown that colonic lamina propria from germ-free mice exhibited lower IL-10 production (142), a reduction later confirmed to be a 50% reduction in steady-state IL-10 levels (142–144). To elucidate the mechanism by which gut microbiota regulate intestinal macrophage IL-10 production, Hayashi et al. used macrophage-specific IL-10-deficient mice to demonstrate that *Clostridium butyricum* (CB), a distinct cluster I *Clostridium* strain, induces IL-10 production to ultimately prevent acute experimental colitis. However, while CB treatment had no effects on IL-10 production by T cells, IL-10-producing $F4/80^{\pm}CD11b^{\pm}CD11c^{int}$ macrophages accumulated within inflamed mucosa after CB treatment. Subsequently, more rigorous examination demonstrated that CB directly triggered IL-10 production by intestinal macrophages there via the TLR2/MyD88 pathway (144). Meanwhile, Ochi et al. recently found that dietary amino acids directly regulate Il-10 production by small intestine (SI) macrophages. Using mice fed via total parenteral nutrition, a significant decrease of IL-10-producing macrophages in the SI was observed, while IL-10-producing $CD4^{\pm}$ T cells remained intact. Likewise, enteral nutrient deprivation selectively decreased IL-10 production by the monocyte-derived $F4/80^{\pm}$ macrophage population, but had no effect on non-monocytic precursor-derived $CD103^{\pm}$ dendritic cells. Notably, in contrast to regulation of colonic macrophages, replenishment of SI macrophages and their IL-10 production were not regulated by gut microbiota (145). Contrary to results obtained under steady-state conditions, an injury model used to study participation of microbiota to explain observed IL-10 increases post-injury yielded different results. Specifically, comparison of *Il10* mRNA levels in uninjured intact tissue and day-2 post-wound tissue isolated from SPF or GF mice indicated that IL-10 mRNA was induced in post-wound colonic tissue isolated from both SPF and GF mice. Therefore, injury-triggered IL-10 increases appeared to be largely microbiota independent (146), although the reasons

remain unclear regarding the differing effects of the gut microbiota observed in different model systems. Nevertheless, we hypothesize that local damage-associated molecular proteins (DAMPs) may regulate immune cells more rapidly and strongly post-intestinal damage, resulting in either a failure of gut microbiota to temporally adjust or a masking of any microbiota-based regulatory effect.

CONCLUDING REMARKS

Gut microbiota resists colonization and growth of invading pathogens through the induction of expression of antimicrobial peptides, IL-22, IL-17, and IL-10 while eliciting inflammasome activation. Because the underlying mechanisms of how the gut microbiota resists pathogenic invasion still remain obscure, future studies are clearly needed to identify gut microbiota functions against various pathogens toward the development of promising strategies to treat infectious diseases. For instance, E. coli Nissle 1917 can induce β-defensin expression mediated by NF-κB- and MAPK/AP-1-dependent pathways (39), while Lactobacillus spp. activate IL-22 production against opportunistic pathogens to reduce colonization (147, 148). Therefore, transplanting suitable specific gut microbiota to compete with specific pathogens could be an effective defense strategy. However, since this strategy poses new disease risks, strategies that restore intestinal homeostasis and promote host immune system may serve to more safely clear pathogens. To this end, identifying specific gut microbiota functions and defining normal gut microbiota populations are necessary first steps toward development of safer strategies for strengthening host defenses against pathogens. Moreover, research on the function and mechanisms of gut microbiota metabolites may facilitate development of novel therapeutic strategies to combat drug-resistant pathogens.

AUTHOR CONTRIBUTIONS

D-KC, W-TM, H-YC, and M-XN designed the structure of the mini-review. H-YC and M-XN wrote the manuscript and drafted the first version of the manuscript. M-XN and W-TM helped revise the manuscript. All authors have reviewed the final version of the manuscript.

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REFERENCES

1. Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Nageshwar Reddy D. Role of the normal gut microbiota. *World J Gastroenterol.* (2015) 21:8787–803. doi: 10.3748/wjg.v21.i29.8787
2. Luthold RV, Fernandes GR, Franco-de-Moraes AC, Folchetti LG, Ferreira SR. Gut microbiota interactions with the immunomodulatory role of vitamin D in normal individuals. *Metabolism.* (2017) 69:76–86. doi: 10.1016/j.metabol.2017.01.007
3. Ayres JS. Cooperative microbial tolerance behaviors in host-microbiota mutualism. *Cell.* (2016) 165:1323–31. doi: 10.1016/j.cell.2016.05.049
4. Foca A, Liberto MC, Quirino A, Marascio N, Zicca E, Pavia G. Gut inflammation and immunity: what is the role of the human gut virome? *Mediators Inflamm.* (2015) 2015:326032. doi: 10.1155/2015/326032
5. Chen B, Chen H, Shu X, Yin Y, Li J, Qin J, et al. Presence of segmented filamentous bacteria in human children and its potential role in the modulation of human gut immunity. *Front Microbiol.* (2018) 9:1403. doi: 10.3389/fmicb.2018.01403
6. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell.* (2014) 157:121–41. doi: 10.1016/j.cell.2014.03.011
7. Okumura R, Takeda K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Exp Mol Med.* (2017) 49:e338. doi: 10.1038/emm.2017.20
8. Okumura R, Takeda K. Maintenance of intestinal homeostasis by mucosal barriers. *Inflamm Regen.* (2018) 38:5. doi: 10.1186/s41232-018-0063-z
9. Ubeda C, Djukovic A, Isaac S. Roles of the intestinal microbiota in pathogen protection. *Clin Transl Immunol.* (2017) 6:e128. doi: 10.1038/cti.2017.2
10. Biedermann L, Rogler G. The intestinal microbiota: its role in health and disease. *Eur J Pediatr.* (2015) 174:151–67. doi: 10.1007/s00431-014-2476-2
11. Goulet O. Potential role of the intestinal microbiota in programming health and disease. *Nutr Rev.* (2015) 73 Suppl. 1:32–40. doi: 10.1093/nutrit/nuv039
12. Tojo R, Suarez A, Clemente MG, de los Reyes-Gavilan CG, Margolles A, Gueimonde M, et al. Intestinal microbiota in health and disease: role of bifidobacteria in gut homeostasis. *World J Gastroenterol.* (2014) 20:15163–76. doi: 10.3748/wjg.v20.i41.15163
13. Kataoka K. The intestinal microbiota and its role in human health and disease. *J Med Invest.* (2016) 63:27–37. doi: 10.2152/jmi.63.27
14. Gevers D, Knight R, Petrosino JF, Huang K, McGuire AL, Birren BW, et al. The human microbiome project: a community resource for the healthy human microbiome. *PLoS Biol.* (2012) 10:e1001377. doi: 10.1371/journal.pbio.1001377
15. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. *Genome Med.* (2016) 8:51. doi: 10.1186/s13073-016-0307-y
16. Lim MY, You HJ, Yoon HS, Kwon B, Lee JY, Lee S, et al. The effect of heritability and host genetics on the gut microbiota and metabolic syndrome. *Gut.* (2017) 66:1031–8. doi: 10.1136/gutjnl-2015-311326
17. Monedero V, Buesa J, Rodriguez-Diaz J. The interactions between host glycobiology, bacterial microbiota, and viruses in the gut. *Viruses.* (2018) 10:96. doi: 10.3390/v10020096
18. Diamond G, Beckloff N, Weinberg A, Kisich KO. The roles of antimicrobial peptides in innate host defense. *Curr Pharm Des.* (2009) 15:2377–92. doi: 10.2174/138161209788682325
19. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science.* (2012) 336:1268–73. doi: 10.1126/science.1223490
20. Kim D, Zeng MY, Nunez G. The interplay between host immune cells and gut microbiota in chronic inflammatory diseases. *Exp Mol Med.* (2017) 49:e339. doi: 10.1038/emm.2017.24
21. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol.* (2000) 1:113–8. doi: 10.1038/77783
22. Elphick DA, Mahida YR. Paneth cells: their role in innate immunity and inflammatory disease. *Gut.* (2005) 54:1802–9. doi: 10.1136/gut.2005.068601
23. Tanabe H, Ayabe T, Bainbridge B, Guina T, Ernst RK, Darveau RP, et al. Mouse paneth cell secretory responses to cell surface glycolipids of virulent

and attenuated pathogenic bacteria. *Infect Immun.* (2005) 73:2312–20. doi: 10.1128/IAI.73.4.2312-2320.2005
24. Miani M, Le Naour J, Waeckel-Enee E, Verma SC, Straube M, Emond P, et al. Gut microbiota-stimulated innate lymphoid cells support beta-defensin 14 expression in pancreatic endocrine cells, preventing autoimmune diabetes. *Cell Metab.* (2018) 28:557–72.e6. doi: 10.1016/j.cmet.2018.06.012
25. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci USA*. (2008) 105:20858–63. doi: 10.1073/pnas.0808723105
26. Menendez A, Willing BP, Montero M, Wlodarska M, So CC, Bhinder G, et al. Bacterial stimulation of the TLR-MyD88 pathway modulates the homeostatic expression of ileal Paneth cell alpha-defensins. *J Innate Immun.* (2013) 5:39–49. doi: 10.1159/000341630
27. MacDonald TT, Monteleone I, Fantini MC, Monteleone G. Regulation of homeostasis and inflammation in the intestine. *Gastroenterology*. (2011) 140:1768–75. doi: 10.1053/j.gastro.2011.02.047
28. Kong S, Zhang YH, Zhang W. Regulation of intestinal epithelial cells properties and functions by amino acids. *Biomed Res Int.* (2018) 2018:2819154. doi: 10.1155/2018/2819154
29. Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci USA*. (2009) 106:15813–8. doi: 10.1073/pnas.0907722106
30. Ramanan D, Tang MS, Bowcutt R, Loke P, Cadwell K. Bacterial sensor Nod2 prevents inflammation of the small intestine by restricting the expansion of the commensal *Bacteroides vulgatus*. *Immunity.* (2014) 41:311–24. doi: 10.1016/j.immuni.2014.06.015
31. Shanahan MT, Carroll IM, Grossniklaus E, White A, von Furstenberg RJ, Barner R, et al. Mouse Paneth cell antimicrobial function is independent of Nod2. *Gut.* (2014) 63:903–10. doi: 10.1136/gutjnl-2012-304190
32. Tan G, Zeng B, Zhi FC. Regulation of human enteric alpha-defensins by NOD2 in the Paneth cell lineage. *Eur J Cell Biol.* (2015) 94:60–6. doi: 10.1016/j.ejcb.2014.10.007
33. Keestra-Gounder AM, Byndloss MX, Seyffert N, Young BM, Chavez-Arroyo A, Tsai AY, et al. NOD1 and NOD2 signalling links ER stress with inflammation. *Nature.* (2016) 532:394–7. doi: 10.1038/nature17631
34. Nieuwenhuis EE, Matsumoto T, Lindenbergh D, Willemsen R, Kaser A, Simons-Oosterhuis Y, et al. Cd1d-dependent regulation of bacterial colonization in the intestine of mice. *J Clin Invest.* (2009) 119:1241–50. doi: 10.1172/JCI36509
35. Sugi Y, Takahashi K, Kurihara K, Nakano K, Kobayakawa T, Nakata K, et al. a-Defensin 5 gene expression is regulated by gut microbial metabolites. *Biosci Biotechnol Biochem.* (2017) 81:242–8. doi: 10.1080/09168451.2016.1246175
36. Jerzynska J, Stelmach W, Balcerak J, Woicka-Kolejwa K, Rychlik B, Blauz A, et al. Effect of *Lactobacillus rhamnosus* GG and vitamin D supplementation on the immunologic effectiveness of grass-specific sublingual immunotherapy in children with allergy. *Allergy Asthma Proc.* (2016) 37:324–34. doi: 10.2500/aap.2016.37.3958
37. Shang M, Sun J. Vitamin D/VDR, probiotics, and gastrointestinal diseases. *Curr Med Chem.* (2017) 24:876–87. doi: 10.2174/0929867323666161202150008
38. Su D, Nie Y, Zhu A, Chen Z, Wu P, Zhang L, et al. Vitamin D signaling through Induction of Paneth cell defensins maintains gut microbiota and improves metabolic disorders and hepatic steatosis in animal models. *Front Physiol.* (2016) 7:498. doi: 10.3389/fphys.2016.00498
39. Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C, et al. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun.* (2004) 72:5750–8. doi: 10.1128/IAI.72.10.5750-5758.2004
40. Schlee M, Wehkamp J, Altenhoefer A, Oelschlaeger TA, Stange EF, Fellermann K. Induction of human beta-defensin 2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin. *Infect Immun.* (2007) 75:2399–407. doi: 10.1128/IAI.01563-06
41. Steubesand N, Kiehne K, Brunke G, Pahl R, Reiss K, Herzig KH, et al. The expression of the beta-defensins hBD-2 and hBD-3 is differentially regulated by NF-kappaB and MAPK/AP-1 pathways in an *in vitro* model of Candida esophagitis. *BMC Immunol.* (2009) 10:36. doi: 10.1186/1471-2172-10-36
42. Seo EJ, Weibel S, Wehkamp J, Oelschlaeger TA. Construction of recombinant *E. coli* Nissle 1917 (EcN) strains for the expression and secretion of defensins. *Int J Med Microbiol.* (2012) 302:276–87. doi: 10.1016/j.ijmm.2012.05.002
43. Etienne-Mesmin L, Chassaing B, Gewirtz AT. Tryptophan: A gut microbiota-derived metabolites regulating inflammation. *World J Gastrointest Pharmacol Ther.* (2017) 8:7–9. doi: 10.4292/wjgpt.v8.i1.7
44. Devlin AS, Marcobal A, Dodd D, Nayfach S, Plummer N, Meyer T, et al. Modulation of a circulating uremic solute via rational genetic manipulation of the gut microbiota. *Cell Host Microbe.* (2016) 20:709–15. doi: 10.1016/j.chom.2016.10.021
45. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. *Nat Commun.* (2018) 9:3294. doi: 10.1038/s41467-018-05470-4
46. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol.* (2011) 13:144–51. doi: 10.1038/ni.2187
47. Duan J, Chung H, Troy E, Kasper DL. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. *Cell Host Microbe.* (2010) 7:140–50. doi: 10.1016/j.chom.2010.01.005
48. Salzman NH. Paneth cell defensins and the regulation of the microbiome: detente at mucosal surfaces. *Gut Microbes.* (2010) 1:401–6. doi: 10.4161/gmic.1.6.14076
49. Fusco A, Savio V, Cammarota M, Alfano A, Schiraldi C, Donnarumma G. Beta-Defensin-2 and Beta-Defensin-3 reduce intestinal damage caused by *Salmonella typhimurium* modulating the expression of cytokines and enhancing the probiotic activity of *Enterococcus faecium*. *J Immunol Res.* (2017) 2017:6976935. doi: 10.1155/2017/6976935
50. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem.* (2001) 276:5707–13. doi: 10.1074/jbc.M008557200
51. O'Neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, et al. Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol.* (1999) 163:6718–24.
52. Sotolongo J, Ruiz J, Fukata M. The role of innate immunity in the host defense against intestinal bacterial pathogens. *Curr Infect Dis Rep.* (2012) 14:15–23. doi: 10.1007/s11908-011-0234-4
53. Katakura K, Watanabe H, Ohira H. Innate immunity and inflammatory bowel disease: a review of clinical evidence and future application. *Clin J Gastroenterol.* (2013) 6:415–9. doi: 10.1007/s12328-013-0436-4
54. Thaiss CA, Levy M, Suez J, Elinav E. The interplay between the innate immune system and the microbiota. *Curr Opin Immunol.* (2014) 26:41–8. doi: 10.1016/j.coi.2013.10.016
55. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science.* (2011) 334:255–8. doi: 10.1126/science.1209791
56. Thomas M, Pierson M, Uprety T, Zhu L, Ran Z, Sreenivasan CC, et al. Comparison of porcine airway and intestinal epithelial cell lines for the susceptibility and expression of pattern recognition receptors upon influenza virus infection. *Viruses.* (2018) 10:312. doi: 10.3390/v10060312
57. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol.* (2010) 10:131–44. doi: 10.1038/nri2707
58. Gallo RL, Hooper LV. Epithelial antimicrobial defence of the skin and intestine. *Nat Rev Immunol.* (2012) 12:503–16. doi: 10.1038/nri3228
59. Mukherjee S, Hooper LV. Antimicrobial defense of the intestine. *Immunity.* (2015) 42:28–39. doi: 10.1016/j.immuni.2014.12.028
60. Dessein R, Gironella M, Vignal C, Peyrin-Biroulet L, Sokol H, Secher T, et al. Toll-like receptor 2 is critical for induction of Reg3 beta expression and intestinal clearance of *Yersinia pseudotuberculosis*. *Gut.* (2009) 58:771–6. doi: 10.1136/gut.2008.168443
61. Burger-van Paassen N, Loonen LM, Witte-Bouma J, Korteland-van Male AM, de Bruijn AC, van der Sluis M, et al. Mucin Muc2 deficiency and weaning influences the expression of the innate defense genes

Reg3beta, Reg3gamma and angiogenin-4. *PLoS ONE.* (2012) 7:e38798. doi: 10.1371/journal.pone.0038798
62. Bel S, Pendse M, Wang Y, Li Y, Ruhn KA, Hassell B, et al. Paneth cells secrete lysozyme via secretory autophagy during bacterial infection of the intestine. *Science.* (2017) 357:1047–52. doi: 10.1126/science.aal4677
63. Valentini M, Piermattei A, Di Sante G, Migliara G, Delogu G, Ria F. Immunomodulation by gut microbiota: role of Toll-like receptor expressed by T cells. *J Immunol Res.* (2014) 2014:586939. doi: 10.1155/2014/586939
64. Earle KA, Billings G, Sigal M, Lichtman JS, Hansson GC, Elias JE, et al. Quantitative imaging of gut microbiota spatial organization. *Cell Host Microbe.* (2015) 18:478–88. doi: 10.1016/j.chom.2015.09.002
65. Larsson E, Tremaroli V, Lee YS, Koren O, Nookaew I, Fricker A, et al. Analysis of gut microbial regulation of host gene expression along the length of the gut and regulation of gut microbial ecology through MyD88. *Gut.* (2012) 61:1124–31. doi: 10.1136/gutjnl-2011-301104
66. Ju T, Shoblak Y, Gao Y, Yang K, Fouhse J, Finlay BB, et al. Initial gut microbial composition as a key factor driving host response to antibiotic treatment, as exemplified by the presence or absence of commensal *Escherichia coli*. *Appl Environ Microbiol.* (2017) 83:e01107-17. doi: 10.1128/AEM.01107-17
67. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science.* (2012) 336:1314–7. doi: 10.1126/science.1221789
68. Eriksson M, Johannssen T, von Smolinski D, Gruber AD, Seeberger PH, Lepenies B. The C-type lectin receptor SIGNR3 binds to fungi present in commensal microbiota and influences immune regulation in experimental colitis. *Front Immunol.* (2013) 4:196. doi: 10.3389/fimmu.2013.00196
69. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol.* (2009) 10:241–7. doi: 10.1038/ni.1703
70. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1beta secretion. *Cytokine Growth Factor Rev.* (2011) 22:189–95. doi: 10.1016/j.cytogfr.2011.10.001
71. Sahoo M, Ceballos-Olvera I, del Barrio L, Re F. Role of the inflammasome, IL-1beta, and IL-18 in bacterial infections. *ScientificWorldJournal.* (2011) 11:2037–50. doi: 10.1100/2011/212680
72. Schmidt RL, Lenz LL. Distinct licensing of IL-18 and IL-1beta secretion in response to NLRP3 inflammasome activation. *PLoS ONE.* (2012) 7:e45186. doi: 10.1371/journal.pone.0045186
73. Kanneganti TD. The inflammasome: firing up innate immunity. *Immunol Rev.* (2015) 265:1–5. doi: 10.1111/imr.12297
74. He Y, Hara H, Nunez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci.* (2016) 41:1012–21. doi: 10.1016/j.tibs.2016.09.002
75. Crowley SM, Vallance BA, Knodler LA. Noncanonical inflammasomes: antimicrobial defense that does not play by the rules. *Cell Microbiol.* (2017) 19. doi: 10.1111/cmi.12730
76. Knodler LA, Crowley SM, Sham HP, Yang H, Wrande M, Ma C, et al. Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. *Cell Host Microbe.* (2014) 16:249–56. doi: 10.1016/j.chom.2014.07.002
77. Thinwa J, Segovia JA, Bose S, Dube PH. Integrin-mediated first signal for inflammasome activation in intestinal epithelial cells. *J Immunol.* (2014) 193:1373–82. doi: 10.4049/jimmunol.1400145
78. Harrison OJ, Srinivasan N, Pott J, Schiering C, Krausgruber T, Ilott NE, et al. Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3(+) Treg cell function in the intestine. *Mucosal Immunol.* (2015) 8:1226–36. doi: 10.1038/mi.2015.13
79. Rauch I, Deets KA, Ji DX, von Moltke J, Tenthorey JL, Lee AY, et al. NAIP-NLRC4 inflammasomes coordinate intestinal epithelial cell expulsion with eicosanoid and IL-18 release via activation of caspase-1 and−8. *Immunity.* (2017) 46:649–59. doi: 10.1016/j.immuni.2017.03.016
80. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, et al. The NLRP3 inflammasome mediates *in vivo* innate immunity to influenza A virus through recognition of viral RNA. *Immunity.* (2009) 30:556–65. doi: 10.1016/j.immuni.2009.02.005
81. Franchi L, Kamada N, Nakamura Y, Burberry A, Kuffa P, Suzuki S, et al. NLRC4-driven production of IL-1beta discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. *Nat Immunol.* (2012) 13:449–56. doi: 10.1038/ni.2263
82. Nordlander S, Pott J, Maloy KJ. NLRC4 expression in intestinal epithelial cells mediates protection against an enteric pathogen. *Mucosal Immunol.* (2014) 7:775–85. doi: 10.1038/mi.2013.95
83. Levy M, Thaiss CA, Katz MN, Suez J, Elinav E. Inflammasomes and the microbiota–partners in the preservation of mucosal homeostasis. *Semin Immunopathol.* (2015) 37:39–46. doi: 10.1007/s00281-014-0451-7
84. Endimiani A, Luzzaro F, Brigante G, Perilli M, Lombardi G, Amicosante G, et al. Proteus mirabilis bloodstream infections: risk factors and treatment outcome related to the expression of extended-spectrum beta-lactamases. *Antimicrob Agents Chemother.* (2005) 49:2598–605. doi: 10.1128/AAC.49.7.2598-2605.2005
85. Hardt WD. Journal club. An infection biologist points out an outstanding issue in mucosal immunology. *Nature.* (2009) 459:893. doi: 10.1038/459893e
86. Seo SU, Kamada N, Munoz-Planillo R, Kim YG, Kim D, Koizumi Y, et al. Distinct commensals induce interleukin-1beta via NLRP3 inflammasome in inflammatory monocytes to promote intestinal inflammation in response to injury. *Immunity.* (2015) 42:744–55. doi: 10.1016/j.immuni.2015.03.004
87. Ignacio A, Morales CI, Camara NO, Almeida RR. Innate sensing of the gut microbiota: modulation of inflammatory and autoimmune diseases. *Front Immunol.* (2016) 7:54. doi: 10.3389/fimmu.2016.00054
88. Chen KW, Gross CJ, Sotomayor FV, Stacey KJ, Tschopp J, Sweet MJ, et al. The neutrophil NLRC4 inflammasome selectively promotes IL-1beta maturation without pyroptosis during acute Salmonella challenge. *Cell Rep.* (2014) 8:570–82. doi: 10.1016/j.celrep.2014.06.028
89. Karki R, Lee E, Place D, Samir P, Mavuluri J, Sharma BR, et al. IRF8 regulates transcription of naips for NLRC4 inflammasome activation. *Cell.* (2018) 173:920–33.e13. doi: 10.1016/j.cell.2018.02.055
90. Chi W, Li F, Chen H, Wang Y, Zhu Y, Yang X, et al. Caspase-8 promotes NLRP1/NLRP3 inflammasome activation and IL-1beta production in acute glaucoma. *Proc Natl Acad Sci USA.* (2014) 111:11181–6. doi: 10.1073/pnas.1402819111
91. Kaushal V, Dye R, Pakavathkumar P, Foveau B, Flores J, Hyman B, et al. Neuronal NLRP1 inflammasome activation of Caspase-1 coordinately regulates inflammatory interleukin-1-beta production and axonal degeneration-associated Caspase-6 activation. *Cell Death Differ.* (2015) 22:1676–86. doi: 10.1038/cdd.2015.16
92. Chen GY, Liu M, Wang F, Bertin J, Nunez G. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol.* (2011) 186:7187–94. doi: 10.4049/jimmunol.1100412
93. Levy M, Thaiss CA, Zeevi D, Dohnalova L, Zilberman-Schapira G, Mahdi JA, et al. Microbiota-modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. *Cell.* (2015) 163:1428–43. doi: 10.1016/j.cell.2015.10.048
94. Micaroni M, Stanley AC, Khromykh T, Venturato J, Wong CX, Lim JP, et al. Rab6a/a′ are important Golgi regulators of pro-inflammatory TNF secretion in macrophages. *PLoS ONE.* (2013) 8:e57034. doi: 10.1371/journal.pone.0057034
95. Brough D, Rothwell NJ. Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *J Cell Sci.* (2007) 120(Pt 5):772–81. doi: 10.1242/jcs.03377
96. Pallett MA, Crepin VF, Serafini N, Habibzay M, Kotik O, Sanchez-Garrido J, et al. Bacterial virulence factor inhibits caspase-4/11 activation in intestinal epithelial cells. *Mucosal Immunol.* (2017) 10:602–12. doi: 10.1038/mi.2016.77
97. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat Med.* (2015) 21:677–87. doi: 10.1038/nm.3893
98. Thi HTH, Hong S. Inflammasome as a therapeutic target for cancer prevention and treatment. *J Cancer Prev.* (2017) 22:62–73. doi: 10.15430/JCP.2017.22.2.62
99. Maes M, Kubera M, Leunis JC. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. *Neuro Endocrinol Lett.* (2008) 29:117–24. doi: 10.1016/j.jad.2012.02.023

100. Roselletti E, Perito S, Gabrielli E, Mencacci A, Pericolini E, Sabbatini S, et al. NLRP3 inflammasome is a key player in human vulvovaginal disease caused by *Candida albicans*. *Sci Rep.* (2017) 7:17877. doi: 10.1038/s41598-017-17649-8
101. Joly S, Ma N, Sadler JJ, Soll DR, Cassel SL, Sutterwala FS. Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. *J Immunol.* (2009) 183:3578–81. doi: 10.4049/jimmunol.0901323
102. Gombault A, Baron L, Couillin I. ATP release and purinergic signaling in NLRP3 inflammasome activation. *Front Immunol.* (2012) 3:414. doi: 10.3389/fimmu.2012.00414
103. Amores-Iniesta J, Barbera-Cremades M, Martinez CM, Pons JA, Revilla-Nuin B, Martinez-Alarcon L, et al. Extracellular ATP activates the NLRP3 inflammasome and is an early danger signal of skin allograft rejection. *Cell Rep.* (2017) 21:3414–26. doi: 10.1016/j.celrep.2017.11.079
104. Franchi L, Kanneganti TD, Dubyak GR, Nunez G. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J Biol Chem.* (2007) 282:18810–8. doi: 10.1074/jbc.M610762200
105. Dubyak GR. P2X7 receptor regulation of non-classical secretion from immune effector cells. *Cell Microbiol.* (2012) 14:1697–706. doi: 10.1111/cmi.12001
106. Franceschini A, Capece M, Chiozzi P, Falzoni S, Sanz JM, Sarti AC, et al. The P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein. *FASEB J.* (2015) 29:2450–61. doi: 10.1096/fj.14-268714
107. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res.* (2013) 54:2325–40. doi: 10.1194/jlr.R036012
108. Grebe A, Hoss F, Latz E. NLRP3 inflammasome and the IL-1 pathway in atherosclerosis. *Circ Res.* (2018) 122:1722–40. doi: 10.1161/CIRCRESAHA.118.311362
109. Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM, Nunez G. K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity.* (2013) 38:1142–53. doi: 10.1016/j.immuni.2013.05.016
110. Mabuchi T, Takekoshi T, Hwang ST. Epidermal CCR6$^+$ gammadelta T cells are major producers of IL-22 and IL-17 in a murine model of psoriasiform dermatitis. *J Immunol.* (2011) 187:5026–31. doi: 10.4049/jimmunol.1101817
111. Malhotra N, Yoon J, Leyva-Castillo JM, Galand C, Archer N, Miller LS, et al. IL-22 derived from gammadelta T cells restricts *Staphylococcus aureus* infection of mechanically injured skin. *J Allergy Clin Immunol.* (2016) 138:1098–107.e93. doi: 10.1016/j.jaci.2016.07.001
112. Steinbach S, Vordermeier HM, Jones GJ. CD4$^+$ and gammadelta T cells are the main producers of IL-22 and IL-17A in lymphocytes from *Mycobacterium bovis*-infected cattle. *Sci Rep.* (2016) 6:29990. doi: 10.1038/srep29990
113. Tyler CJ, McCarthy NE, Lindsay JO, Stagg AJ, Moser B, Eberl M. Antigen-presenting human gammadelta T cells promote intestinal CD4(+) T cell expression of IL-22 and mucosal release of calprotectin. *J Immunol.* (2017) 198:3417–25. doi: 10.4049/jimmunol.1700003
114. Xu X, Weiss ID, Zhang HH, Singh SP, Wynn TA, Wilson MS, et al. Conventional NK cells can produce IL-22 and promote host defense in *Klebsiella pneumoniae* pneumonia. *J Immunol.* (2014) 192:1778–86. doi: 10.4049/jimmunol.1300039
115. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med.* (2008) 14:282–9. doi: 10.1038/nm1720
116. Ota N, Wong K, Valdez PA, Zheng Y, Crellin NK, Diehl L, et al. IL-22 bridges the lymphotoxin pathway with the maintenance of colonic lymphoid structures during infection with *Citrobacter rodentium*. *Nat Immunol.* (2011) 12:941–8. doi: 10.1038/ni.2089
117. Kinnebrew MA, Ubeda C, Zenewicz LA, Smith N, Flavell RA, Pamer EG. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. *J Infect Dis.* (2010) 201:534–43. doi: 10.1086/650203
118. Abt MC, Buffie CG, Susac B, Becattini S, Carter RA, Leiner I, et al. TLR-7 activation enhances IL-22-mediated colonization resistance against vancomycin-resistant enterococcus. *Sci Transl Med.* (2016) 8:327ra325. doi: 10.1126/scitranslmed.aad6663
119. Sellau J, Alvarado CF, Hoenow S, Mackroth MS, Kleinschmidt D, Huber S, et al. IL-22 dampens the T cell response in experimental malaria. *Sci Rep.* (2016) 6:28058. doi: 10.1038/srep28058
120. Behnsen J, Jellbauer S, Wong CP, Edwards RA, George MD, Ouyang W, et al. The cytokine IL-22 promotes pathogen colonization by suppressing related commensal bacteria. *Immunity.* (2014) 40:262–73. doi: 10.1016/j.immuni.2014.01.003
121. Stelter C, Kappeli R, Konig C, Krah A, Hardt WD, Stecher B, et al. Salmonella-induced mucosal lectin RegIIIbeta kills competing gut microbiota. *PLoS ONE.* (2011) 6:e20749. doi: 10.1371/journal.pone.0020749
122. Hayden JA, Brophy MB, Cunden LS, Nolan EM. High-affinity manganese coordination by human calprotectin is calcium-dependent and requires the histidine-rich site formed at the dimer interface. *J Am Chem Soc.* (2013) 135:775–87. doi: 10.1021/ja3096416
123. Feinen B, Russell MW. Contrasting roles of IL-22 and IL-17 in murine genital tract infection by *Neisseria gonorrhoeae*. *Front Immunol.* (2012) 3:11. doi: 10.3389/fimmu.2012.00011
124. Quintana FJ. The aryl hydrocarbon receptor: a molecular pathway for the environmental control of the immune response. *Immunology.* (2013) 138:183–9. doi: 10.1111/imm.12046
125. Murray IA, Perdew GH. Ligand activation of the Ah receptor contributes to gastrointestinal homeostasis. *Curr Opin Toxicol.* (2017) 2:15–23. doi: 10.1016/j.cotox.2017.01.003
126. Gao J, Xu K, Liu H, Liu G, Bai M, Peng C, et al. Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Front Cell Infect Microbiol.* (2018) 8:13. doi: 10.3389/fcimb.2018.00013
127. Million M, Tomas J, Wagner C, Lelouard H, Raoult D, Gorvel J-P. New insights in gut microbiota and mucosal immunity of the small intestine. *Hum Microbiome J.* (2018) 7–8:23–32. doi: 10.1016/j.humic.2018.01.004
128. Yitbarek A, Taha-Abdelaziz K, Hodgins DC, Read L, Nagy E, Weese JS, et al. Gut microbiota-mediated protection against influenza virus subtype H9N2 in chickens is associated with modulation of the innate responses. *Sci Rep.* (2018) 8:13189. doi: 10.1038/s41598-018-31613-0
129. Natividad JM, Agus A, Planchais J, Lamas B, Jarry AC, Martin R, et al. Impaired aryl hydrocarbon receptor ligand production by the gut microbiota is a key factor in metabolic syndrome. *Cell Metab.* (2018) 28:737–49.e4. doi: 10.1016/j.cmet.2018.07.001
130. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity.* (2013) 39:372–85. doi: 10.1016/j.immuni.2013.08.003
131. Valeri M, Raffatellu M. Cytokines IL-17 and IL-22 in the host response to infection. *Pathog Dis.* (2016) 74:ftw111. doi: 10.1093/femspd/ftw111
132. Krishnan S, Ding Y, Saedi N, Choi M, Sridharan GV, Sherr DH, et al. Gut microbiota-derived tryptophan metabolites modulate inflammatory response in hepatocytes and macrophages. *Cell Rep.* (2018) 23:1099–111. doi: 10.1016/j.celrep.2018.03.109
133. Ismail AS, Severson KM, Vaishnava S, Behrendt CL, Yu X, Benjamin JL, et al. Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. *Proc Natl Acad Sci USA.* (2011) 108:8743–8. doi: 10.1073/pnas.1019574108
134. Witter AR, Okunnu BM, Berg RE. The essential role of neutrophils during infection with the intracellular bacterial pathogen listeria monocytogenes. *J Immunol.* (2016) 197:1557–65. doi: 10.4049/jimmunol.1600599
135. Nielsen MM, Witherden DA, Havran WL. gammadelta T cells in homeostasis and host defence of epithelial barrier tissues. *Nat Rev Immunol.* (2017) 17:733–45. doi: 10.1038/nri.2017.101
136. Krishnan S, Prise IE, Wemyss K, Schenck LP, Bridgeman HM, McClure FA, et al. Amphiregulin-producing gammadelta T cells are vital for safeguarding oral barrier immune homeostasis. *Proc Natl Acad Sci USA.* (2018) 115:10738–43. doi: 10.1073/pnas.1802320115
137. Heiss CN, Olofsson LE. The role of the gut microbiota in development, function and disorders of the central nervous system and the enteric nervous system. *J Neuroendocrinol.* (2019) e12684. doi: 10.1111/jne.12684

138. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell.* (2005) 122:107–18. doi: 10.1016/j.cell.2005.05.007
139. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol.* (2008) 180:5771–7. doi: 10.4049/jimmunol.180.9.5771
140. Cyktor JC, Turner J. Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens. *Infect Immun.* (2011) 79:2964–73. doi: 10.1128/IAI.00047-11
141. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol.* (2012) 32:23–63. doi: 10.1615/CritRevImmunol.v32.i1.30
142. Ueda Y, Kayama H, Jeon SG, Kusu T, Isaka Y, Rakugi H, et al. Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. *Int Immunol.* (2010) 22:953–62. doi: 10.1093/intimm/dxq449
143. Rivollier A, He J, Kole A, Valatas V, Kelsall BL. Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. *J Exp Med.* (2012) 209:139–55. doi: 10.1084/jem.20101387
144. Hayashi A, Sato T, Kamada N, Mikami Y, Matsuoka K, Hisamatsu T, et al. A single strain of *Clostridium butyricum* induces intestinal IL-10-producing macrophages to suppress acute experimental colitis in mice. *Cell Host Microbe.* (2013) 13:711–22. doi: 10.1016/j.chom.2013.05.013
145. Ochi T, Feng Y, Kitamoto S, Nagao-Kitamoto H, Kuffa P, Atarashi K, et al. Diet-dependent, microbiota-independent regulation of IL-10-producing lamina propria macrophages in the small intestine. *Sci Rep.* (2016) 6:27634. doi: 10.1038/srep27634
146. Quiros M, Nishio H, Neumann PA, Siuda D, Brazil JC, Azcutia V, et al. Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. *J Clin Invest.* (2017) 127:3510–20. doi: 10.1172/JCI90229
147. Sassone-Corsi M, Raffatellu M. No vacancy: how beneficial microbes cooperate with immunity to provide colonization resistance to pathogens. *J Immunol.* (2015) 194:4081–7. doi: 10.4049/jimmunol.1403169
148. Crouzet L, Derrien M, Cherbuy C, Plancade S, Foulon M, Chalin B, et al. *Lactobacillus paracasei* CNCM I-3689 reduces vancomycin-resistant *Enterococcus persistence* and promotes *Bacteroidetes resilience* in the gut following antibiotic challenge. *Sci Rep.* (2018) 8:5098. doi: 10.1038/s41598-018-23437-9

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Microbiota Inhibit Epithelial Pathogen Adherence by Epigenetically Regulating C-Type Lectin Expression

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Numerous bacterial pathogens infect the mammalian host by initially associating with epithelial cells that line the intestinal lumen. Recent work has revealed that commensal bacteria that reside in the intestine promote defense against pathogenic infection, however whether the microbiota direct host pathways that alter pathogen adherence is not well-understood. Here, by comparing germ-free mice, we identify that the microbiota decrease bacterial pathogen adherence and dampen epithelial expression of the cell surface glycoprotein C-type lectin 2e (Clec2e). Functional studies revealed that overexpression of this lectin promotes adherence of intestinal bacterial pathogens to mammalian cells. Interestingly, microbiota-sensitive downregulation of Clec2e corresponds with decreased histone acetylation of the Clec2e gene in intestinal epithelial cells. Histone deacetylation and transcriptional regulation of Clec2e depends on expression and recruitment of the histone deacetylase HDAC3. Thus, commensal bacteria epigenetically instruct epithelial cells to decrease expression of a C-type lectin that promotes pathogen adherence, revealing a novel mechanism for how the microbiota promote innate defense against infection.

Keywords: microbiota, HDAC, CLEC, intestine epithelial cells, citrobacter, epigenetic

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INTRODUCTION

Infections of the gastrointestinal tract are a major cause of morbidity and mortality worldwide. Specifically, enteric infections caused by bacterial pathogens account for well over 200 million individual cases of enteritis resulting in an estimated 5 million deaths annually (1, 2). In addition to local intestinal infections, the gastrointestinal tract is the initial site of adhesion and entry for several pathogens that disseminate to cause systemic disease (3). Thus, adherence and invasion are critical steps in the pathogenesis of both enteric and systemic bacterial infections. In order to establish disease, pathogens can interact with host cells by expressing adhesin molecules which recognize various components such as extracellular matrix proteins, integral membrane adhesion receptors, and cell membrane associated glycoproteins (4). These interactions between bacterial pathogens and host cells are not only critical for initiating infection, but also direct tissue tropism, species specificity, and host susceptibility to infection (4–7). Therefore, understanding how pathogenic adherence is mediated is critical for directing effective approaches that prevent and treat enteric infections.

In addition to pathogenic bacteria, the mammalian gastrointestinal tract harbors trillions of innocuous commensal bacteria. These commensal microbes, collectively termed the microbiota, are required for healthy intestinal development and immune cell activation (8). Importantly, the presence of the microbiota has also been consistently shown to be essential for host defense against pathogenic infections (8, 9). While several mechanisms have been proposed to account for microbiota-dependent protection against infection, many pathways indicate that commensal bacteria can potentiate host-cell intrinsic defenses (10–12). Intestinal epithelial cells (IECs) reside at the direct interface between the microbiota and underlying host immune cells and are in constant contact with both beneficial as well as invading bacteria. Thus, IECs are a key cell type to which enteric pathogens often directly associate with in order to infect and invade the host. In addition to mediating binding and sensing of microbial components, these critically located cells can actively respond to pathogenic challenges by secreting antimicrobial peptides, mucins, and cytokines that prime and regulate innate and adaptive immune cell compartments (13–16). However, it is not clear whether the microbiota restrict enteric infection by regulating pathogen binding to the intestinal epithelium.

In mammalian cells, DNA is packaged around histone proteins that are condensed into a higher order structure called chromatin. In general, chromatin structure itself restricts access of transcriptional machinery to the genome thereby repressing gene expression. However, covalent modifications of the amino-terminal tails of histones, specifically, acetylation, methylation, phosphorylation, SUMOylation, and ubiquitination are associated with conformational changes in the chromatin landscape. For example, histone acetylation is known to generate an open chromatin structure that contributes to active transcription (17–19). These modifications are mediated by epigenetic modifying enzymes such as histone acetyltransferases and histone deacetylases (HDACs). The balance and pattern of these modifications on specific histone tails regulate chromatin reorganization and direct transcriptional machinery. Thus, epigenetic modifications enable environmental signals to trigger transcriptional changes without altering underlying DNA sequence (20–22).

In this study, we aimed to test whether the microbiota affect IEC-intrinsic pathways that alter the ability of pathogens to adhere to the IECs. *Citrobacter rodentium*, a murine enteric pathogen with a similar pathogenesis to enteropathogenic *E. coli* in humans, infects the host by initially adhering to IECs. By employing germ-free (GF) mice, we identified that the microbiota reduce pathogen colonization with *C. rodentium* during infection and instruct decreased IEC interactions with the pathogen. Global gene expression analyses revealed that the microbiota highly suppressed IEC expression of the cell-surface C-type lectin 2e (Clec2e). Interestingly, functional studies showed that overexpression of Clec2e enhanced pathogen bacterial binding to the mammalian cell membrane. Furthermore, microbiota-dependent transcriptional suppression of Clec2e in IECs correlated with decreased histone acetylation and recruitment of the histone deacetylase, HDAC3. Collectively, these data demonstrate a novel mechanism by which commensal bacteria in the intestine epigenetically regulate expression of a pathogen-binding glycoprotein to promote host defense against infection.

MATERIALS AND METHODS

Mice and *in vivo* Infections

Conventionally-housed C57Bl/6J mice were purchased from Jackson Laboratories and maintained in our specific-pathogen free colony at CCHMC. Germ-free (GF) mice were maintained in plastic isolators in the CCHMC Gnotobiotic Mouse Facility, fed autoclaved feed and water, and monitored to ensure absence of microbes. HDAC3^{FF} mice (23) were crossed to C57Bl/6J mice expressing Cre recombinase under control of the IEC-specific villin promoter (24) to generate HDAC3$^{\Delta IEC}$ mice (25). Mice were housed up to 4 per cage in a ventilated cage system in a 12 h light/dark cycle, with free access to water and food. For *C. rodentium* infection, age- and gender- matched mice were orally inoculated with 10^9 colony forming units (CFUs) of *C. rodentium* (26, 27). To enumerate intestinal bacterial burdens, stool was collected in PBS and homogenized in a TissueLyser II at 30 Hz for 3 min. Homogenates were serially diluted and plated on MacConkey agar. CFUs were counted and normalized to stool weight after 18 h. All experiments were performed according to the animal guidelines upon approval of the Institutional Animal Care and Use Committee at CCHMC.

IEC Harvest, RNA Analyses, Western Blotting

IECs were harvested from mouse intestine as described previously (25, 27, 28). IECs from the small intestine were harvested from the most distal 12 cm section. RNA was isolated from cells using the RNeasy Kit (Qiagen) then subjected to reverse transcription with Verso reverse transcriptase (Thermo Fisher). Directional polyA RNA-seq for IECs from the small intestine was performed by the Sequencing Core at the University of Cincinnati (28). Sequence reads were aligned by using Illumina sequence analysis pipeline by the Laboratory for Statistical Genomics and Systems Biology at the University of Cincinnati. Real-time PCR was performed using SYBR (Applied Biosystems) and analyzed with a threshold in the linear range of amplification using primer sequences as follows: Clec2eF: 5′-AGCAAGG TTCACAGCTCTCC-3′; Clec2eR: 5′-GCTGCTATGGAGTGA TCATGG-3′; RegIIIγF: 5′-TTCCTGTCCTCCATGATCAAA-3′; RegIIIγR: 5′-CATCCACCTCTGTTGGGTTC-3′; HPRTF: 5′-GATTAGCGATGAACCAGGT-3′; HPRTR: 5′-CCTCCCATC TCCTTCATGACA-3′. Expression analysis in IECs from large intestine of HDAC3^{FF} and HDAC3$^{\Delta IEC}$ mice by microarray was described previously (25). For western blot analyses, total cell lysates were probed with anti-histone H3 (Santa Cruz) and anti-DDK (FLAG) (Origene) and imaged using an Odyssey Fc imager (LICOR). Global expression data has been deposited in NCBI's Gene Expression Omnibus (GEO) and is accessible through accession number GSE128362.

ChIP-Sequencing

ChIP was performed as described previously with few modifications (29). Briefly, cells were fixed in 1% PFA for 10 min and quenched with glycine. Total cell extracts were sonicated using a Covaris S220 Focused-ultrasonicator and nuclear extracts were immunoprecipitated with rabbit anti-H3K9Ac (Millipore, 06-942) or rabbit anti-HDAC3 (Abcam, ab7030) using a SX-8G IP-STAR robot. Sequencing was performed using Illumina HiSeq 2500, mapped to mus musculus genome mm10 with Bowtie and peaks were identified with MACS (30, 31) and visualized in Biowardrobe (32). ChIP-qPCR was performed using SYBR (Applied Biosystems) and analyzed as fold difference normalized to an unaffected control gene. Reactions were run on a real-time PCR system (QuantStudio3; Applied Biosystems) with custom made primer pairs: Clec2e-ChIPF: 5′-ACACAAGATGCAGCGGAGAT-3′; Clec2e-ChIPR: 5′-GTGAAGGGGTTTTCACTAGGGG-3′; Insl-ChIPF: 5′-CAG AGACCATCAGCAAGCAG-3′; Insl-ChIPR: 5′-TTCTCCCTA AAGTCGCTGGA-3′; Albumin-ChIPF: 5′-AGAGCGATCTTT CTGCACACA-3′; Albumin-ChIPR: 5′-AGGAGAAAGGTT ACCCACTTCATTT-3′. ChIP-seq data is accessible through GEO series accession numbers GSE50453 and GSE128369.

Cell Culture and Immunofluorescence

HEK293T cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C and 5% CO_2. Cells were transiently transfected with pCMV6-Clec2e-myc-DDK (FLAG) vector (Origene, MR202134) using Lipofectamine 3000 (Thermo Fisher). Transfected cells were seeded onto Retronectin (Takara Bio) coated chamber slides (Ibidi) and infected with 10^6 CFUs of GFP-expressing *C. rodentium* for 6 h in antibiotic-free media (26, 27). Cells were washed in PBS 3 times and fixed in 4% paraformaldehyde for 20 min. Fixed cells were blocked with 2% BSA for 1 h at room temperature and stained in 0.5% BSA with 488-anti-GFP (Thermo Fisher, 1:300), Phalloidin (Invitrogen, 1:200) and DAPI (Invitrogen, 1:1,000) for 1 h at room temperature. Stained cells were visualized using Nikon A1R LUN-V inverted confocal microscope.

Intestinal Organoids

Murine organoids were generated from colonic crypts isolated from germ-free and conventionally-housed mice as previously described (33). Briefly, dissected colons were opened longitudinally, scrapped to remove intestinal contents and outer cells, washed repeatedly in ice-cold PBS, and cut into 1-cm pieces. Colonic pieces were incubated in chelation buffer (2 mM EDTA in PBS) for 30 min at 4°C with rotation. The tissue was transferred into a new tube containing Shaking Buffer (PBS, 43.3 mM sucrose, 54.9 mM sorbitol) and gently shaken by hand for 2–4 min. Colonic crypts were resuspended and plated in Matrigel (Corning) with organoid culture media (60% Advanced DMEM/F12 media supplemented with 10 mM HEPES, 2 mM L-glutamate, 40% L-WRN conditioned media, 1x N2 supplement, 1x B27 supplement, 50 ng/mL murine EGF, and 10 μM Y-27632 ROCK inhibitor) overlaid. Culture media was changed every 3–4 days. Organoid cultures were infected with GFP-*C. rodentium* at a concentration of 10^6 CFUs for 24 h. After incubation, organoids were washed 3 times in ice-cold PBS, dislodged from plate and Matrigel, and fixed in 1% PFA for 1 h at 4°C. GF organoids were stimulated with 10 ng/mL of *E. coli* LPS for 24 h.

Flow Cytometry

Cells were stained for flow cytometry using the following fluorescence-conjugated antibodies diluted in FACS Buffer (2% FBS, 0.01% Sodium Azide, PBS): PE anti-CD326 (EpCAM) (Clone: G8.8, eBioscience), BUV395 anti-CD45.2 (Clone: 104, BD Biosciences), 488 anti-GFP (Clone; FM264G, BioLegend). Dead cells were excluded with the Fixable Violet Dead Cell Stain Kit (Invitrogen). Samples were acquired on the BD LSRFortessa and analyzed with FlowJo Software (Treestar). The geometric mean fluorescence intensity (MFI) for GFP-*C. rodentium* expression was assessed and the background MFI determined in uninfected controls was subtracted from infected samples.

Bacterial Adhesion Assay

Adhesion of pathogenic bacteria to mammalian cells was determined as previously described (34). Briefly, HEK293T cells were seeded at 70–90% confluency and incubated with GFP-*C. rodentium* or wild-type *Salmonella enterica* serovar Typhimurium at a multiplicity of infection (MOI) of 5:1 (bacteria:cells) for 6 h in antibiotic-free media. Cells were washed 3 times with PBS and adherent bacteria were detached using a 1% triton-X 100 lysis buffer and plated onto MacConkey agar in 10-fold serial dilutions. Colony forming units (CFUs) were counted after 16 h.

Statistical Analyses

Results are expressed as mean ± SEM. Statistical significance was determined with the Student's *t*-test, with all data meeting the assumptions of the statistical test used. Results were considered significant at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Statistical significance was calculated using Prism version 7.0 (GraphPad Software).

RESULTS

Microbiota Decrease Pathogen Adherence to Intestinal Epithelial Cells

Citrobacter rodentium (*C. rodentium*) is a murine bacterial pathogen with similar pathogenesis to enteropathogenic *E. coli* in humans. Germ-free (GF) mice infected with *C. rodentium* exhibited significantly higher pathogen burdens compared to conventionally-housed (CNV) mice (**Figure 1A**), indicating that the microbiota enhance defense against pathogenic colonization. Interestingly, microbiota-sensitive protection against infection was detected as early as day 4 post-infection, suggesting the presence of the microbiota affect the initial establishment of *C. rodentium* colonization. Intestinal epithelial cells (IECs) produce antimicrobial peptides and consistent with previous studies (35), the microbiota induced IEC expression of the antimicrobial peptide RegIIIγ that targets bacterial pathogens

(**Figure 1B**) (36). In order to investigate how the microbiota induce epithelial-intrinsic defense, intestinal epithelial organoids that are devoid of immune cells were compared. Interestingly, intestinal epithelial organoids generated from CNV-housed mice expressed significantly reduced levels of RegIIIγ (**Figure 1B**). However, despite this impairment in RegIIIγ expression, organoids from CNV mice exhibited lower adherent GFP-expressing *C. rodentium* compared to GF organoids as measured by flow cytometry (**Figure 1C**). These data suggest that other mechanisms, aside from RegIIIγ, contribute to microbiota-sensitive IEC-intrinsic resistance against pathogenic bacterial adherence.

Microbiota Exposure Downregulates C-Type Lectin 2e Expression in Intestinal Epithelial Cells

In order to identify potential mediators of pathogen adherence that are regulated by the microbiota, we compared genes expressed in IECs harvested from the intestine of GF and CNV mice by RNA-sequencing. These analyses identified C-type lectin 2 member e (Clec2e; Clr-a) as one of the most significantly downregulated genes in IECs in response to microbial exposure (**Figure 2A**). Clec2e expression was confirmed to be decreased by quantitative PCR (qPCR) in IECs from independent cohorts of GF and CNV mice in both the small intestine (**Figure 2B**) and large intestine (**Figure 2C**). To investigate whether microbiota-suppressed Clec2e expression was maintained in the absence of immune cells or persistent microbial stimulation, intestinal organoid cultures were generated from colonic crypts isolated from GF and CNV mice (**Figure 2D**). Consistent with IECs *in vivo*, Clec2e expression was repressed in organoids derived from CNV mice (**Figure 2E**), suggesting prior exposure to the microbiota led to sustained downregulation of Clec2e. To determine how the microbiota may suppress Clec2e expression, intestinal organoids derived from GF mice were incubated with LPS (**Figure 2F**). LPS reduced Clec2e expression in IECs, although less than observed in CNV organoids, suggesting that microbial-derived components may collectively regulate epithelial Clec2e expression.

Expression of Clec2e Increases Cellular Adherence of Enteric Bacterial Pathogens

Clec2e is a homodimeric cell surface glycoprotein expressed in the intestinal epithelium that shares homology with other C-type lectins (37, 38). However, unlike other CLEC2 family members, Clec2e does not interact with Nkrp1 receptors (37, 39). C-type lectin receptor family members contain extracellular carbohydrate binding domains that associate with common pathogen-associated molecular patterns including mannose, fucose, and β-glycan (40, 41), provoking the hypothesis that Clec2e may facilitate bacterial adhesion to host cells. To test whether Clec2e plays a role in bacterial adhesion to mammalian cells, Clec2e-FLAG was overexpressed in HEK293T cells followed by incubation with either *Salmonella* or *C. rodentium*, enteric bacterial pathogens that directly bind to IECs. Expression of transfected Clec2e was confirmed by Western analyses (**Figure 3A**). Interestingly, Clec2e-expressing cells exhibited significantly greater adherence of *Salmonella* (**Figure 3B**) and *C. rodentium* (**Figure 3C**) compared to negative control cells. Bacterial adherence of GFP-expressing *C. rodentium* to Clec2e-expressing cells was confirmed at the cellular level by immunofluorescence (**Figure 3D**) and flow cytometry (**Figure 3E**). Together, these data indicate that Clec2e expression promotes adherence of bacterial pathogens to mammalian cells.

Microbiota Induce Loss of Histone Acetylation and HDAC3 Recruitment Within Regulatory Regions of Clec2e

Environmental factors can regulate mammalian gene expression through epigenetic modifications of the chromatin, such as DNA methylation and histone acetylation. Consistent with this, recent studies have revealed that epigenetic pathways may be essential in mediating host-microbe dynamics (17, 42, 43). Therefore, to determine whether the microbiota epigenetically modify chromatin at the Clec2e gene, histone acetylation was compared in primary IECs harvested from GF and CNV mice. For these analyses, chromatin immunoprecipitation (ChIP)-sequencing (seq) was performed for the histone mark H3K9Ac, which is associated with permissive and actively transcribed chromatin (17). Remarkably, ChIP-seq analyses revealed that H3K9Ac levels were significantly decreased at multiple sites within the Clec2e locus in IECs isolated from CNV mice compared to GF controls (**Figure 4A**). This loss of histone acetylation in regulatory regions of Clec2e due to the microbiota was confirmed by ChIP-qPCR for H3K9Ac (**Figure 4B**). Previous studies have demonstrated that histone acetylation in IECs can be regulated by epigenetic-modifying enzymes called histone deacetylase (HDACs) (44, 45). The class I histone deacetylase 3 (HDAC3) deacetylates histone H3K9Ac and mediates microbiota-dependent regulation of epithelial gene expression (25, 27). Thus, to determine whether HDAC3 epigenetically regulates Clec2e, HDAC3 recruitment was first examined by ChIP. HDAC3 was enriched at the site of differential H3K9Ac in Clec2e (**Figure 4B**) relative to a negative non-HDAC3 target (**Figure 4C**), supporting that Clec2e is a direct target of HDAC3. Interestingly, IECs from CNV mice exhibited significantly higher HDAC3 recruitment to Clec2e compared to IECs from GF mice (**Figure 4D**). Collectively, these data indicate that the microbiota direct epigenetic regulation of Clec2e through HDAC3.

HDAC3 Regulates Epithelial Clec2e Expression and Pathogen Adherence

The microbiota-dependent increase in HDAC3 recruitment to Clec2e suggests that loss of H3K9Ac and decreased expression of Clec2e in response to the microbiota could be mediated by HDAC3. Thus, to directly test whether IEC-intrinsic HDAC3 regulates histone acetylation within the Clec2e gene, ChIP-seq for H3K9Ac was performed in IECs harvested from mice that lack HDAC3 expression specifically in IECs ($HDAC3^{\Delta IEC}$) compared to floxed $HDAC3^{FF}$ control mice (25). Consistent with histone deacetylation by HDAC3, IECs harvested from the large intestine of mice lacking IEC-HDAC3 ($HDAC3^{\Delta IEC}$) exhibited

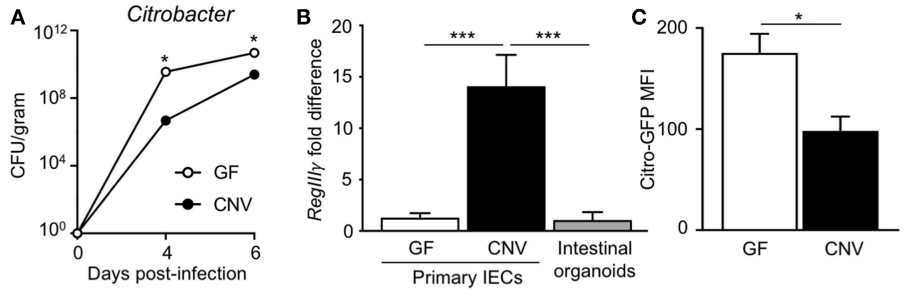

FIGURE 1 | Microbiota exposure decreases pathogen adherence to intestinal epithelial cells. **(A)** *C. rodentium* colony forming units (CFUs) in stool of infected germ-free (GF) and conventionally-housed (CNV) mice during early course of infection (day 4–6). Data are representative of at least 3 independent experiments with 3–4 mice per group. **(B)** Relative RegIIIγ mRNA expression in IECs isolated from the large intestine of GF and CNV mice compared to intestinal organoid cultures. **(C)** MFI of GFP-*C. rodentium* infected intestinal organoids derived from GF and CNV mice. Data represent two independent experiments with 3–4 mice per group. Results are mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

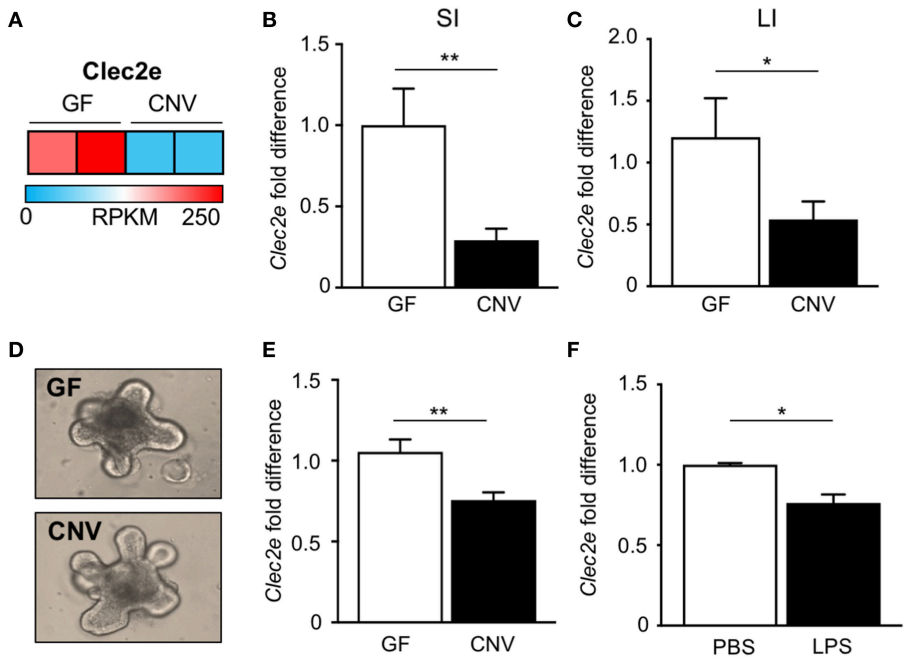

FIGURE 2 | Microbiota downregulate intestinal epithelial expression of C-type lectin Clec2e. **(A)** Relative Clec2e expression levels in IECs harvested from small intestine of GF and CNV mice, represented as reads per kilobase per million mapped reads (RPKM). **(B,C)** Real-time quantitative PCR analysis of Clec2e in IECs from GF and CNV mice isolated from **(B)** small intestine (SI) or **(C)** large intestine (LI). **(D)** Intestinal organoids derived from GF and CNV mice. **(E)** Clec2e mRNA expression in GF and CNV organoids. **(F)** Clec2e mRNA expression in GF intestinal organoids in the absence or presence of LPS. Data represent two independent experiments with 3–4 mice per group. Results are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

significantly higher levels of H3K9Ac in Clec2e compared to IECs from HDAC3^{FF} mice (**Figure 5A**). Increased H3K9Ac enrichment within the microbiota-sensitive regulatory region in the Clec2e gene was also identified in IECs from the small intestine of HDAC3$^{\Delta IEC}$ mice (**Figure 5B**), indicating that Clec2e histone acetylation is regulated by epithelial HDAC3. HDAC3 recruitment is associated with transcriptional repression of bound genes. Thus, to test whether elevated H3K9Ac with HDAC3 depletion corresponds with altered expression, Clec2e mRNA expression was measured in IECs from HDAC3^{FF} and HDAC3$^{\Delta IEC}$ mice. Consistent with the role of HDAC3 as a transcriptional repressor of direct targets, these analyses revealed significantly increased Clec2e expression in IECs harvested from the small and large intestine of HDAC3$^{\Delta IEC}$ mice compared to IECs from HDAC3^{FF} controls (**Figure 5C**), indicating that Clec2e expression in HDAC3-deficient IECs results from impaired HDAC3-mediated deacetylation within the Clec2e gene. Collectively, these studies demonstrate that epigenetic and transcriptional regulation of Clec2e broadly depends on epithelial HDAC3 expression in the intestine.

To next test whether HDAC3-dependent regulation of IECs affects pathogen adhesion, HDAC3^{FF} and HDAC3$^{\Delta IEC}$ mice

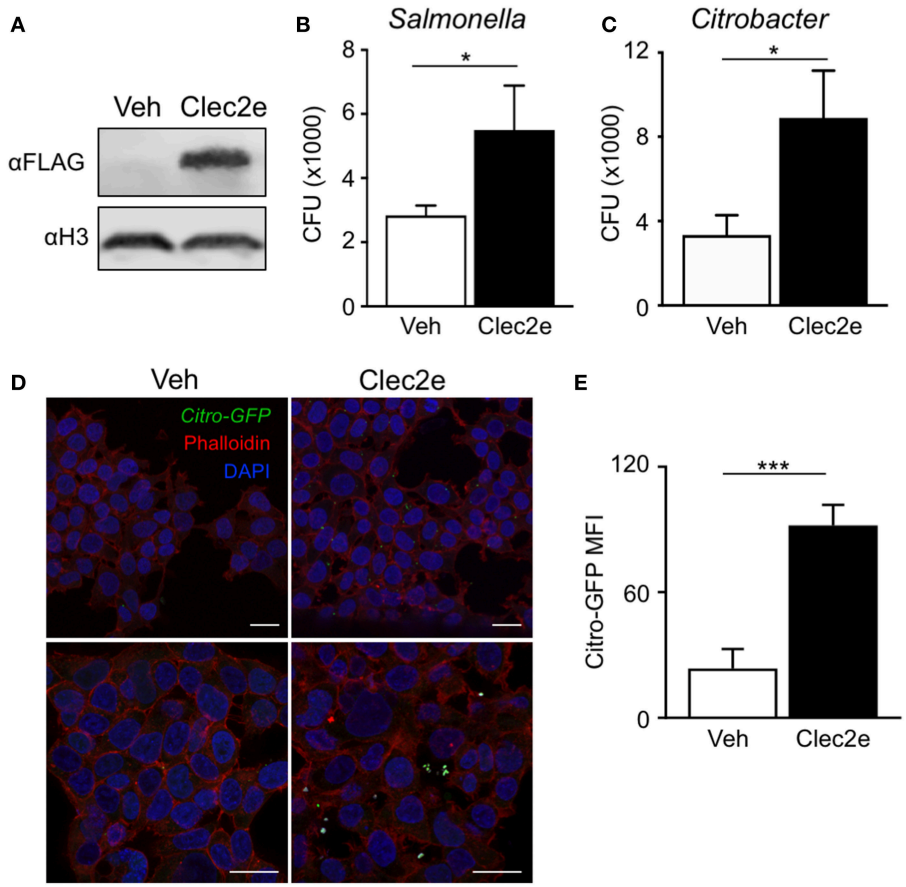

FIGURE 3 | Expression of Clec2e enables increased adherence of bacterial pathogens. Clec2e overexpressed in HEK293T cells using a FLAG-tagged Clec2e expression vector. **(A)** Western blot analysis of Clec2e-FLAG expression in vehicle or Clec2e-FLAG transfected HEK293T cell lysates. **(B,C)** *In vitro* bacterial adhesion assay of infected HEK293T cells, expressed as adherent CFUs 6 h post infection. **(D)** Immunofluorescent staining of HEK293T cells infected with GFP-expressing *C. rodentium* (green). Mammalian cell membrane and nuclei are stained with Phalloidin (red) and DAPI (blue), respectively. Scale bars, 25 μm. **(E)** MFI of GFP-*C. rodentium* bound to vehicle-treated or Clec2e-transfected HEK293T cells. Data represent 2 independent experiments with $n = 3$ per group. Results are mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

were infected with *C. rodentium*. Interestingly, *C. rodentium*-infected HDAC3$^{\Delta IEC}$ mice exhibited higher pathogen burden (**Figure 5D**) and increased GFP-*C. rodentium* adherence to IECs (**Figure 5E**) relative to infected HDAC3^{FF} control mice, confirming increased pathogen adhesion in HDAC3$^{\Delta IEC}$ mice. Further, to test this in the absence of immune cells, intestinal organoids were generated from the colon of control HDAC^{FF} mice and mice lacking HDAC3 in IECs. Consistent with the *in vivo* findings, HDAC3$^{\Delta IEC}$ organoids exhibited elevated GFP-*C. rodentium* binding compared to cells from floxed controls (**Figure 5F**). Taken together, these data indicate that regulation of Clec2e expression in IECs by HDAC3-mediated histone deacetylation can promote decreased bacterial pathogen adherence to the intestinal epithelium.

DISCUSSION

The intestinal epithelium not only maintains intestinal homeostasis to innocuous commensals, but it also defends against invading pathogens (13, 46). Our data indicate that the microbiota can promote epithelial defense by epigenetically suppressing Clec2e-mediated pathogen adherence. Consistent with previously published data (10, 27), we show that GF mice are more susceptible to enteric infection relative to microbiota-replete mice. Previous studies have focused on investigating how the microbiota impact immune cell activation and antibacterial immunity (11, 47, 48), however we observed very early susceptibility to *C. rodentium* infection in GF mice suggesting an important role for innate responses. By employing intestinal organoid cultures, we determined that the microbiota directly impact IEC-intrinsic defense and identified that Clec2e downregulation by the microbiota can reduce pathogen colonization. The microbiota influence several aspects of IEC biology and microbiota-sensitive alterations in IEC composition can also impact host resistance to enteric infection. However, Clec2e is expressed throughout the intestinal epithelium including progenitor and differentiated epithelial cells (37), suggesting

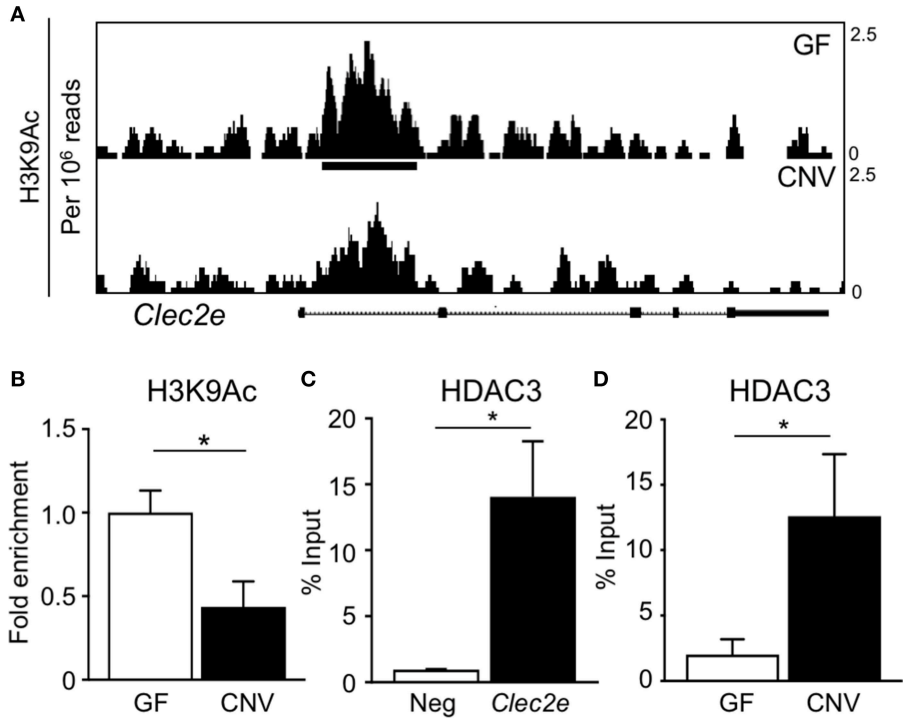

FIGURE 4 | Microbiota induce histone deacetylation and HDAC3 enrichment at Clec2e gene. **(A)** ChIP-seq for H3K9Ac in primary IECs isolated from the small intestine of GF and CNV mice. **(B)** ChIP-qPCR for H3K9Ac in Clec2e from IECs. **(C)** ChIP-qPCR for HDAC3 in Clec2e as percent of input, relative to a negative control gene (Insulin 1). **(D)** HDAC3 ChIP-qPCR in Clec2e from GF and CNV IECs. Data represent two independent experiments with 3-4 mice per group. Results are mean ± SEM. $^*p < 0.05$.

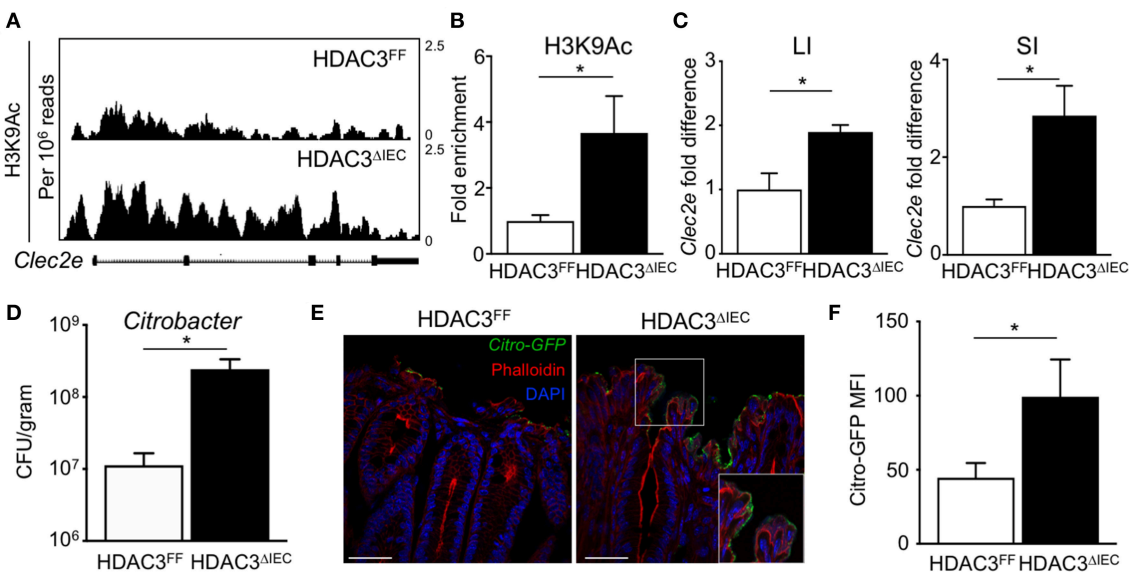

FIGURE 5 | HDAC3 regulates epithelial Clec2e expression and pathogen adherence. **(A)** ChIP-seq for H3K9Ac in primary IECs isolated from the large intestine of HDAC3^{FF} and HDAC3$^{\Delta IEC}$ mice. **(B)** ChIP-qPCR for H3K9Ac in Clec2e in IECs from the small intestine. **(C)** Clec2e expression in IECs from large and small intestine of HDAC3^{FF} and HDAC3$^{\Delta IEC}$ mice. **(D)** *C. rodentium* CFUs in stool at day 6 post infection. **(E)** Fluorescence microscopy of colon from HDAC3^{FF} and HDAC3$^{\Delta IEC}$ mice infected with GFP-*C. rodentium*. (Green: GFP-*C. rodentium*, Red: Phalloidin, Blue: DAPI). Scale bars, 25 μm. **(F)** MFI of GFP-*C. rodentium* infected intestinal organoids derived from HDAC3^{FF} and HDAC3$^{\Delta IEC}$ mice. Data are representative of at least 2 independent experiments with 3–4 mice per group. Results are mean ± SEM. $^*p < 0.05$.

that differences in epithelial composition induced by the microbiota are unlikely to be a main mechanism regulating Clec2e expression.

Consistent with our data, studies using GF mice and mouse models that are deficient for pattern recognition receptor mediators have revealed that mucins and antimicrobial peptides require microbial stimulation for expression (35, 49–52). These proteins work in concert to prevent intestinal infection by restricting bacterial adhesion and invasion, limiting microbial growth and colonization, and directly killing bacteria. For example, mucins function by forming a protective barrier that limits access of microbes to underlying IECs and can bind to several enteric pathogens including *C. rodentium* to prevent adhesion (26, 53). In addition, expression of cathelicidin-related antimicrobial peptide by IECs plays an important and non-redundant role in preventing *C. rodentium* adhesion and colonization, especially in early stages of infection (54). Another member of the C-type lectin family with structural similarity to Clec2e, RegIIIγ, binds intestinal bacteria via interactions with peptidoglycan carbohydrates and directly lyses bacteria (35). These studies, combined with our Clec2e data, demonstrate that the microbiota direct multiple IEC-intrinsic host defenses that alter bacterial access to IECs and limit adhesion and colonization.

Similar to enteropathogenic *E. coli* and enterohemorrhagic *E. coli*, *C. rodentium* employs a type 3 secretion system and other virulence strategies to attach to the apical plasma membrane of IECs (55). *Salmonella* is also equipped with a type 3 secretion system and several fimbriae proteins that enable adherence and invasion to colonic IECs (3, 4, 56). Genetic deletion of type 3 secretion systems or fimbriae molecules drastically reduces bacterial colonization, confirming the necessity of these molecules for pathogenesis (26, 55, 56). Fimbriae and other filopodia-like extensions that enable bacterial adhesion often interact with host plasma membrane associated proteins. *Salmonella* fimbriae bind to a specific glycosylated moiety that is abundantly expressed in the intestinal epithelium (56). Interestingly, Clec2e is a heavily glycosylated protein whose expression is restricted to the intestinal epithelium and is downregulated with LPS or Poly:(IC) stimulation (37), suggesting it may play a functional role in regulating intestinal host defense. Future investigation will require GF and CNV Clec2e$^{\Delta IEC}$ knockout models in combination with mono-association studies to dissect the contribution of specific commensal microbes or microbial components that influence *in vivo* regulation of pathogen control by Clec2e.

Through global RNA-sequencing analysis we identified a drastic reduction in the expression of the C-type lectin protein, Clec2e, in IECs isolated from CNV mice compared to GF controls. Clec2e (Clr-a) is an orphan C-type lectin molecule that closely resembles the natural killer (NK) gene complex receptor, Clec2h (Clr-f). However, unlike Clec2h, Clec2e does not bind any known NK cell receptors (37, 39, 57). In addition to being signaling partners for NK cell receptors, C-type lectin molecules play a crucial role in recognition of conserved pathogen-associated molecular patterns. Specifically, C-type lectin receptors bind carbohydrate structures commonly associated with microbial cell wall components including mannose, fucose, and β-glucans (35, 40, 41). Further, expression of Clec2e is restricted to the intestinal epithelium and is downregulated with LPS and Poly:(IC) in a TLR3-dependent manner (37). Here, we demonstrate that overexpression of Clec2e is sufficient to promote bacterial adherence to mammalian cells. While the ligand of Clec2e remains unknown, its structural similarities to Dectin-1 and RegIII microbial binding proteins, along with our bacterial adhesion data, suggests Clec2e may bind conserved microbial cell wall components or bacterial pili and fimbria necessary for cellular adherence. Although expanded studies are needed to fully interrogate how Clec2e interacts with commensal bacterial populations and pathogens beyond *Salmonella* and *C. rodentium*, a lack of Clec2e suppression may contribute to heightened susceptibility of GF or antibiotic-treated mice to infection (48).

Epigenetic modifications enable host cells to alter gene expression without modifying the genetic sequence and changes in the host epigenome occur downstream of external environmental signals (20–22). Recent studies, focused predominantly on immune cells types, have supported that the microbiota may imprint or epigenetically prime genes in the host through enzymes such as DNA methyltransferases (58), histone methyltransferases (29, 59), and HDACs (25, 60–63). In addition to HDAC3, other class I HDACs are expressed in IECs (44, 45) and the role of these HDACs as well as other regulatory proteins may further alter epigenetic regulation of the Clec2e gene in IECs. IECs are equipped to sense and respond to common microbial moieties such as LPS, and previous studies showed TLR4 expression in IECs was epigenetically primed by the microbiota (64). Specifically, DNA methylation of TLR4 was decreased in GF mice compared to CNV controls, consistent with reduced TLR4 expression with microbial exposure (64). Histone acetylation is associated with permissive and actively transcribed chromatin. Our data using intestinal organoids revealed that the microbiota mediate durable changes in IECs that remain even after microbial stimulation has been removed. In the presence of the microbiota, H3K9Ac was reduced in the Clec2e gene which directly corresponds with reduced expression of Clec2e in IECs from CNV mice, indicating that the microbiota epigenetically regulate Clec2e expression. This study further demonstrates that H3K9Ac in Clec2e is regulated by HDAC3 as loss of HDAC3 expression leads to increased histone acetylation and loss of transcriptional repression of Clec2e. Taken together, our data demonstrate a novel mechanism by which the microbiota promote host defense through suppression of IEC-intrinsic pathways that are coopted for pathogen adherence and highlights that epigenetic regulation of innate pathways in IECs may represent a potent, long-lasting mechanism by which the microbiota prime host defense.

DATA AVAILABILITY

The datasets generated for this study can be found in Gene Expression Omnibus (GEO), GSE128362, GSE50453, GSE128369.

ETHICS STATEMENT

All experiments were performed according to the animal guidelines upon approval of the Institutional Animal Care and Use Committee at CCHMC.

AUTHOR CONTRIBUTIONS

TA, VW, and EE designed the studies and analyzed the data. VW, EE, TR, and JW carried out experiments. BV provided bacterial strains. TA, VW, and EE wrote the manuscript.

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REFERENCES

1. Dupont HL. Acute infectious diarrhea in immunocompetent adults. *N Engl J Med.* (2014) 370:1532–40. doi: 10.1056/NEJMra1301069
2. Dupont HL. Bacterial diarrhea. *N Engl J Med.* (2009) 361:1560–9. doi: 10.1056/NEJMcp0904162
3. Cossart P, Sansonetti PJ. Bacterial invasion : the paradigms of enteroinvasive pathogens. *Science.* (2004) 304:242–9. doi: 10.1126/science.1090124
4. Pizarro-Cerdá J, Cossart P. Bacterial adhesion and entry into host cells. *Cell.* (2006) 124:715–27. doi: 10.1016/j.cell.2006.02.012
5. Fitzhenry RJ, Reece S, Trabulsi LR, Heuschkel R, Murch S, Thomson M, et al. Tissue tropism of enteropathogenic *Escherichia coli* strains belonging to the O55 serogroup. *Infect Immun.* (2002) 70:4362–8. doi: 10.1128/IAI.70.8.4362
6. Flores J, Okhuysen PC. Genetics of susceptibility to infection with enteric pathogens. *Curr Opin Infect Dis.* (2009) 22:471–6. doi: 10.1097/QCO.0b013e3283304eb6.Genetics
7. Baumler A, Fang FC. Host specificity of bacterial pathogens. *Cold Spring Harb Perscpectives Med.* (2013) 3:a010041. doi: 10.1101/cshperspect.a010041
8. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell.* (2014) 157:121–41. doi: 10.1016/j.cell.2014.03.011
9. Libertucci J, Young VB. The role of the microbiota in infectious diseases. *Nat Microbiol.* (2019) 4:35–45. doi: 10.1038/s41564-018-0278-4
10. Kamada N, Kim YG, Sham HP, Vallance BA, Puente JL, Martens EC, et al. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. *Science.* (2012) 336:1325–30. doi: 10.1126/science.1222195
11. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell.* (2009) 139:485–98. doi: 10.1016/j.cell.2009.09.033
12. Ganal SC, Sanos SL, Kallfass C, Oberle K, Johner C, Kirschning C, et al. Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. *Immunity.* (2012) 37:171–86. doi: 10.1016/j.immuni.2012.05.020
13. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol.* (2014) 14:141–53. doi: 10.1038/nri3608
14. Gallo RL, Hooper LV. Epithelial antimicrobial defence of the skin and intestine. *Nat Rev Immunol.* (2012) 12:503–16. doi: 10.1038/nri3228
15. Giacomin PR, Moy RH, Noti M, Osborne LC, Siracusa MC, Alenghat T, et al. Epithelial-intrinsic IKKα expression regulates group 3 innate lymphoid cell responses and antibacterial immunity. *J Exp Med.* (2015) 212:1513–28. doi: 10.1084/jem.20141831
16. Ramanan D, Cadwell K. Intrinsic defense mechanisms of the intestinal epithelium. *Cell Host Microbe.* (2016) 19:434–41. doi: 10.1016/j.chom.2016.03.003
17. Woo V, Alenghat T. Host – microbiota interactions : epigenomic regulation. *Curr Opin Immunol.* (2017) 44:52–60. doi: 10.1016/j.coi.2016.12.001
18. Alenghat T. Epigenomics and the microbiota. *Toxicol Pathol.* (2015) 43:101–6. doi: 10.1177/0192623314553805
19. Grunstein M. Histone acetylation in chromatin structure and transcription. *Nature.* (1997) 389:349–52. doi: 10.1038/38664
20. Renz H, von Mutius E, Brandtzaeg P, Cookson WO, Autenrieth IB, Haller D. Gene-environment interactions in chronic inflammatory disease. *Nat Immunol.* (2011) 12:273–7. doi: 10.1038/ni0411-273
21. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. *Genes Dev.* (2009) 23:781–3. doi: 10.1101/gad.1787609.Copyright
22. Happel N, Doenecke D. Histone H1 and its isoforms: contribution to chromatin structure and function. *Gene.* (2009) 431:1–12. doi: 10.1016/j.gene.2008.11.003
23. Mullican SE, Gaddis CA, Alenghat T, Nair MG, Giacomin PR, Everett LJ, et al. Histone deacetylase 3 is an epigenomic brake in macrophage alternative activation. *Genes Dev.* (2011) 25:2480–8. doi: 10.1101/gad.175950.111
24. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem.* (2002) 277:33275–83. doi: 10.1074/jbc.M204935200
25. Alenghat T, Osborne LC, Saenz SA, Kobuley D, Ziegler CG, Mullican SE, et al. Histone deacetylase 3 coordinates commensal-bacteria-dependent intestinal homeostasis. *Nature.* (2013) 504:153–7. doi: 10.1038/nature12687
26. Bergstrom KSB, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, et al. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathog.* (2010) 6:e1000902. doi: 10.1371/journal.ppat.1000902
27. Navabi N, Whitt J, Wu S, Woo V, Moncivaiz J, Jordan MB, et al. Epithelial histone deacetylase 3 instructs intestinal immunity by coordinating local lymphocyte activation. *Cell Rep.* (2017) 19:1165–75. doi: 10.1016/j.celrep.2017.04.046
28. Whitt J, Woo V, Lee P, Moncivaiz J, Haberman Y, Denson L, et al. Disruption of epithelial HDAC3 in intestine prevents diet-induced obesity in mice. *Gastroenterology.* (2018) 155:501–13. doi: 10.1053/j.gastro.2018.04.017
29. Kelly D, Kotliar M, Woo V, Jagannathan S, Whitt J, Moncivaiz J, et al. Microbiota-sensitive epigenetic signature predicts inflammation in Crohn's disease. *JCI Insight.* (2018) 3:122104. doi: 10.1172/jci.insight.122104
30. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* (2009) 10:R25. doi: 10.1186/gb-2009-10-3-r25
31. Zhang Y, Liu T, Ca M, Eeckhoute J, Berstein B, Nusbaum C, et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* (2008) 9:R137. doi: 10.1186/gb-2008-9-9-r137

32. Kartashov AV, Barski A. Biowardrobe: an integrated platform for analysis of epigenomics and transcriptomics data. *Genome Biol.* (2015) 2015:16. doi: 10.1186/s13059-015-0720-3
33. Waddell A, Vallance JE, Hummel A, Alenghat T, Rosen MJ. IL-33 induces murine intestinal goblet cell differentiation indirectly via innate lymphoid cell IL-13 secretion. *J Immunol.* (2018) 2018:ji1800292. doi: 10.4049/jimmunol.1800292
34. Letourneau J, Levesque C, Berthiaume F, Jacques M, Mourez M. *In vitro* assay of bacterial adhesion onto mammalian epithelial cells. *J Vis Exp.* (2011) 1:3–6. doi: 10.3791/2783
35. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science.* (2006) 1126–30. doi: 10.1126/science.1127119.Symbiotic
36. Mukherjee S, Zheng H, Derebe MG, Callenberg KM, Partch C, Rollins D, et al. Antibacterial membrane attack by a pore-forming intestinal C-type lectin. *Nature.* (2014) 505:103–7. doi: 10.1038/nature12729
37. Rutkowski E, Leibelt S, Born C, Friede ME, Bauer S, Weil S, et al. Clr-a: a novel immune-related C-type lectin-like molecule exclusively expressed by mouse gut epithelium. *J Immunol.* (2017) 198:916–26. doi: 10.4049/jimmunol.1600666
38. Miki T, Holst O, Hardt WD. The bactericidal activity of the C-type lectin RegIIIβ against gram-negative bacteria involves binding to lipid a.pdf. *J Biol Chem.* (2012) 287:34844–55. doi: 10.1074/jbc.M112.399998
39. Zhang Q, Rahim MMA, Allan DSJ, Tu MM, Belanger S, Abou-Samra E, et al. Mouse Nkrp1-Clr gene cluster sequence and expression analyses reveal conservation of tissue-specific MHC-independent immunosurveillance. *PLoS ONE.* (2012) 7:e50561. doi: 10.1371/journal.pone.0050561
40. Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity.* (2011) 34:651–64. doi: 10.1016/j.immuni.2011.05.001
41. Hoving JC, Wilson GJ, Brown GD, Town C, Africa S. Signalling C-type lectin receptors, microbial recognition and immunity. *Cell Microbiol.* (2014) 16:185–94. doi: 10.1111/cmi.12249
42. Obata Y, Furusawa Y, Hase K. Epigenetic modifications of the immune system in health and disease. *Immunol Cell Biol.* (2015) 93:226–32. doi: 10.1038/icb.2014.114
43. Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. *Nature.* (2016) 535:65–74. doi: 10.1038/nature18847
44. Wilson EM, Rotwein P. Control of MyoD function during initiation of muscle differentiation by an autocrine signaling pathway activated by insulin-like growth factor-II. *J Biol Chem.* (2006) 281:29962–71. doi: 10.1074/jbc.M605445200
45. Turgeon N, Blais M, Gagné JM, Tardif V, Boudreau F, Perreault N, et al. HDAC1 and HDAC2 restrain the intestinal inflammatory response by regulating intestinal epithelial cell differentiation. *PLoS ONE.* (2013) 8:e73785. doi: 10.1371/journal.pone.0073785
46. Pott J, Hornef M. Innate immune signalling at the intestinal epithelium in homeostasis and disease. *EMBO Rep.* (2012) 13:684–98. doi: 10.1038/embor.2012.96
47. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity.* (2012) 37:158–70. doi: 10.1016/j.immuni.2012.04.011
48. Abt MC, Pamer EG. Commensal bacteria mediated defenses against pathogens. *Curr Opin Immunol.* (2014) 29:16–22. doi: 10.1016/j.coi.2014.03.003
49. Mukherjee S, Hooper L V. Antimicrobial defense of the intestine. *Immunity.* (2015) 42:28–39. doi: 10.1016/j.immuni.2014.12.028
50. Johansson MEV, Jakobsson HE, Holmén-Larsson J, Schütte A, Ermund A, Rodríguez-Piñeiro AM, et al. Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell Host Microbe.* (2015) 18:582–92. doi: 10.1016/j.chom.2015.10.007
51. Hase K, Eckmann L, Leopard JD, Varki N, Kagnoff MF. Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. *Infect Immun.* (2002) 70:953–63. doi: 10.1128/IAI.70.2.953-963.2002
52. Natividad JMM, Hayes CL, Motta JP, Jury J, Galipeau HJ, Philip V, et al. Differential induction of antimicrobial REGIII by the intestinal microbiota and Bifidobacterium breve NCC2950. *Appl Environ Microbiol.* (2013) 79:7745–54. doi: 10.1128/AEM.02470-13
53. Florin THJ, Mcguckin MA, Linde SK. Mucin dynamics in intestinal bacterial infection. *PLoS ONE.* (2008) 3:e3952. doi: 10.1371/journal.pone.0003952
54. Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF. Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. *J Immunol.* (2005) 174:4901–7. doi: 10.4049/jimmunol.174.8.4901
55. Mundy R, MacDonald TT, Dougan G, Frankel G, Wiles S. Citrobacter rodentium of mice and man. *Cell Microbiol.* (2005) 7:1697–706. doi: 10.1111/j.1462-5822.2005.00625.x
56. Chessa D, Winter MG, Jakomin M, Bäumler AJ. Salmonella enterica serotype Typhimurium Std fimbriae bind terminal α(1,2)fucose residues in the cecal mucosa. *Mol Microbiol.* (2009) 71:864–75. doi: 10.1111/j.1365-2958.2008.06566.x
57. Leibelt S, Friede ME, Rohe C, Gütle D, Rutkowski E, Weigert A, et al. Dedicated immunosensing of the mouse intestinal epithelium facilitated by a pair of genetically coupled lectin-like receptors. *Mucosal Immunol.* (2015) 8:232–42. doi: 10.1038/mi.2014.60
58. Yu D-H, Gadkari M, Zhou Q, Yu S, Gao N, Guan Y, et al. Postnatal epigenetic regulation of intestinal stem cells requires DNA methylation and is guided by the microbiome. *Genome Biol.* (2015) 16:211. doi: 10.1186/s13059-015-0763-5
59. Burgess SL, Saleh M, Cowardin CA, Buonomo E, Noor Z, Watanabe K, et al. Role of serum amyloid A, GM-CSF and bone marrow granulocyte-monocyte precursor expansion in segmented filamentous bacteria-mediated protection from *Entamoeba histolytica*. *Infect Immun.* (2016) 84:IAI.00316-16. doi: 10.1128/IAI.00316-16
60. Schulthess J, Pandey S, Capitani M, Rue-Albrecht K, Arnold I, Franchini F, et al. The short chain fatty acid butyrate imprints an antimicrobial program in macrophages. *Immunity.* (2019) 50:432–45. doi: 10.1016/J.IMMUNI.2018.12.018
61. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci USA.* (2014) 111:2247–52. doi: 10.1073/pnas.1322269111
62. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature.* (2013) 504:446–50. doi: 10.1038/nature12721
63. Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, et al. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. *Mucosal Immunol.* (2015) 8:80–93. doi: 10.1038/mi.2014.44
64. Takahashi K, Sugi Y, Nakano K, Tsuda M, Kurihara K, Hosono A, et al. Epigenetic control of the host gene by commensal bacteria in large intestinal epithelial cells. *J Biol Chem.* (2011) 286:35755–62. doi: 10.1074/jbc.M111.271007

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MINI REVIEW
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Commensal Bacteria: An Emerging Player in Defense Against Respiratory Pathogens

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A diverse community of trillions of commensal bacteria inhabits mucosal and epidermal surfaces in humans and plays an important role in defense against pathogens, including respiratory pathogens. Commensal bacteria act on the host's immune system to induce protective responses that prevent colonization and invasion by pathogens. On the other hand, these bacteria can directly inhibit the growth of respiratory pathogens by producing antimicrobial products/signals and competing for nutrients and adhesion sites. Such mechanisms preserve the niche for commensal bacteria and support the host in containing respiratory infections. Herein, we discuss current evidence on the role of commensal bacteria in conferring protection against respiratory pathogens and the underlying mechanisms by which these bacteria do so. A deeper knowledge of how commensal bacteria interact with the host and pathogens might provide new insights that are poised to aid in the development of vaccines and therapeutics that target infectious diseases.

Keywords: host, commensal, pathogen, lungs, vaccine

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INTRODUCTION

Since the inception of the Human Microbiome Project in 2007, a plethora of knowledge has accumulated that throws light on diverse and crucial roles played by commensal bacteria in homeostasis and disease (1, 2). With the help of advances in omic and systems biology technologies, our knowledge of the composition, genetics, and functional capacity of commensal bacteria is growing at a fast pace. It is becoming clear that commensal bacteria, which reside in various parts of the human body, such as the gut and airways, correspond approximately to the total number of human cells (about 1:1 ratio), and exert a profound impact on regulation of immunophysiological functions, including but not limited to, metabolism, ontogeny, and pathogen defense (3, 4). Several recent studies have shown that commensals promote resistance to gut pathogens that is mutually beneficial to the host and the commensal microbiota (5–7). However, imbalances in the microbial communities can occur, and are linked to many diseases, such as inflammatory bowel disease, allergies, asthma, diabetes, and obesity (8). It remains scantily understood how these bacteria execute their functional activities against respiratory pathogens. Respiratory infections are of utmost importance, as they inflict substantial social and economic burden on people across the world in general and in low and lower-middle income countries in particular (9–11). Additionally, current therapeutic and prophylactic interventions against respiratory diseases have major constraints, such as rapid emergence of anti-microbial resistance and disruption of the normal microbiota by use of antibiotics. Unraveling the interactions between commensals and pathogens may allow the exploitation of inhibitory properties of commensals

to combat pathogens causing respiratory diseases. In this review article, we provide an overview of the current state of knowledge about the role of commensal bacteria in protective immunity to respiratory pathogens and the mechanisms involved in commensal bacteria mediated defenses. Understanding the relationship between commensal bacteria, host, and pathogen is a way forward to develop safe and effective prophylactics and therapeutics against pathogens.

COMMENSAL BACTERIA MEDIATED PROTECTION

Protection in Mouse Models

Much of what is known about the direct role of commensal bacteria in protection against respiratory pathogens stems from studies using various mouse models, including germ-free and antibiotic-treated mice (12–23). Following lung infection with *Streptococcus pneumoniae*, numerous studies have shown that antibiotic-treated mice display significantly higher pathogen loads and increased pathologies in the lungs compared with sham-treated mice (15, 21). Likewise, germ-free mice showed enhanced levels of bacterial burden when subjected to *S. pneumoniae* and *Klebsiella pneumoniae* lung infections (15). Similar to the protection conferred by fecal microbiota transplant (FMT) against intestinal pathogens, FMT in gut microbiota-depleted mice restored pulmonary bacterial clearance early after *S. pneumoniae* infection (21). In case of mouse models of *Mycobacterium tuberculosis* infection, gut microbiota disruption after pre- and post-antibiotic treatment showed decreased resistance to infection in the lungs, which was associated with severe histopathological changes, such as pulmonary granulomas (24). Furthermore, antibiotic-induced dysbiosis changed the microbiota diversity in the gut and promoted lung colonization by *M. tuberculosis* (25). Similar protective effect was conferred after mice having antibiotic-induced disrupted microbiota received an intranasal infectious dose of influenza A virus (12). In a different study, FMT into germ-free mice led to reconstitution of the gut microbiota that facilitated increased survival against lethal influenza A virus infection (26). Overall, these studies employing multiple experimental approaches provide *in vivo* evidence that underscores a profound contribution of commensal bacteria in defense against diverse respiratory pathogens.

Even though antibiotic-treated and germ-free mice have proven to be a crucial tool in understanding the role of the microbiota in pathogen defense, there are potential pitfalls that need consideration while interpreting results from studies involving these animals. Germ-free animals lack all detectable microbes in different organs and have an impaired immune system, whereas antibiotics are used to deplete specific microbiota (27). Although these two approaches provide crucial information on the function of the microbiota in general, the specific contribution of the microbiota found in different body compartments, such as the lung microbiota, in immunity to respiratory infections is unclear. This is important because the lung microbiota, which in healthy adults seems to mainly consist of a small number of bacteria originating from the oral cavity, plays a significant role in respiratory health, and disease (28–30). Additionally, there is a need for models that can answer a more direct question about protection in the presence of a fully developed immune system. To address this issue, researchers have attempted to deplete the lung microbiota in mice by a combination of aerosolized vancomycin and neomycin via nasal route, which resulted in a significant reduction in the lung commensal microbiota, with the advantage of minimally affecting the gut microbiota (31, 32). But the possibility of antibiotic spread to the nearby tissues/organs harboring different microbiota remains, requiring future studies to focus on developing better models to fill in this pitfall.

The microbiota consists of a large number of bacterial species, and therefore, it is of great interest to specifically identify commensal species that protect from respiratory pathogens. Recent studies have evaluated the protective efficacy of commensal bacterial species in respiratory infections. Oral administration of *Bifidobacterium longum* (BB536), but not saline, in mice significantly reduced viral loads, pulmonary pathology, and body weight loss following intranasal challenge with influenza virus, suggesting a protective role for this commensal bacterium in influenza infection (33). Similarly, oral or nasal inoculation of mice with different strains of *Lactobacillus*, e.g., *L. gasseri* (TMC0356), *L. rhamnosus* (CRL 1505), and *L. brevis* (KB290), conferred protective immunity to influenza virus infections (34–36). Furthermore, *L. rhamnosus* (CRL 1505) exerted a protective effect in mice subjected to an intranasal challenge with respiratory syncytial virus infection (37, 38). These data indicate a prophylactic role for commensal bacteria against viral pathogens. In order to assess therapeutic significance, *B. longum* (MM2) was orally administered in mice infected with influenza virus. Mice that received *B. longum* (MM2) ameliorated infection, as determined by decreased body weight loss, viral titers, and inflammation, compared with control mice (39). Protective effect induced by these probiotic bacteria is not confined to respiratory infections with viruses, but can be applicable to bacterial pathogens (37–40). Intake of *B. longum* (5^{1A}) in mice not only demonstrated protective effect against infection with *K. pneumoniae*, but also suppressed inflammatory changes in the lung (40). Very recently, we have demonstrated that intranasal immunization of mice with the commensal *Streptococcus mitis* conferred protection against lung infection with *S. pneumoniae* strains D39 (serotype 2) and TIGR4 (serotype 4), which illuminates the unique ability of *S. mitis* to offer resistance to different pneumococcal serotypes (41). Two recent studies performed by independent groups further show that the gut colonizer bacterium *Helicobacter hepaticus* influences the composition of the gut microbiota and the outcome of *M. tuberculosis* infection in mouse models (42, 43). Mice subjected to intestinal colonization with *H. hepaticus*, when challenged by intranasal route with *M. tuberculosis*, reflected higher mycobacterial burden in the lungs compared with the controls (42). This increased mycobacterial burden in the *H. hepaticus*-colonized mice coincided with severe *M. tuberculosis*-mediated pulmonary pathologies, mainly characterized by granulomas and tissue damage, and accumulation/production of inflammatory

leukocytes/cytokines (42). Similar to these results, mice colonized with *H. hepaticus* eliminated subunit-vaccine-induced protective immunity to lung infection with *M. tuberculosis* (43). Taken together, these findings indicate that commensal bacteria can be harnessed for prophylactic and therapeutic purposes, provided utmost precaution on the possible negative effects of enriching for specific colonizers of the microbiota.

Protection in Humans

Relatively little information is available on whether commensal bacteria can prevent respiratory infections in humans. Oral commensals, such as *Streptococcus oralis* and *Streptococcus salivarius*, can induce protection against middle ear inflammation, referred to as otitis media, which is primarily caused by respiratory pathogens, such as *S. pneumoniae* and *Haemophilus influenzae* (44–46). Upon intranasal administration of *S. salivarius* and *S. oralis*, children susceptible to acute otitis media displayed reduced recurrences of disease with no side effects (44). Contrarily, a nasal spray containing oral commensals, e.g., *S. mitis* and *S. oralis*, in susceptible children under 4 years of age showed no significant effect regarding the number of episodes of recurrent otitis media compared to the placebo group (46). The discrepancy in these studies might be due to differences in bacterial doses, inoculation regimens, and combinations, which need to be analyzed in light of new technologies (e.g., metagenomics and next generation sequencing) and concepts like dysbiosis. The fact that antibiotics were used together with the streptococcal nasal spray in the first study, but not in the second, is also an important factor to consider. In controlled infection studies in humans, nasopharyngeal colonization by the commensal *Neisseria lactamica* provided protection against the respiratory pathogen *Neisseria meningitidis* (47, 48). Furthermore, in a block-randomized challenge trial, 310 healthy individuals (18–25 years) were intranasally inoculated with live *N. lactamica* or sham and the bacterial carriage was monitored for 26 weeks (48). All those who developed nasopharyngeal colonization by *N. lactamica* revealed a significant reduction in the *N. meningitidis* carriage compared with sham-treated ones (48). These studies show that commensal bacteria not only show inhibitory/displacing effects on the carriage of respiratory pathogens but also highlight the ease and safety with which these bacteria can be used to contain infections in humans. It is however notable that most bacteria with high pathogenic potential, such as those in the above examples, are also part of the healthy microbiome (49). The reason as to why these pathogens cause diseases is attributed to various host and microbial factors, including viral infections (49). Dysbiosis in particular, such as a result of antibiotic use has been associated with a reduction in the prevalence of respiratory commensal bacteria like *Corynebacterium* spp. and *Dolosigranulum* spp. in the nasopharynx of healthy infants. These are considered to reduce the colonization by *S. pneumoniae*, *H. influenzae*, and *S. aureus* in the respiratory tract (50). It is further shown that respiratory syncytial virus infection in children below 2 years of age was positively correlated with nasopharyngeal *H. influenzae* and *Streptococcus* microbiota clusters and inversely correlated with *Staphylococcus aureus* (51). Transcriptomic analysis of the children infected with *H. influenzae* and *Streptococcus* clusters presented greater expression of immune components, suggesting that nasopharyngeal microbiota can influence host immunity (51). In line with this, prolonged antibiotic treatment in early life has also been annexed with an increased risk for respiratory infections in infants (52, 53). Thus, these studies shine light on the effect of the microbiota perturbations caused by antibiotics on host susceptibility to respiratory infections, particularly during the critical life period of immune maturation.

MECHANISMS OF COMMENSAL BACTERIA MEDIATED PROTECTION

A pertinent question however remains as to what are the underlying mechanisms by which commensal bacteria perform their protective function against respiratory pathogens. Emerging data thus far indicate that commensal bacteria confer protection in two ways: host-mediated immunity (acting on the host's immune system) and direct action (directly inhibiting/killing pathogens and competing for colonization).

Host-Mediated Immunity

A wealth of emerging evidence indicates that both the lung and gut microbiota are involved in the regulation of immune responses during lung infections (28, 54). However, it is difficult to assess the specific contributions of the lung and gut microbiota to protective immunity to respiratory pathogens, mainly due to three reasons: (1) the gut microbiota is the largest and most diverse community of commensals that significantly influences the outcome of immunity in the lungs as well as gut; (2) the gut is the largest lymphoid organ in the body because of which it occupies a central position in host-microbiota studies; and (3) we do not have optimal models to ascertain their specific roles in immunity. Despite an important role for these commensal bacteria in promoting resistance against respiratory pathogens, the mechanistic basis for this resistance remains unclear. Several studies have shown a potential defect in innate immunity and subsequent adaptive immunity in the lung, when signals from commensal bacteria are abrogated (12, 14, 25, 55). Key innate immune cells that are recruited to the lungs and are involved in protective immunity include macrophages, natural killer (NK) cells, and mucosal-associated invariant T (MAIT) cells (12, 14, 25, 55). The pulmonary macrophages in mice depleted of the microbiota by antibiotics reflected reduced expression of the macrophage-associated antiviral genes, such as Irf 7, Ifnb, Mx1, Tnfa, Il6, and Il1b following influenza virus infection (14). This corresponded with reduced response to IFN-γ, IFN-α, or influenza infection in macrophages from the mice treated with antibiotics. *In vivo* experiments in mice also indicated that the alveolar macrophage response was impaired during viral infection, which was characterized by down-regulation of most of the antiviral genes activated *ex vivo* (14). Wang et al. demonstrated a new mechanism in mice colonized with *S. aureus* where $CD11b^{+}$ M2 alveolar macrophages, stimulated with Toll-like receptor (TLR) 2, play a protective role in influenza

infection (56). Another innate immune cell type is MAIT cell that is shown to play an important role in microbiota mediated mycobacterial immunity (25). Flow cytometric analysis reflected that mice depleted of the microbiota had reduced number of lung MAIT cells, characterized by MR1-5-OP-RU tetramer$^+$TCRβ$^+$ phenotype, which expressed significantly lower IL-17A compared with control mice, suggesting that lung MAIT cells may function to contain early pulmonary *M. tuberculosis* infection (25). Furthermore, NK cells from germ-free mice did not induce anti-influenza immunity because macrophages and dendritic cells failed to produce type 1 IFN in response to infection (55). Cumulatively, these data suggest that microbiota-derived signals provide a stimulus that maintains the potency of the lung innate immune system needed for invoking effective immunity (**Figure 1**).

Adaptive immunity follows innate immunity and is crucial for specific immunity against respiratory pathogens (57–59). Rabbit antisera raised against *S. mitis* show cross-reactivity with *S. pneumoniae* (59). Similar to IgG mediated cross-reactivity, IgA antibodies from the sera, nasal wash, and bronchoalveolar lavage of mice vaccinated with *S. mitis* cross-reacted with *S. pneumoniae* serotypes 2 and 4 (41). On the other hand, human CD4$^+$ T cells expressing IL-17A, which are reactive to *S. mitis*, show cross-reactivity toward *S. pneumoniae* in an *in vitro* culture system (60). Intranasal vaccination of mice with *S. mitis* led to an increased production of IL-17A by CD4$^+$ T cells in the lungs compared to PBS-treated control mice (41). These results are in line with the evidence that the gut commensal segmented filamentous bacteria (SFB) regulate pulmonary Th17 immunity to the fungal pathogen *Aspergillus fumigatus* (19). In a mouse model of influenza viral infection, it is shown that commensal bacteria regulate virus-specific CD4$^+$ and CD8$^+$ T cells and antibodies following lung infection with virus (12). Overall, commensal bacteria mediated adaptive immunity to respiratory pathogens include both humoral (IgG and IgA) and T cell-mediated responses.

Involvement of the gut microbiota in protective immunity to pulmonary pathogens illustrates a pathogenic nexus between the microbiota and the "gut-lung axis," underscoring a profound protective influence of the gut commensals over several pathogens residing at distant anatomical compartments of the body (61). The gut microbiota mediated control of the lung immunopathology is also evident from studies demonstrating the susceptibility of animals with the altered gut microbiota to allergic lung diseases (20). On the other hand, dysbiosis in the lung microbiota can change the composition of the gut microbiota. For example, mice subjected to lung infection with influenza virus infection or intranasal instillation of lipopolysaccharide disturbed the gut microbiota homeostasis, which supports the fact that the gut and lungs are closely linked in a way that they affect each other's microbiology and physiology (62, 63). Moreover, how the gut microbiota controls the lung immunity has recently been explored by few key studies in mouse models of pulmonary bacterial infections. Brown *et al.* performed a well-designed and comprehensive study that sheds light on a major innate immune mechanism used by the microbiota to clear lung infections in mouse models (15). In antibiotic-treated mice, there was an increased growth of *S. pneumoniae* and *K. pneumoniae* in the lungs after bacterial inoculation compared to sham-treated mice, which was associated with reduced production of innate immune factors, such granulocyte–macrophage colony-stimulating factor (GM-CSF) (15). *In vivo* neutralization of GM-CSF into antibiotic-treated mice, which received the microbiota from the sham-treated mice and had restored pulmonary bacterial clearance, resulted in making these mice prone to infections (15). These findings suggest that GM-CSF is essential for the microbiota to execute their functional activities against both Gram-positive (*S. pneumoniae*) and Gram-negative (*K. pneumoniae*) pathogens. It was further demonstrated that GM-CSF programs alveolar macrophage function via an extracellular signal-regulated kinase (ERK)-specific signaling pathway leading to increased pathogen killing via reactive oxygen species (ROS) (15). Several studies have implicated pattern recognition receptor (PRR) ligands produced by the gut microbiota in controlling immune responses outside the intestinal tract (16, 64). Following antibiotic-mediated depletion of the microbiota in mice, early clearance of *K. pneumoniae* was impaired and this could be rescued by injection of bacterial Nod-like receptor (NLR) ligands (the NOD1 ligand MurNAcTri(DAP) and NOD2 ligand muramyl dipeptide [MDP]), but not bacterial TLR ligands (16). Defects in early innate immunity were found to be due to reduced ROS-mediated killing of bacteria by alveolar macrophages (16). Interestingly, upon treatment of mice with antibiotics and NLR ligands orally prior to *S. pneumoniae* lung infection, neutralization of GM-CSF abrogated the rescue of respiratory clearance (15). Taking account of all these data, it is clear that the microbiota and NLR ligands regulate lung innate immunity to respiratory pathogens via GM-CSF, highlighting crucial mechanisms of the gut-lung axis of communication. In addition, the gut commensal SFB has been reported to provide protection in immunocompromised (Rag$^{-/-}$) mice by partially enhancing neutrophil resolution during pneumococcal lung infection, which corresponded with reduced expression of the anti-phagocytic molecule CD47 (65). Like NK cells, another lymphoid cell population referred to as group 3 lymphoid cells that produce IL-22 (IL-22$^+$ILC3), a cytokine involved in pathogen defense, has been implicated in gut commensal bacteria-induced protection against *S. pneumoniae* (66). Disruption of commensal bacteria by antibiotics decreased the influx of IL-22$^+$ILC3 cells into the lungs of new born mice, which made them more prone to pneumococcal infection compared with control mice. This effect was reversed when ILC3 cells were adoptively transferred or exogenous IL-22 administered in mice (66). Thus, these immune mechanisms furnish crucial information on how the gut microbiota controls protective immunity to lung infections (**Figure 1**).

Direct Action

Commensal bacteria resist colonization of pathogens by using wide range of direct mechanisms for niche competition, such as secretion of inhibitory substances and nutrient competition, enlisting the exploitative, and interference modes of competition (67). Recent studies dissected novel mechanisms used by resident commensals to inhibit and contain respiratory pathogens, such as

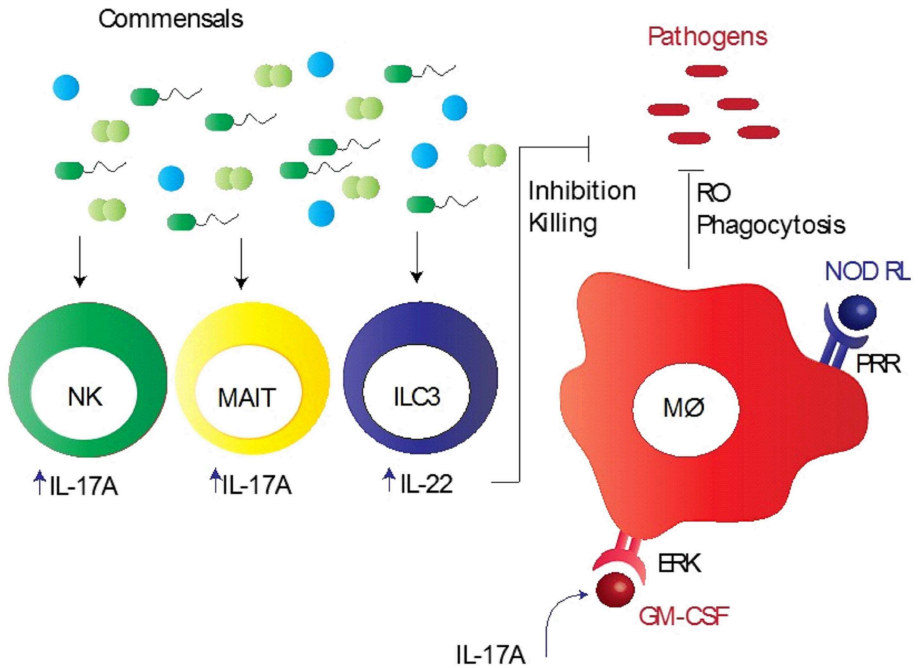

FIGURE 1 | Commensal bacteria mediated innate immunity to respiratory pathogens. Commensal bacteria stimulate various innate immune cells, particularly alveolar macrophages (Mϕ), mucosa-associated invariant T (MAIT) cells, group 3 innate lymphoid cells (ILC3), and natural killer (NK) cells, to induce early protection. These bacteria promote pathogen killing via granulocyte–macrophage colony-stimulating factor (GM-CSF), which stimulates pathogen killing and clearance by alveolar macrophages (Mϕ) through phagocytosis, reduced reactive oxygen species (RO), and extracellular signal regulated kinase (ERK) signaling. Intrapulmonary GM-CSF production in response to infection is regulated by the microbiota via interleukin-17A (IL-17A). Pattern recognition receptor (PRR) expressed by Mϕ recognizes PRR ligands, such as nod-like receptor ligands (NOD RL), leading to the activation of Mϕ. NK and MAIT cells when activated by commensal bacteria produce large quantities of IL-17A, whereas ILC3 cells secrete IL-22, aiding in inhibition/killing of various respiratory pathogens.

disruption of biofilms, exploitation of host resources to generate antimicrobial products, and down-regulation of virulence genes. This highlights the complexity and diversity of mechanisms involved in direct inhibition (68–70). The well-documented mechanism by which commensal bacteria can directly inhibit the pathogen growth and compete with them is the production of ribosomally produced antimicrobials called bacteriocins (71, 72). For example, *S. salivarius* produces a wide range of bacteriocins, which is a major mechanism that antagonizes *S. pneumoniae* (71, 73–75). More recently, it is also demonstrated that *S. salivarius* reduces the *S. pneumoniae* colonization by blocking the adhesion sites, suggesting multiple mechanisms used by this commensal to inhibit pathogens (76). Apart from ribosomally encoded bacteriocins, commensal bacteria encode non-ribosomally produced bioactive antimicrobials to compete with pathogens (77). Zipperer et al. showed that the nasal commensal *Staphylococcus lugdunensis* directly inhibits the growth of *S. aureus* through a novel cyclic antimicrobial peptide named "Lugdunin." Lugdunin possessed bactericidal activity against all tested strains of *S. aureus* in *vitro*. Moreover, in animal model, the co-colonization of *S. lugdunensis* and *S. aureus* resulted in competitive exclusion of *S. aureus* (77). The use of purified antimicrobials or bacterial strains encoding antimicrobials may serve as a source of new generation of antibiotics to deal with multidrug resistant strains, such as methicillin resistant *S. aureus*. One mechanism, which contributes to competitive advantage for colonization of commensal bacteria to preserve their niche and to suppress the growth of pathogens, includes the production of hydrogen peroxide. Epidemiological data show a negative correlation between *S. pneumoniae* and *S. aureus* and presumably, the reason for increased *S. aureus* related otitis media after use of pneumococcal vaccine (78, 79). One possible mechanism implicated to define this negative association is hydrogen peroxide mediated inhibition of *S. aureus* by pneumococcal hydrogen peroxide (80).

Nutrient competition is also a strategy used by commensal bacteria to reduce the fitness of pathogens by competing for the same pool of resources (81). Stubbendieck et al. recently showed that isolates of *Corynebacterium* spp. inhibited *Staphylococcus* spp. *in vitro*. This inhibition was due to reduced iron bioavailability, mediated by siderophore–induced sequestration of iron by *Corynebacterium* spp. (82). Another novel mechanism of commensal mediated inhibition is through the production of secreted enzymes. Iwase et al. first demonstrated the negative correlation between the commensal *Staphylococcus epidermidis* and pathogenic *S. aureus* in human nasal samples. To gain further insight to explain this negative association, they identified the inhibitory factor produced by *S. epidermidis* as serine protease, which inhibits the biofilm formation and

TABLE 1 | Examples of direct mechanisms of colonization resistance used by commensal bacteria against respiratory pathogens.

Commensal bacteria	Anatomical location	Mechanism of inhibition	Respiratory pathogens	References
Streptococcus salivarius	Oral cavity	Ribosomally synthesized antimicrobials (Bacteriocins)	*Streptococcus pneumoniae* *Streptococcus pyogenes*	(71)
Staphylococcus lugdunensis	Skin, and nasal cavity	Non-ribosomally synthesized antimicrobials (Lugdunin)	*Staphylococcus aureus*	(77)
Corynebacterium accolens	Skin, and nasal cavity	Metabolic products with antimicrobial properties (Free fatty acids)	*Streptococcus pneumoniae*	(69)
Staphylococcus epidermidis	Skin and nasal cavity	Secreted enzymes (Serine protease)	*Staphylococcus aureus*	(68)
Streptococcus pneumoniae	Nasopharynx, and oral cavity	Hydrogen peroxide (H_20_2) mediated killing	*Staphylococcus aureus*	(80)
Corynebacterium spp.	Skin and nasal cavity	Nutrient competition (Iron limitation by siderophore production)	*Staphylococcus* spp.	(82)

human nasal colonization by *S. aureus* (68). Follow-up study from the same group showed that intranasal colonization of mice with serine protease producing *S. epidermidis* inhibited colonization with methicillin resistant *S. aureus* (83). Commensal bacteria also exploit the host resources to generate metabolic compounds with antimicrobial properties to suppress the growth of respiratory pathogens. An elegant study by Bomar et al. investigated the mechanistic explanation for correlation between increased abundance of *Corynebacterium* species and reduced *S. pneumoniae* colonization (69). Interestingly, they found that *Corynebacterium accolens* encodes lipase, which catalyzes the hydrolysis of host triacylglycerolsto to produce free fatty acids with antibacterial properties that suppress the growth of *S. pneumoniae* (69). Taken together, the above examples evidently suggest that antagonistic interactions exist in the polymicrobial community utilizing wide range of mechanisms by which commensal bacteria inhibit respiratory pathogens. Advanced understanding of existing mechanisms using both *in vitro* and *in vivo* models and further elucidation of novel mechanisms may enable us to exploit commensals to inhibit respiratory pathogens. Mechanisms used by commensal bacteria to directly inhibit/kill respiratory pathogens are exemplified in **Table 1**.

CONCLUSIONS AND FUTURE INSIGHTS

Advanced research technologies have been applied to evaluate the contribution of commensal bacteria to respiratory infections. Accumulating evidence indicates an important role for commensal bacteria in defense against respiratory pathogens, which paves the way to target these bacteria for the development of vaccines and therapeutics that provide optimal protection with safety and low cost. Moreover, the use of modern experimental tools to decipher the novel mechanisms used by commensals to inhibit pathogens may assist in designing novel therapeutics with targeted approach focusing exclusively on the pathogen inhibition without disrupting the homeostatic microbial community. Future studies are required to address the following questions: (1) What are the underlying mechanisms by which the trio of commensals, pathogens, and host interact with each other? (2) What could be the long-term consequences of using commensal bacteria-based vaccines/therapeutics on the host, pathogens, and the microbiota? (3) What are the effects of medical manipulations, such as antibiotics and probiotics, on the biology of commensal bacteria? (4) How can we use commensal bacteria-expressed bacteriocins for protection against respiratory pathogens? (5) Which specific commensal bacterial species of the microbiota are directly involved in protection immunity to different pathogens? (6) How can we use commensal microbiota/bacteria to correct dysbiosis? A sincere exploration of these questions may have implications for the clinical use of commensal bacteria with inhibitory properties against pathogens. This may be important to bypass the drawbacks associated with currently available options, such as antimicrobial resistance.

AUTHOR CONTRIBUTIONS

RK, FP, and SS wrote and revised the manuscript. All authors assisted in the conception of this review and acquisition of relevant literature. All authors gave approval of the last version to be published.

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REFERENCES

1. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature.* (2007) 449:804–10. doi: 10.1038/nature 06244
2. Gevers D, Knight R, Petrosino JF, Huang K, McGuire AL, Birren BW, et al. The human microbiome project: a community resource for the healthy human microbiome. *PLoS Biol.* (2012) 10:e1001377. doi: 10.1371/journal.pbio.1001377
3. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. *PLoS*

Biol. (2016) 14:e1002533. doi: 10.1371/journal.pbio.1002533
4. Wang BH, Yao MF, Lv LX, Ling ZX, Li LJ. The human microbiota in health and disease. *Eng Prc.* (2017) 3:71–82. doi: 10.1016/J.ENG.2017.01.008
5. Kamada N, Seo SU, Chen GY, Nunez G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol.* (2013) 13:321–35. doi: 10.1038/nri3430
6. Abt MC, Pamer EG. Commensal bacteria mediated defenses against pathogens. *Curr Opin Immunol.* (2014) 29:16–22. doi: 10.1016/j.coi.2014.03.003
7. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat Rev Immunol.* (2013) 13:790–801. doi: 10.1038/nri3535
8. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis.* (2015) 26:26191. doi: 10.3402/mehd.v26.26191
9. Epaud R. An update on paediatric respiratory diseases. *Eur Respir Rev.* (2018) 27:180013. doi: 10.1183/16000617.0013-2018
10. Alimi Y, Lim WS, Lansbury L, Leonardi-Bee J, Nguyen-Van-Tam JS. Systematic review of respiratory viral pathogens identified in adults with community-acquired pneumonia in Europe. *J Clin Virol.* (2017) 95:26–35. doi: 10.1016/j.jcv.2017.07.019
11. Fogel N, Tuberculosis: A disease without boundaries. *Tuberculosis.* (2015) 95:527–531. doi: 10.1016/j.tube.2015.05.017
12. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci USA.* (2011) 108:5354–9. doi: 10.1073/pnas.1019378108
13. Fagundes CT, Amaral FA, Vieira AT, Soares AC, Pinho V, Nicoli JR, et al. Transient TLR activation restores inflammatory response and ability to control pulmonary bacterial infection in germfree mice. *J Immunol.* (2012) 188:1411–20. doi: 10.4049/jimmunol.1101682
14. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity.* (2012) 37:158–70. doi: 10.1016/j.immuni.2012.04.011
15. Brown RL, Sequeira RP, Clarke TB. The microbiota protects against respiratory infection via GM-CSF signaling. *Nat Commun.* (2017) 8:1512. doi: 10.1038/s41467-017-01803-x
16. Clarke TB, Early innate immunity to bacterial infection in the lung is regulated systemically by the commensal microbiota via nod-like receptor ligands. *Infect Immun.* (2014) 82:4596–606. doi: 10.1128/IAI.02212-14
17. Gauguet S, D'Ortona S, Ahnger-Pier K, Duan B, Surana NK, Lu R, et al. Intestinal microbiota of mice influences resistance to Staphylococcus *aureus* pneumonia. *Infect Immun.* (2015) 83:4003–14. doi: 10.1128/IAI.00037-15
18. Lankelma JM, Birnie E, Weehuizen AF, Scicluna BP, Belzer C, Houtkooper RH, et al. The gut microbiota as a modulator of innate immunity during melioidosis. *PLoS Neglect Trop D.* (2017) 11:e0005548. doi: 10.1371/journal.pntd.0005548
19. McAleer JP, Nguyen LH, Chen K, Kumar P, Ricks DM, Binnie M, et al. Pulmonary Th17 antifungal immunity is regulated by the gut microbiome. *J Immunol.* (2016) 197:97–107. doi: 10.4049/jimmunol.1502566
20. Samuelson DR, Welsh DA, Shellito JE. Regulation of lung immunity and host defense by the intestinal microbiota. *Front Microbiol.* (2015) 6:1085. doi: 10.3389/fmicb.2015.01085
21. Schuijt TJ, Lankelma JM, Scicluna BP, de Sousa e Melo F, Roelofs JJ, de Boer JD, et al. The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. *Gut.* (2016) 65:575–83. doi: 10.1136/gutjnl-2015-309728
22. Wu S, Jiang ZY, Sun YF, Yu B, Chen J, Dai CQ, et al. Microbiota regulates the TLR7 signaling pathway against respiratory tract influenza A virus infection. *Curr Microbiol.* (2013) 67:414–22. doi: 10.1007/s00284-013-0380-z
23. Chen LW, Chen PH, Hsu CM. Commensal microflora contribute to host defense against escherichia coli pneumonia through Toll-Like Receptors. *Shock.* (2011) 36:67–75. doi: 10.1097/SHK.0b013e3182184ee7
24. Khan N, Vidyarthi A, Nadeem S, Negi S, Nair G, Agrewala JN. Alteration in the gut microbiota provokes susceptibility to tuberculosis. *Front Immunol.* (2016) 7:529. doi: 10.3389/fimmu.2016.00529
25. Dumas A, Corral D, Colom A, Levillain F, Peixoto A, Hudrisier D, et al., The host microbiota contributes to early protection against lung colonization by *Mycobacterium tuberculosis*. *Front Immunol.* (2018) 9:2656. doi: 10.3389/fimmu.2018.02656
26. Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al. Wild mouse gut microbiota promotes host fitness and improves disease resistance. *Cell.* (2017) 171:1015–28.e13. doi: 10.1016/j.cell.2017.09.016
27. Kennedy EA, King KY, Baldridge MT. Mouse microbiota models: comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Front Physiol.* (2018) 9:1534. doi: 10.3389/fphys.2018.01534
28. Mathieu E, Escribano-Vazquez U, Descamps D, Cherbuy C, Langella P, Riffault S, et al. Paradigms of lung microbiota functions in health and disease, particularly, in asthma. *Front Physiol.* (2018) 9:1168. doi: 10.3389/fphys.2018.01168
29. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. *PLoS ONE.* (2010) 5:e8578. doi: 10.1371/journal.pone.0008578
30. Venkataraman A, Bassis CM, Beck JM, Young VB, Curtis JL, Huffnagle GB, et al. Application of a neutral community model to assess structuring of the human lung microbiome. *MBio.* (2015) 6:e02284-14. doi: 10.1128/mBio.02284-14
31. Le Noci V, Guglielmetti S, Arioli S, Camisaschi C, Bianchi F, Sommariva M, et al. Modulation of pulmonary microbiota by antibiotic or probiotic aerosol therapy: a strategy to promote immunosurveillance against lung metastases. *Cell Rep.* (2018) 24:3528–38. doi: 10.1016/j.celrep.2018.08.090
32. Zarogoulidis P, Kioumis I, Porpodis K, Spyratos D, Tsakiridis K, Huang H, et al. Clinical experimentation with aerosol antibiotics: current and future methods of administration. *Drug Des Devel Ther.* (2013) 7:1115–34. doi: 10.2147/DDDT.S51303
33. Iwabuchi N, Xiao JZ, Yaeshima T, Iwatsuki K. Oral administration of bifidobacterium longum ameliorates influenza virus infection in mice. *Biol Pharm Bull.* (2011) 34:1352–5. doi: 10.1248/bpb.34.1352
34. Kawase M, He F, Kubota A, Yoda K, Miyazawa K, Hiramatsu M. Heat-killed *Lactobacillus gasseri* TMC0356 protects mice against influenza virus infection by stimulating gut and respiratory immune responses. *FEMS Immunol Med Microbiol.* (2012) 64:280–8. doi: 10.1111/j.1574-695X.2011.00903.x
35. Zelaya H, Tada A, Vizoso-Pinto MG, Salva S, Kanmani P, Aguero G, et al. Nasal priming with immunobiotic Lactobacillus rhamnosus modulates inflammation-coagulation interactions and reduces influenza virus-associated pulmonary damage. *Inflamm Res.* (2015) 64:589–602. doi: 10.1007/s00011-015-0837-6
36. Waki N, Yajima N, Suganuma H, Buddle BM, Luo D, Heiser A, et al. Oral administration of Lactobacillus brevis KB290 to mice alleviates clinical symptoms following influenza virus infection. *Lett Appl Microbiol.* (2014) 58:87–93. doi: 10.1111/lam.12160
37. Chiba E, Tomosada Y, Vizoso-Pinto MG, Salva S, Takahashi T, Tsukida K, et al. Immunobiotic *Lactobacillus rhamnosus* improves resistance of infant mice against respiratory syncytial virus infection. *Int Immunopharmacol.* (2013) 17:373–82. doi: 10.1016/j.intimp.2013.06.024
38. Tomosada Y, Chiba E, Zelaya H, Takahashi T, Tsukida K, Kitazawa H, et al. Nasally administered *Lactobacillus rhamnosus* strains differentially modulate respiratory antiviral immune responses and induce protection against respiratory syncytial virus infection. *BMC Immunol.* (2013) 14:40. doi: 10.1186/1471-2172-14-40
39. Kawahara T, Takahashi T, Oishi K, Tanaka H, Masuda M, Takahashi S, et al. Consecutive oral administration of Bifidobacterium longum MM-2 improves the defense system against influenza virus infection by enhancing natural killer cell activity in a murine model. *Microbiol Immunol.* (2015) 59:1–12. doi: 10.1111/1348-0421.12210
40. Vieira AT, Rocha VM, Tavares L, Garcia CC, Teixeira MM, Oliveira SC, et al. Control of Klebsiella pneumoniae pulmonary infection and immunomodulation by oral treatment with the commensal probiotic Bifidobacterium longum 5(1A). *Microbes Infect.* (2016) 18:180–9. doi: 10.1016/j.micinf.2015.10.008
41. Shekhar S, Khan R, Schenck K, Petersen FC. Intranasal immunization with the commensal Streptococcus mitis confers protective immunity against pneumococcal lung infection. *Appl Environ Microbiol.* (2019). 85:e02235-18. doi: 10.1128/AEM.02235-18

42. Majlessi L, Sayes F, Bureau JF, Pawlik A, Michel V, Jouvion G, et al. Colonization with Helicobacter is concomitant with modified gut microbiota and drastic failure of the immune control of *Mycobacterium tuberculosis*. *Mucosal Immunol.* (2017) 10:1178–89. doi: 10.1038/mi.2016.140
43. Arnold IC, Hutchings C, Kondova I, Hey A, Powrie F, Beverley P, et al. Helicobacter hepaticus infection in BALB/c mice abolishes subunit-vaccine-induced protection against *M. tuberculosis*. *Vaccine.* (2015) 33:1808–14. doi: 10.1016/j.vaccine.2015.02.041
44. La Mantia I, Varricchio A, Ciprandi G. Bacteriotherapy with *Streptococcus salivarius* 24SMB and Streptococcus oralis 89a nasal spray for preventing recurrent acute otitis media in children: a real-life clinical experience. *Int J Gen Med.* (2017) 10:171–5. doi: 10.2147/IJGM.S137614
45. Marchisio P, Santagati M, Scillato M, Baggi E, Fattizzo M, Rosazza C, et al. *Streptococcus salivarius* 24SMB administered by nasal spray for the prevention of acute otitis media in otitis-prone children. *Eur J Clin Microbiol Infect Dis.* (2015) 34:2377–83. doi: 10.1007/s10096-015-2491-x
46. Tano K, Hakansson EG, Holm SE, Hellstrom S. A nasal spray with alpha-haemolytic streptococci as long term prophylaxis against recurrent otitis media. *Int J Pediatr Otorhi.* (2002) 62:17–23. doi: 10.1016/S0165-5876(01)00581-X
47. Evans CM, Pratt CB, Matheson M, Vaughan TE, Findlow J, Borrow R, et al. Nasopharyngeal colonization by *Neisseria lactamica* and induction of protective immunity against *Neisseria meningitidis*. *Clin Infect Dis.* (2011) 52:70–7. doi: 10.1093/cid/ciq065
48. Deasy AM, Guccione E, Dale AP, Andrews N, Evans CM, Bennett JS, et al. Nasal inoculation of the commensal neisseria lactamica inhibits carriage of neisseria meningitidis by young adults: a controlled human infection study. *Clin Infect Dis.* (2015) 60:1512–20. doi: 10.1093/cid/civ098
49. Henriques-Normark B, Normark S. Commensal pathogens, with a focus on Streptococcus pneumoniae, and interactions with the human host. *Exp Cell Res.* (2010) 316:1408–14. doi: 10.1016/j.yexcr.2010.03.003
50. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe.* (2015) 17:704–15. doi: 10.1016/j.chom.2015.03.008
51. de Steenhuijsen Piters WA, Heinonen S, Hasrat R, Bunsow E, Smith B, Suarez-Arrabal MC, et al. Nasopharyngeal microbiota, host transcriptome, and disease severity in children with respiratory syncytial virus infection. *Am J Respir Crit Care Med.* (2016) 194:1104–15. doi: 10.1164/rccm.201602-0220OC
52. Arboleya S, Sanchez B, Milani C, Duranti S, Solis G, Fernandez N, et al. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. *J Pediatr.* (2015) 166:538–44. doi: 10.1016/j.jpeds.2014.09.041
53. Kuppala VS, Meinzen-Derr J, Morrow AL, Schibler KR. Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants. *J Pediatr.* (2011) 159:720–5. doi: 10.1016/j.jpeds.2011.05.033
54. Man WH, de Steenhuijsen Piters WA, Bogaert D, The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol.* (2017) 15:259–70. doi: 10.1038/nrmicro.2017.14
55. Ganal SC, Sanos SL, Kallfass C, Oberle K, Johner C, Kirschning C, et al. Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. *Immunity.* (2012) 37:171–86. doi: 10.1016/j.immuni.2012.05.020
56. Wang J, Li F, Sun R, Gao X, Wei H, Li LJ, et al. Bacterial colonization dampens influenza-mediated acute lung injury via induction of M2 alveolar macrophages. *Nat Commun.* (2013) 4:2106. doi: 10.1038/ncomms3106
57. Shekhar S, Khan R, Ferreira DM, Mitsi E, German E, Rorvik GH, et al. Antibodies reactive to commensal *Streptococcus mitis* show cross-reactivity with virulent *Streptococcus pneumoniae* serotypes. *Front Immunol.* (2018) 9:747. doi: 10.3389/fimmu.2018.00747
58. Shekhar S, Schenck K, Petersen FC. Exploring host-commensal interactions in the respiratory tract. *Front Immunol.* (2017) 8:1971. doi: 10.3389/fimmu.2017.01971
59. Skov Sorensen UB, Yao K, Yang Y, Tettelin H, Kilian M. Capsular polysaccharide expression in commensal streptococcus species: genetic and antigenic similarities to *Streptococcus pneumoniae*. *MBio.* (2016) 7: e01844–16. doi: 10.1128/mBio.01844-16
60. Engen SA, Rukke HV, Becattini S, Jarrossay D, Blix IJ, Petersen FC, et al. The oral commensal streptococcus mitis shows a mixed memory Th cell signature that is similar to and cross-reactive with *Streptococcus pneumoniae*. *PLoS ONE.* (2014) 9:e104306. doi: 10.1371/journal.pone.0104306
61. Dumas A, Bernard L, Poquet Y, Lugo-Villarino G, Neyrolles O. The role of the lung microbiota and the gut-lung axis in respiratory infectious diseases. *Cell Microbiol.* (2018) 20:e12966. doi: 10.1111/cmi.12966
62. Looft T, Allen HK. Collateral effects of antibiotics on mammalian gut microbiomes. *Gut Microbes.* (2012) 3:463–7. doi: 10.4161/gmic.21288
63. Sze MA, Tsuruta M, Yang SW, Oh Y, Man SF, Hogg JC, et al. Changes in the bacterial microbiota in gut, blood, and lungs following acute LPS instillation into mice lungs. *PLoS ONE.* (2014) 9:e111228. doi: 10.1371/journal.pone.0111228
64. Chu H, Mazmanian SK. Innate immune recognition of the microbiota promotes host-microbial symbiosis. *Nat Immunol.* (2013) 14:668–75. doi: 10.1038/ni.2635
65. Felix KM, Jaimez IA, Nguyen TV, Ma H, Raslan WA, Klinger CN, et al. Gut microbiota contributes to resistance against pneumococcal pneumonia in immunodeficient Rag(-/-) mice. *Front Cell Infect Microbiol.* (2018) 8:118. doi: 10.3389/fcimb.2018.00118
66. Gray J, Oehrle K, Worthen G, Alenghat T, Whitsett J, Deshmukh H. Intestinal commensal bacteria mediate lung mucosal immunity and promote resistance of newborn mice to infection. *Sci Transl Med.* (2017) 9:eaaf9412. doi: 10.1126/scitranslmed.aaf9412
67. Stubbendieck RM, Straight PD. Multifaceted interfaces of bacterial competition. *J Bacteriol.* (2016) 198:2145–55. doi: 10.1128/JB.00275-16
68. Iwase T, Uehara Y, Shinji H, Tajima A, Seo H, Takada K, et al. Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. *Nature.* (2010) 465:346-U100. doi: 10.1038/nature09074
69. Bomar L, Brugger SD, Yost BH, Davies SS, Lemon KP. *Corynebacterium accolens* releases antipneumococcal free fatty acids from human nostril and skin surface triacylglycerols. *Mbio.* (2016) 7:e01725-15. doi: 10.1128/mBio.01725-15
70. Ramsey MM, Freire MO, Gabrilska RA, Rumbaugh KP, Lemon KP. *Staphylococcus aureus* shifts toward commensalism in response to corynebacterium species. *Front Microbiol.* (2016) 7:1230. doi: 10.3389/fmicb.2016.01230
71. Santagati M, Scillato M, Patane F, Aiello C, Stefani S. Bacteriocin-producing oral streptococci and inhibition of respiratory pathogens. *FEMS Immunol Med Microbiol.* (2012) 65:23–31. doi: 10.1111/j.1574-695X.2012.00928.x
72. Janek D, Zipperer A, Kulik A, Krismer B, Peschel A. High frequency and diversity of antimicrobial activities produced by nasal staphylococcus strains against bacterial competitors. *PLoS Pathog.* (2016) 12:e1005812. doi: 10.1371/journal.ppat.1005812
73. Tagg JR, Streptococcal bacteriocin-like inhibitory substances: some personal insights into the bacteriocin-like activities produced by streptococci good and bad. *Probiot Antimicro.* (2009) 1:60–6. doi: 10.1007/s12602-008-9002-7
74. Walls T, Power D, Tagg J. Bacteriocin-like inhibitory substance (BLIS) production by the normal flora of the nasopharynx: potential to protect against otitis media? *J Med Microbiol.* (2003) 52:829–33. doi: 10.1099/jmm.0.05259-0
75. Wescombe PA, Heng CK, Burton JP, Chilcott CN, Tagg JR. *Streptococcal bacteriocins* and the case for *Streptococcus salivarius* as model oral probiotics. *Future Microbiol.* (2009) 4:819–35. doi: 10.2217/fmb.09.61
76. Manning J, Dunne EM, Wescombe PA, Hale JD, Mulholland EK, Tagg JR, et al. Investigation of *Streptococcus salivarius*-mediated inhibition of pneumococcal adherence to pharyngeal epithelial cells. *BMC Microbiol.* (2016) 16:225. doi: 10.1186/s12866-016-0843-z
77. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, et al. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature.* (2016) 535:511–6. doi: 10.1038/nature18634
78. Regev-Yochay G, Dagan R, Raz M, Carmeli Y, Shainberg B, Derazne E, et al. Association between carriage of *Streptococcus pneumoniae* and *Staphylococcus aureus* in children. *Jama-J Am Med Assoc.* (2004) 292:716–20. doi: 10.1001/jama.292.6.716
79. Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rumke HC, et al. Colonisation by *Streptococcus pneumoniae* and

Staphylococcus aureus in healthy children. *Lancet.* (2004) 363:1871–2. doi: 10.1016/S0140-6736(04)16357-5

80. Regev-Yochay G, Trzcinski K, Thompson CM, Malley R, Lipsitch M. Interference between *Streptococcus pneumoniae* and *Staphylococcus aureus*: *in vitro* hydrogen peroxide-mediated killing by *Streptococcus pneumoniae*. *J Bacteriol.* (2006) 188:4996–5001. doi: 10.1128/JB.00317-06
81. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol.* (2013) 14:685–90. doi: 10.1038/ni.2608
82. Stubbendieck RM, May DS, Chevrette MG, Temkin MI, Wendt-Pienkowski E, Cagnazzo J, et al. Competition among nasal bacteria suggests a role for siderophore-mediated interactions in shaping the human nasal microbiota. *Appl Environ Microbiol.* (2018) AEM.02406–18. doi: 10.1128/AEM.02406-18
83. Park B, Iwase T, Liu GY. Intranasal application of S. epidermidis prevents colonization by methicillin-resistant *Staphylococcus aureus* in mice. *PLoS ONE.* (2011) 6:e25880. doi: 10.1371/journal.pone.0025880

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Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* Uses the VraSR Regulatory System to Modulate Autophagy for Increased Intracellular Survival in Macrophage-Like Cell Line RAW264.7

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The VraSR two-component system is a vancomycin resistance-associated sensor/regulator that is upregulated in vancomycin-intermediate *Staphylococcus aureus* (VISA) and heterogeneous VISA (hVISA) strains. VISA/hVISA show reduced susceptibility to vancomycin and an increased ability to evade host immune responses, resulting in enhanced clinical persistence. However, the underlying mechanism remains unclear. Recent studies have reported that *S. aureus* strains have developed some strategies to survive within the host cell by using autophagy processes. In this study, we confirmed that clinical isolates with high *vraR* expression showed increased survival in murine macrophage-like RAW264.7 cells. We constructed isogenic *vraSR* deletion strain Mu3Δ*vraSR* and *vraSR*-complemented strain Mu3Δ*vraSR*-C to ascertain whether *S. aureus* uses the VraSR system to modulate autophagy for increasing intracellular survival in RAW264.7. Overall, the survival of Mu3ΔvraSR in RAW264.7 cells was reduced at all infection time points compared with that of the Mu3 wild-type strain. Mu3Δ*vraSR*-infected RAW264.7 cells also showed decreased transcription of autophagy-related genes *Becn1* and *Atg5*, decreased LC3-II turnover and increased p62 degradation, and fewer visible punctate LC3 structures. In addition, we found that inhibition of autophagic flux significantly increased the survival of Mu3Δ*vraSR* in RAW264.7 cells. Together, these results demonstrate that *S. aureus* uses the VraSR system to modulate host-cell autophagy processes for increasing its own survival within macrophages. Our study provides novel insights into the impact of VraSR on bacterial

infection and will help to further elucidate the relationship between bacteria and the host immune response. Moreover, understanding the autophagic pathway in *vraSR* associated immunity has potentially important implications for preventing or treating VISA/hVISA infection.

Keywords: *Staphylococcus aureus*, autophagy, VraSR, regulation, macrophage

INTRODUCTION

Staphylococcus aureus is an important human pathogen responsible for both hospital-associated and community-acquired infections (Monaco et al., 2017). *S. aureus* causes a wide range of diseases, from minor skin and soft tissue infections to severe systemic illnesses such as pneumonia, arthritis, endocarditis, and bacteremia (Lowy, 1998). In recent years, indiscriminate and extensive use of vancomycin has resulted in the emergence and development of vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) strains, many of which show an increased ability to evade host immune surveillance and enhanced clinical persistence (Gardete et al., 2012; Cameron et al., 2017; Katayama et al., 2017).

Autophagy is a basic physiological process in eukaryotes and plays an important role in cellular repair and homeostasis (Ohsumi, 2014). Degraded intracellular components are removed and recycled into newly emerging double-membrane vacuoles called autophagosomes (Mizushima et al., 2010). These autophagosomes mature to fuse with lysosomes and are digested. This process of autophagosome formation and eventual degradation is termed autophagic flux (Mizushima et al., 2010). Studies have demonstrated that many pathogens have evolved strategies to harness autophagic processes for survival inside the host cell (Campoy and Colombo, 2009; Mostowy, 2013; Gomes and Dikic, 2014; Mostowy, 2014; Soong et al., 2015; Siqueira et al., 2018). In particular, Neumann et al. and Schnaith et al. reported that *S. aureus* can take advantage of the autophagic mechanism to aid in its own replication or intracellular survival (Schnaith et al., 2007; Neumann et al., 2016).

The two-component regulatory system VraSR is a vancomycin resistance-associated sensor (VraS)/regulator (VraR) that is highly expressed in VISA/hVISA strains. In this study, we found that clinical isolates with high *vraR* expression showed increased survival in murine macrophage-like RAW264.7 cells and deletion of *vraSR* in *S. aureus* resulted in decreased survival in RAW264.7, indicating that VraSR could enhance intracellular *S. aureus* survival. We hypothesize that the VraSR regulatory system could be involved in regulation of host autophagy pathways to promote the survival of *S. aureus*. To examine this hypothesis, the autophagic responses of RAW264.7 cells infected with hVISA reference strain Mu3, *vraSR* deletion mutant Mu3Δ*vraSR*, or *vraSR*-complemented strain Mu3Δ*vraSR*-C were investigated. The results showed that *S. aureus* uses the VraSR regulatory system to induce autophagy and inhibit autophagic flux, thereby increasing bacterial intracellular survival in RAW264.7. This finding provides novel insights into the impact of VraSR on bacterial infection and may help to further elucidate the relationship between bacteria and the host immune response.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in **Supplementary Table S1**. All *S. aureus* strains were cultured with shaking (200 rpm) at 37°C in tryptic soy broth (TSB). *Escherichia coli* strains were cultured with shaking (200 rpm) in Luria-Bertani medium at 37°C. The culture media were supplemented with appropriate antibiotics when required (ampicillin, 100 μg/l; chloramphenicol, 10 μg/l; and anhydrotetracycline, 1 μg/ml).

Construction of the *vraSR* Mutant Strain

The *vraSR* deletion mutant strain was constructed as described previously (Li et al., 2017). Briefly, the upstream and downstream fragments of *vraSR* were amplified from *S. aureus* Mu3 genomic DNA using the *vraSR*-UF/*vraSR*-UR and *vraSR*-DF/*vraSR*-DR primer sets, respectively, and ligated by overlap extension polymerase chain reaction (PCR) to form an up-down fragment. The resulting fragment was recombined into the temperature-sensitive shuttle plasmid pKOR1 using Gateway® BP ClonaseTM II Enzyme Mix (Thermo Fisher Scientific) to generate recombinant plasmid pKOR1-*vraSR*. pKOR1-*vraSR* was then transformed into *S. aureus* strain RN4220 by electroporation for modification and then transformed into *S. aureus* strain Mu3. The mutant strains that had allelic replacement were screened via high temperature and anhydrotetracycline-resistant and chloramphenicol-sensitive colonies and were further confirmed by PCR, quantitative reverse-transcriptase PCR (qRT-PCR) and sequencing. All primers used in this study are listed in **Supplementary Table S2**.

Complementation of the *vraSR* Deletion Strain

To generate a complementation strain, *vraSR* and its promoter region were amplified and cloned into the shuttle plasmid pLI50, producing recombinant plasmid pLI50-*vraSR*. The recombinant plasmid was then transferred into *E. coli* DH5α and DC10B successively, and finally electroporated into the *S. aureus* Mu3Δ*vraSR* strain. Successful uptake of the complementation plasmid was confirmed by restriction mapping, PCR, and sequencing of PCR fragments. The presence of *vraSR* transcripts within the transformants was verified by qRT-PCR analysis.

Growth Curve Analysis

S. aureus strains were incubated overnight in 5 ml of TSB at 37°C with shaking at 200 rpm. The overnight cultures were diluted 1/100 in 30 ml of fresh TSB and incubated at 37°C with shaking

at 220-rpm. The optical densities ($OD_{600\ nm}$) of the *S. aureus* cultures were then monitored at 1 h intervals for a total of 18 h.

Cell Culture

Murine macrophage-like cell line RAW264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM high glucose; HyClone) supplemented with 10% (v/v) fetal calf serum (FCS; HyClone). Cells were cultured in a humidified incubator containing 5% CO_2 at 37°C.

Assessment of Bacterial Intracellular Survival

Intracellular killing assays were performed as described previously (Munzenmayer et al., 2016). For *S. aureus* infection, early stationary phase bacteria ($OD_{600\ nm}$ = 1.0–1.5) were harvested and washed once in cold phosphate-buffered saline (PBS). RAW264.7 cells were then infected with *S. aureus* at a multiplicity of infection (MOI) of 10. Following incubation for 1 h, infected cells were washed three times with PBS before the addition of 10% (v/v) FCS-DMEM supplemented with 10 μg/ml lysostaphin (Sigma-Aldrich) and 100 μg/ml gentamicin (Sigma-Aldrich) to each well. Plates were then incubated for 1 h to kill extracellular bacteria. Following incubation, the cells were washed with PBS and further incubated in fresh 10% (v/v) FCS-DMEM. At 0, 3, 6, 12, and 24 h post-infection (hpi), infected cells were washed three times with PBS to remove extracellular bacteria and dead cells and lysed by the addition of 0.5% (v/v) Triton X-100 (Sigma-Aldrich). The number of intracellular bacteria (expressed as colony-forming units, CFU) was determined by serial dilution and plating on TSB agar. In addition, replicate plates were incubated with 1.25 mM 3-methyladenine (3-MA, Sigma) or 100 nM bafilomycin A1 (Baf A1, Sigma) for 2 h prior to infection to block autophagy in the RAW264.7 cells.

Transmission Electron Microscopy

RAW264.7 cells were incubated with the individual *S. aureus* strains at a MOI of 50 for 3 h before being collected by centrifugation and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. Cells were then post-fixed in 1% osmium tetroxide and dehydrated through a series of graded acetone washes. The dehydrated cells were embedded in epoxy resin, sectioned, and stained with uranyl acetate and lead citrate in preparation for observation under a transmission electron microscope (HT7700; Hitachi Co.). Autophagosomes were counted as described previously. (Yla-Anttila et al., 2009).

RNA Extraction and qRT-PCR Assays

S. aureus RNA was extracted as described previously (Dai et al., 2017) to examine levels of *vraR* expression. RAW264.7 cells (2.5×10^6 cells/well) in 6-well plates were infected with *S. aureus* at a MOI of 50 and incubated at 37°C with 5% CO_2. At 0, 1.5, 3, 4.5, and 6 hpi, the culture medium was removed and RAW264.7 cells were washed twice with ice-cold PBS. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and RNA quantity and quality were measured using a NanoDrop ND-1000 spectrophotometer (PeqLab). RNA was reverse-transcribed into cDNA using PrimeScript Reverse Transcriptase as per the manufacturer's instructions (TaKaRa).

qRT-PCR assays were performed using aliquots of cDNA and SYBR Premix EX TaqTM II (Takara) in an ABI 7500 qPCR instrument (Foster). Primers used for expression analysis are described in **Supplementary Table S1**. All gene expression was normalized against that of *Actb* (β-actin) and the 16S rRNA gene was used as an internal control. All qRT-PCR assays were repeated three times.

Western Blot Analysis

RAW264.7 (2.5×10^6 cells/well) were infected with *S. aureus* at a MOI of 50 in 6-well plates for 3 h and then collected and washed twice with ice-cold PBS. The total protein from the cells was extracted using a RIPA lysis buffer solution (Wuhan Boster), with the total protein concentration determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Cell lysis solutions were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% skim milk in tris-buffered saline Tween 20 buffer for 2 h at room temperature and then incubated overnight at 4°C with primary antibodies against LC3-I/II and p62/SQSTM1 (1:1000, Cell Signaling Technology). The membranes were then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Densitometric analysis of the western blot was performed using Image Gauge software (Fujifilm). β-actin was used as an internal control, and the ratio of the intensity of the protein of interest to β-actin was calculated.

mRFP-GFP-LC3 Puncta Formation Assays

RAW264.7 cells were plated in 6-well plates and allowed to reach 70% confluence by the time of transfection. The cells were then infected with mRFP-GFP-LC3 adenoviral vector (HanBio) at a MOI of 100 for 36 h, cultured with *S. aureus* (MOI = 50) for 3 h, and then fixed in 4% (w/v) polyoxymethylene for 30 min at 37°C. Images were captured on a Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss AG) using a 63× oil objective lens, followed by image analysis using Zeiss ZEN acquisition software. For quantification of LC3 punctate, mRFP-GFP-LC3 and mRFP-LC3 punctate dots were counted in 100 cells. Punctate dots were measured using Image J software (NIH).

Statistical Analysis

All statistical analyses were performed using SPSS (Version 16.0; SPSS for Windows). When appropriate, one-way analysis of variance (ANOVA) and unpaired *t*-tests were analyzed to determine statistical significance, with a *P*-value cutoff of <0.05 to establish significance. Where appropriate, Bonferroni posttests were performed to directly compare experimental means.

RESULTS

The VraSR Regulatory System Is Important for Bacterial Intracellular Survival and Proliferation

In our previous study, a total of 24 clinical *S. aureus* isolates were screened on brain heart infusion agar containing 3 mg/l vancomycin (BHI-V3) (Chang et al., 2015). Based on the resulting data, we selected 12 isolates that grew on BHI-V3 (GV3) were selected as the test group, and other 12 isolates that did not grow on BHI-V3 (NGV3) for examination in the current study as the test and control groups, respectively. The relative ability of the two groups of clinical strains to persist intracellularly was quantified using a lysostaphin/gentamicin protection assay in RAW264.7 cells. We observed that the GV3 group showed increased survival rates in RAW264.7 cells at 24 hpi compared with the NGV3 group (**Figures 1A,B**). Therefore, to further investigate the impact of *vraR* expression on *S. aureus* intracellular growth, we constructed isogenic *vraSR* deletion strain Mu3Δ*vraSR* and *vraSR*-complemented strain Mu3Δ*vraSR*-C. We examined the mRNA expression and protein levels of VraR in the three *S. aureus* strains by q PCR analysis and western blotting to confirm the successful construction of the mutant strain (**Figures 1C,D**). To rule out the potential influence of the *vraSR* deletion on *S. aureus* growth rate, the growth of strains Mu3, Mu3Δ*vraSR*, and Mu3Δ*vraSR*-C was monitored hourly for 18 h. Overall, there were no substaintial differences in the growth of the three strains.

For *S. aureus* infection assay, we selected a low MOI (MOI = 10) instead of a high MOI (MOI = 50), because a high bacterial burden may cause earlier death of RAW264.7 cells, preventing intracellular replication of *S. aureus*. Our results showed that, in comparison with wild-type strain Mu3, fewer Mu3Δ*vraSR* cells were present within the infected RAW264.7 cells, and that the survival of Mu3Δ*vraSR* continued to decrease throughout the infection process. However, complemented mutant Mu3Δ*vraSR*-C showed similar survival rates to the wild-type strain Mu3 in RAW264.7 cells, indicating that VraSR is important for intracellular *S. aureus* survival and proliferation (**Figure 1E**).

The VraSR Regulatory System Contributes to the Formation of Autophagic Vesicles in *S. aureus*-Infected Cells

Ultrastructural features of infected cells were examined by transmission electron microscopy. Double-membraned structures, characteristic of autophagosomes, containing undigested *S. aureus* cells were observed within the RAW264.7 cells (**Figure 2A**). Quantification of *S. aureus*-containing autophagosome-like vacuoles per 50 infected cells showed that Mu3Δ*vraSR*-infected cells displayed significantly fewer *S. aureus*-containing autophagosome-like vacuoles compared with cells infected with Mu3 or Mu3Δ*vraSR*-C ($P < 0.01$), indicating that the VraSR regulatory system contributes to the formation of *S. aureus*-containing autophagosome-like vacuoles in *S. aureus*-infected cells (**Figure 2B**).

The VraSR Regulatory System Contributes to the Expression of Autophagy-Related Genes

As autophagy is a dynamic process, we performed a series of biochemical assays to examine the activation of autophagy at the molecular level (Levine et al., 2011). qRT-PCR assays were used to determine the transcriptional levels of autophagy-related genes *Ulk1*, *Becn1*, and *Atg5*. As shown in **Figure 3**, the transcriptional levels of the three genes were gradually upregulated between 0 and 3 hpi but declined after 4.5 hpi. Compared with Mu3-infected cells, the Mu3Δ*vraSR*-infected cells showed significantly lower transcriptional levels of *Becn1* and *Atg5* at 3 hpi, while gene expression in the Mu3Δ*vraSR*-C-infected cells was comparable with that in Mu3-infected cells, indicating that the VraSR regulatory system contributes to increase expression of autophagy-related genes.

The VraSR Regulatory System Participates in Modulation of Autophagic Protein LC3 Turnover and p62 Degradation

LC3-II is a commonly used marker for autophagosome formation (Barth et al., 2010). Western blot analysis revealed that the levels of LC3-II in the Mu3Δ*vraSR*-infected cells were significantly lower than those in Mu3- and Mu3Δ*vraSR*-C-infected cells at 3 hpi (**Figures 4A,B**), indicating that the VraSR regulatory system is important for autophagic activity. Levels of other autophagic substrates that are degraded by autolysosomes, such as p62, can be used to monitor autophagic flux. We therefore monitored levels of p62 degradation by autolysosomes. At 3 hpi, the amount of p62 in Mu3Δ*vraSR*-infected cells was significantly lower than that in Mu3- and Mu3Δ*vraSR*C-infected cells (**Figures 4C,D**), indicating that the VraSR regulatory system may inhibit autophagic flux in RAW264.7.

The VraSR Regulatory System Is Involved in Autophagic Flux Inhibition in *S. aureus* Infected Cells

Autophagic flux was morphologically traced to distinguish between the two stages. mRFP-GFP-LC3 can be used as an indicator of autophagic flux because it appears yellow (mRFP and GFP signals merged) in autophagosomes and red (only mRFP signals) in autolysosomes, as a result of quenching of the GFP signal by the acidic pH of the lysosomes, while the RFP signal remains stable at an acidic pH. In this study, RAW264.7 cells stably expressing mRFP-GFP-LC3 were infected with different *S. aureus* strains. All autophagic structures could be measured by confocal microscope. Results showed that Mu3Δ*vraSR*-infected cells had fewer punctate LC3 structures (yellow dots) at 3 hpi compared with Mu3-infected cells, while the Mu3Δ*vraSR*-C-infected cells appeared similar to the Mu3-infected cells (**Figures 5A,B**), indicating that the VraSR regulatory system

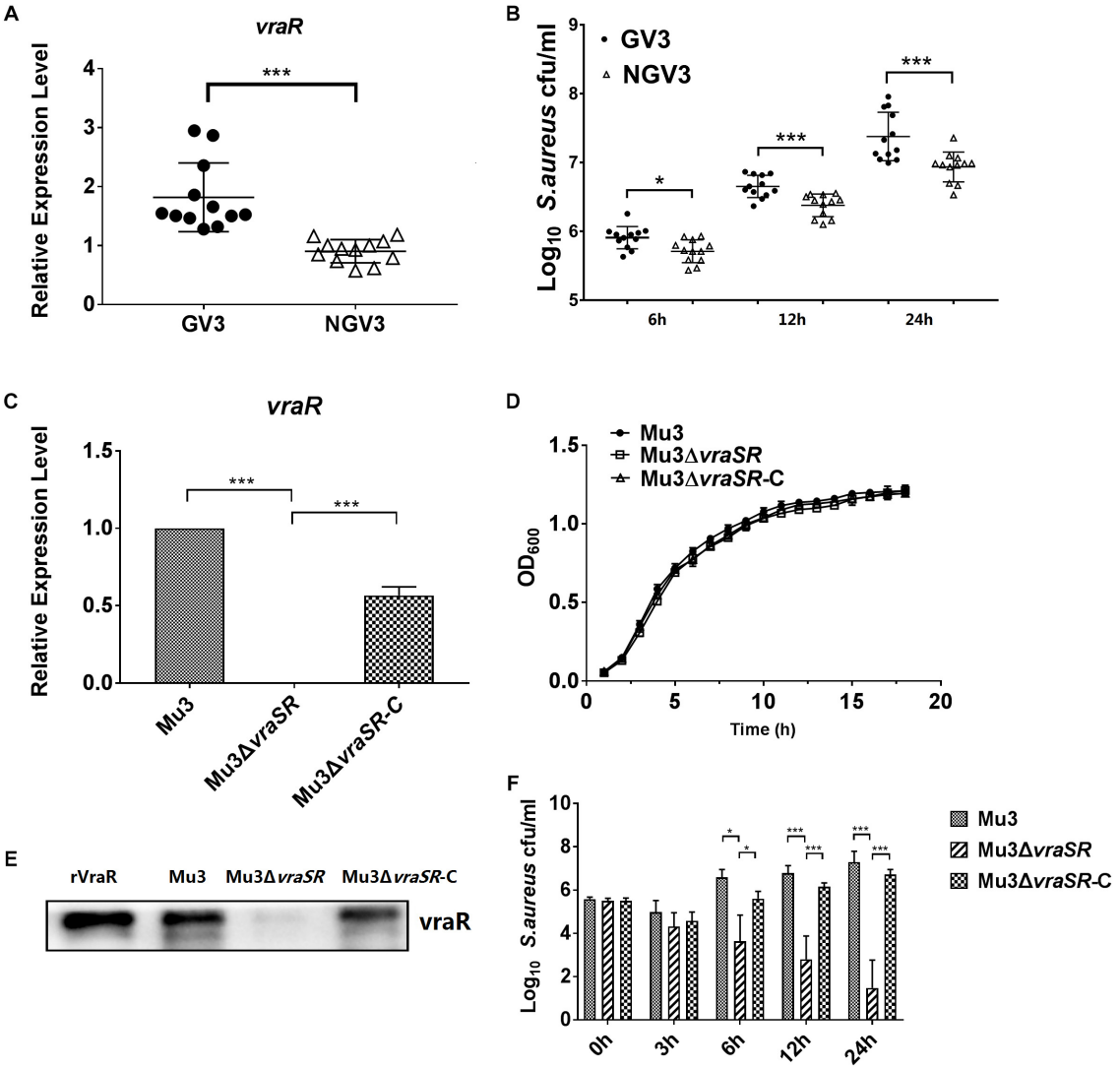

FIGURE 1 | The VraSR regulatory system is important for intracellular survival and proliferation of *S. aureus*. **(A)** Comparison of the expression levels of *vraR* between the GV3 (12 isolates) and NGV3 (12 isolates) groups of clinical isolates by qRT-PCR assay. The data are the mean ± SD from three independent experiments ($n = 3$). Statistical significance was determined using a Student's unpaired *t* test ($P < 0.01$). **(B)** Comparison of intracellular survival of *S. aureus* between GV3 (12 isolates) and NGV3 (12 isolates) clinical *S. aureus* isolates at each time point. The data are the mean ± SD from three independent experiments ($n = 3$). Statistical significance was determined using a Student's unpaired *t*-test ($P < 0.01$). **(C)** Comparison of the expression levels of *vraR* in *S. aureus* strains Mu3, Mu3Δ*vraSR*, and Mu3Δ*vraSR*-C by qRT-PCR assay. The data are the mean ± SD from three independent experiments ($n = 3$). Statistical significance was determined by one-way ANOVA with Bonferroni posttest ($P < 0.01$). **(D)** Growth curve analysis of Mu3, Mu3Δ*vraSR*, and Mu3Δ*vraSR*-C. Bacteria were cultured in TSB at 37°C with shaking at 200 rpm. OD_{600} values were measured three times at each time point ($n = 3$). Statistical significance was determined by a one-way ANOVA with a Bonferroni posttest ($P > 0.05$). **(E)** Comparison of the levels of VraR expression in strains Mu3, Mu3Δ*vraSR*, and Mu3Δ*vraSR*-C by western blot. Gels are representative of three independent experiments ($n = 3$). **(F)** Survival of intracellular *S. aureus* were determined at each time point. The *X*-axis represents hours post-infection and the *Y*-axis represents $\log_{10}$ CFU/ml *S. aureus*. The data are presented as the mean ± SD. The SD was calculated from experiments performed in triplicate ($n = 3$). Statistical significance was determined by one-way ANOVA with Bonferroni posttest ($P < 0.01$). $^*P < 0.05$; $^{***}P < 0.01$.

promotes the formation of autophagosomes. Moreover, we observed that Mu3Δ*vraSR*-infected cells had more single, red LC3 puncta compared with the other two groups, indicating that the VraSR regulatory system could block or delay the fusion of the autophagosome with the lysosome.

To further confirm the effect of the VraSR regulatory system on the autophagic pathway and to investigate whether the autophagic pathway can target intracellular *S. aureus*, RAW264.7 cells were pretreated with 3-MA (a well-established phosphatidylinositol 3-kinase (PI3K) inhibitor that prevents the induction of autophagy) or Baf A1 (a selective inhibitor of vacuolar H^+-ATPase that prevents lysosomal acidification) for 2 h prior to infection with *S. aureus*. And we examined the effects of 3-MA and Baf A1 on cells viability by Cell Counting Kit-8 (CCK-8) assay. RAW264.7 cells viability was not affected at 12 h treatment with 3-MA or Baf A1 at concentrations up to

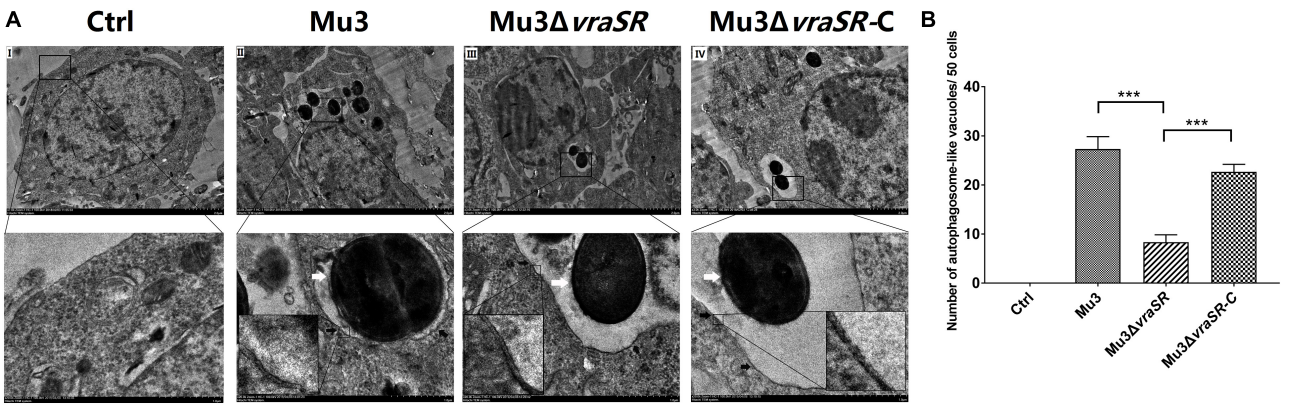

FIGURE 2 | The VraSR regulatory system contributes to the formation of autophagosome-like vacuoles in *S. aureus*-infected cells. **(A)** Ultrastructural features were examined by transmission electron microscopy (TEM) under 3000× magnification. Uninfected **(I)** RAW264.7 cells were compared with RAW264.7 cells infected with Mu3 **(II)**, Mu3Δ*vraSR* **(III)**, or Mu3Δ*vraSR*-C **(IV)** for 3 h. Boxed areas from I, II, III, and IV are magnified from the respective main images (white arrow, intracellular *S. aureus*; black arrow, autophagosome-like vacuole). **(B)** Number of autophagosome-like vacuoles observed by TEM. The data are presented as the mean ± SD. The SD was calculated from experiments performed in triplicate ($n = 3$). Statistical significance was determined by a one-way ANOVA with Bonferroni posttest. ***$P < 0.01$.

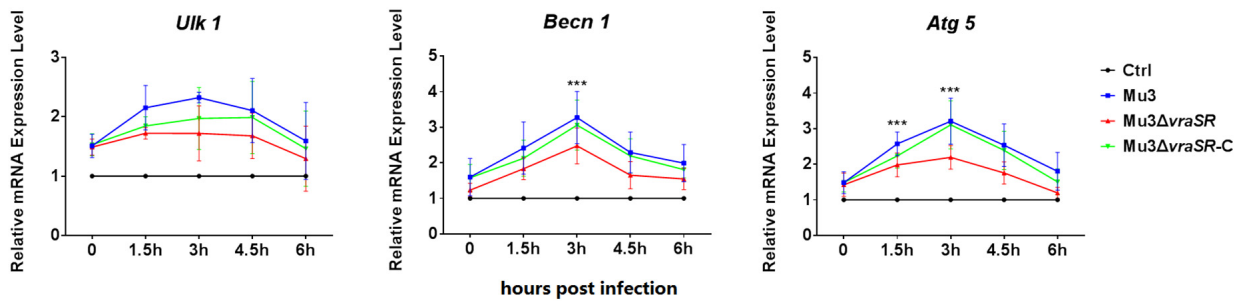

FIGURE 3 | The VraSR regulatory system contributes to the expression of autophagy-related genes. qRT-PCR was performed to analyze the transcriptional levels of *Ulk1*, *Atg5*, and *Becn1* in Mu3-, Mu3Δ*vraSR*-, or Mu3Δ*vraSR*-C-infected cells at each time point. The data are presented as the mean ± SD. The SD was calculated from experiments performed five times ($n = 5$). Statistical significance was determined by one-way ANOVA with Bonferroni posttest. ***$P < 0.01$.

1.25 mM or 100 nM, respectively (**Supplementary Figure S1**). And the results of growth curve assay showed that 3-MA and Baf A1 had no effect on the growth of *S. aureus* (**Supplementary Figure S2**). We observed that 3-MA treatment decreased the levels of LC3-II (**Figure 4C**) and significantly decreased the intracellular survival of Mu3 and Mu3Δ*vraSR*-C in RAW264.7 cells (**Figure 4D**). In comparison, Baf A1 treatment significantly increased the accumulation of autophagic substrate p62 protein in all infected groups (**Figure 4C**), suggesting that autophagic flux was decreased in infected cells. We also determined that the intracellular survival of Mu3 and Mu3Δ*vraSR*-C was significantly decreased following pretreatment 3-MA while the survival of Mu3Δ*vraSR* inside cells pretreated with Baf A1 was significantly increased (**Figure 4D**). These results further demonstrated that the autophagy pathway of RAW264.7 cells is inhibited by 3-MA, which decreased the intracellular survival of *S. aureus*. Baf A1 inhibited autophagic flux and enhanced the survival of Mu3Δ*vraSR*. Taken together, these results indicate that *S. aureus* uses the VraSR regulatory system to block autophagic flux for increasing intracellular survival.

DISCUSSION

While autophagy is a cytosolic catabolic process in eukaryotic cells, it is also an innate defense mechanism against invading pathogenic bacteria (Gong et al., 2012). However, recent reports have shown that autophagy may play different roles during the infection of different bacterial pathogens, in addition to its known involvement in bacterial clearance, coordinating autonomous cell signaling, and, in some cases, promoting bacterial replication (Mostowy, 2013). Although autophagosomes are reported to be intracellular niches for *S. aureus*, the underlying mechanisms by which *S. aureus* triggers the autophagy machinery were poorly understood (Schnaith et al., 2007; Neumann et al., 2016). In the current study, we showed that *S. aureus* uses the VraSR regulatory system to enhance intracellular survival and increase the number of autophagic vesicles in *S. aureus*-infected cells, indicating that *S. aureus* uses the VraSR regulatory system to modulate autophagy in RAW264.7 cells.

The formation of the autophagosome involves the assembly of 36 autophagy-related (ATG) proteins into complexes

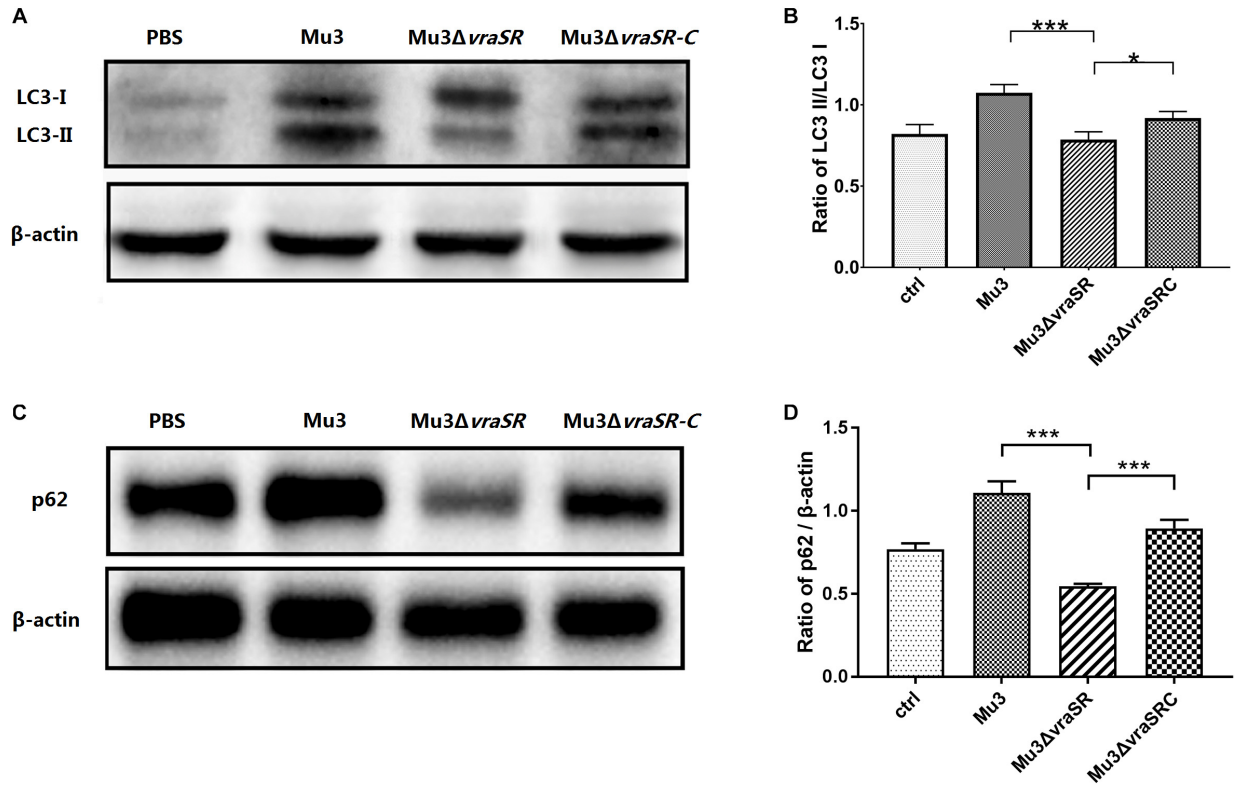

FIGURE 4 | The VraSR regulatory system is involved in the regulation of autophagic protein LC3 turnover and P62 degradation. **(A)** Western blot analysis of the expression of LC3-I and LC3-II in RAW264.7 cells. Gels are representative of three independent experiments ($n = 3$). **(B)** Semi-quantitative analyses of LC3-II/LC3-I expression based on the density of bands on the western blot. The data are presented as the mean ± SD. The SD was calculated from experiments performed in triplicate ($n = 3$). Statistical significance was determined by one-way ANOVA with Bonferroni posttest. **(C)** Western blot analysis of the expression of p62 in RAW264.7 cells. β-actin was used as the loading control. Gels are representative of three independent experiments ($n = 3$). **(D)** Semi-quantitative analyses of p62/β-actin expression based on the density of bands on the western blot. The data are presented as the mean ± SD. The SD was calculated from experiments performed in triplicate ($n = 3$). Statistical significance was determined by one-way ANOVA with Bonferroni posttest. $^*P < 0.05$; $^{***}P < 0.01$.

that are essential for different steps of the process: the ULK1 complex triggers autophagy, the beclin 1 and class III phosphatidylinositol 3-OH kinase (PI3KC3) complex generates an essential lipid component of autophagosomes, and the ATG12-ATG5-ATG16L1 ubiquitin-like conjugation system mediates formation and elongation of the autophagosome. As autophagy is a dynamic process, we selected *Ulk1*, *Becn1*, and *Atg5* as markers of the three stages of autophagy, qRT-PCR analysis was then performed to examine the mRNA levels of each of the three genes. The transcript levels of *Becn1* and *Atg5* were significantly decreased in Mu3Δ*vraSR*-infected cells, indicating that the VraSR regulatory system contributes to increase the expression of autophagy-related genes. At the same time, we observed that the VraSR regulatory system participates in modulation of autophagic protein LC3 turnover and P62 degradation. Confocal microscopy-based morphological analyses revealed that the VraSR regulatory system plays an important role in *S. aureus*-induced autophagosome maturation and inhibition of autolysosome formation. Therefore, our findings suggest that the VraSR regulatory system positively contributes to bacterial survival in RAW264.7 cells in two ways: first, hVISA strains use the VraSR regulatory system to promote autophagy, thereby recruiting LC3 protein to develop the autophagosomes; and second, hVISA strains use the VraSR regulatory system to interfere with autophagosome and lysosome fusion, thereby enhancing the intracellular survival *S. aureus*.

According to the literature, various bacterial species exploit autophagy and promote the formation of autophagic vacuoles in which to multiply by regulating their cell wall components or virulence factors (Campoy and Colombo, 2009; Irving et al., 2014; Castrejon-Jimenez et al., 2015). For example, peptidoglycan is one of the most components of the *Listeria monocytogenes* cell wall. As such, peptidoglycan and its cleavage products are recognized by peptidoglycan-recognition protein, which induces autophagy (Yano et al., 2008). Intracellular *L. monocytogenes* then hijacks autophagy in macrophages by secreting the virulence factor listeriolysin O to evade killing (Zhang et al., 2019). In *S. aureus* strains, two-component system VraSR can up-regulate the synthesis of peptidoglycan, resulting in an increase in D-alanine-D-alanine residues (Kuroda et al., 2003). However, whether VraSR can induce autophagy via thickening of the peptidoglycan layer and increasing its cleavage products as well as *L. monocytogenes* needs further verification.

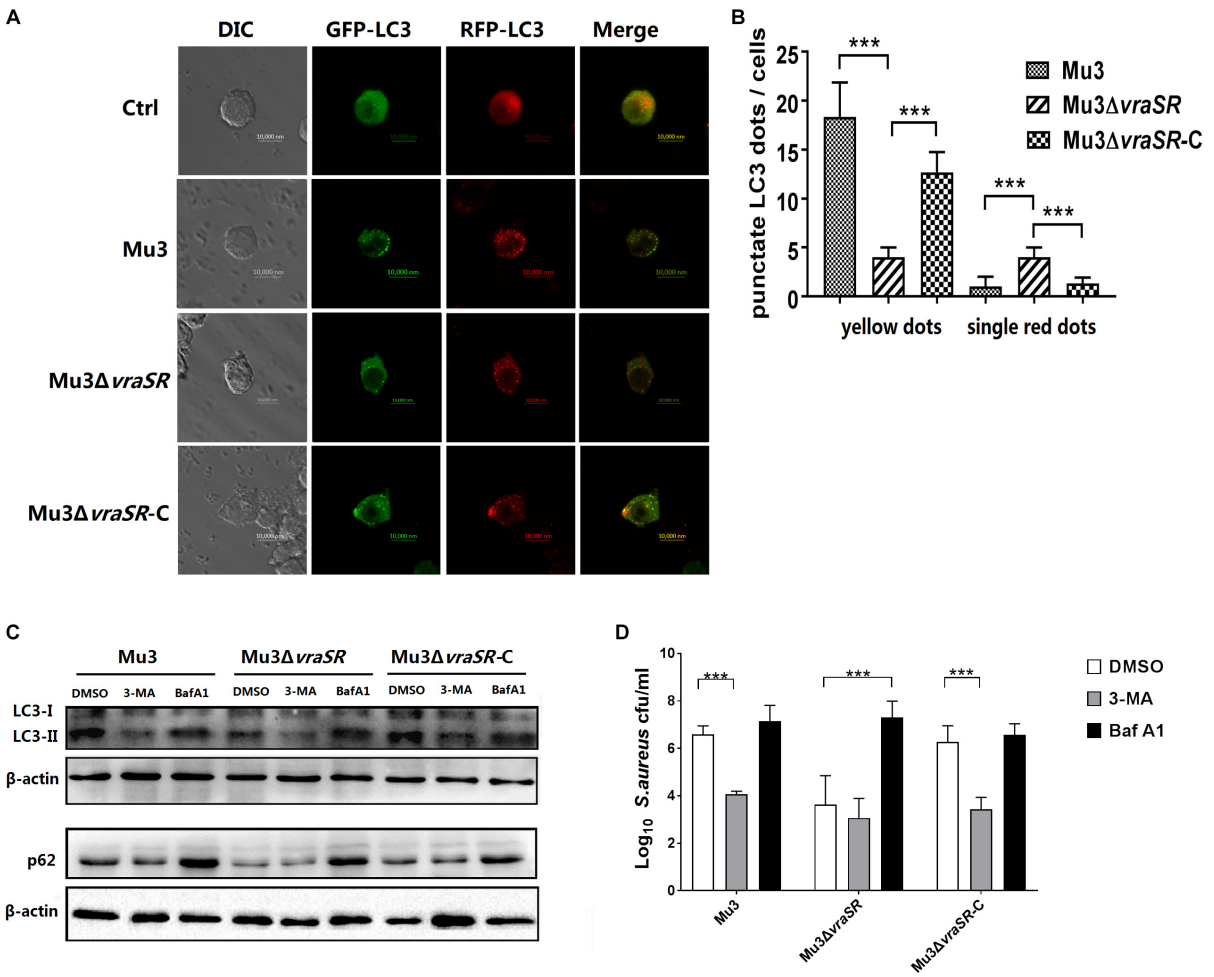

FIGURE 5 | The VraSR regulatory system is involved in autophagic flux inhibition in *S. aureus*-infected cells. **(A)** RAW264.7 cells stably expressing mRFP-GFP-LC3 were infected with Mu3, Mu3Δ*vraSR*, or Mu3Δ*vraSR*-C for 3 h. Representative differential interference contrast (DIC) and corresponding GFP and RFP fluorescence images are shown. Bars, 10 μm. **(B)** Quantitative measurement of autophagic flux. The number of punctate dots was enumerated in at least 100 cells at 3 hpi. The *X*-axis represents the average number of puncta/cell. The data are presented as the mean ± SD. The SD was calculated from experiments performed in triplicate ($n = 3$). Statistical significance was determined by one-way ANOVA with Bonferroni posttest. **(C)** RAW264.7 cells were pretreated with 3-MA or Baf A1 for 2 h before being infected with *S. aureus* for 3 h. Cells were then lysed to examine LC3 and p62 protein levels by western blot. β-actin are used as the loading control. Gels were representative of three independent experiments ($n = 3$). **(D)** Quantification of intracellular bacteria in RAW264.7 cells infected with *S. aureus* for 6 h in the presence or absence of 3-MA or Baf A1. The *X*-axis represents different strains and the *Y*-axis represents $\log_{10}$ CFU/ml *S. aureus*. The data are presented as the mean ± SD. The SD was calculated from experiments performed in triplicate ($n = 3$). Statistical significance was determined by one-way ANOVA with Bonferroni posttest. ***$P < 0.01$.

Our previous study demonstrated the capacity of VraSR to modulate *S. aureus* virulence by binding the P2–P3 intergenic region of the *agr* promoter (Dai et al., 2017). The Agr quorum-sensing system is a key regulatory system in Staphylococci, controlling the expression of a number of virulence factors. It is also essential for *S. aureus* survival within macrophages (Kubica et al., 2008). Some reports have also suggested that factors regulated by Agr are required for an autophagic response to *S. aureus* infection (Schnaith et al., 2007; O'Keeffe et al., 2015; Soong et al., 2015). Therefore, we speculate that the mechanism via which the VraSR regulatory system regulates autophagy may be related to the expression of Agr, which we plan to investigate in future.

In this work, we determined that *S. aureus* uses the VraSR regulatory system to block autophagic flux and delay the early stage of autophagosome formation, thereby promoting bacterial survival inside RAW264.7 cells. Although this study is limited by the use of only a single murine cell lineage and one *S. aureus* strain, to our knowledge, this is first report showing that the VraSR two-component system is responsible for the onset of autophagy in eukaryotic cells. Our findings provide new insights into the impact of VraSR on bacterial infection and will help to further elucidate the relationship between bacteria and host immune response. Importantly, our results suggest that VraSR may be a potential target for preventing or treating VISA/hVISA infection.

AUTHOR CONTRIBUTIONS

YD, XM, and JL contributed conception and design of the study. YD organized the database. CG and WC performed the statistical analysis. YD wrote the first draft of the manuscript. LC, WC, WY, and XM wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01222/full#supplementary-material

FIGURE S1 | CCK8 analysis of cell viability of RAW264.7 cells treated with different concentrations of 3-MA **(A)** or Baf A1 **(B)** for 12 h. 3-MA did not show cytotoxicity at concentrations of up to 1.25 mM and Baf A1 did not show cytotoxicity at concentrations of up to 100 nM.

FIGURE S2 | Growth curves of Mu3 **(A)**, Mu3ΔvraSR **(B)**, and Mu3ΔvraSR-C **(C)** cultured in presence or absence of 3-MA or Baf A1. Bacteria were grown in TSB at 37°C with shaking at 200 rpm. Values of $OD_{600\ nm}$ were measured every 1 h. Values are from three biological replicates ± SEM. Statistical significance was determined by a one-way ANOVA with Bonferroni posttest ($P > 0.05$).

TABLE S1 | Strains an plasmids used in this study.

TABLE S2 | Primers used in this study.

REFERENCES

Barth, S., Glick, D., and Macleod, K. F. (2010). Autophagy: assays and artifacts. *J. Pathol.* 221, 117–124. doi: 10.1002/path.2694

Cameron, D. R., Lin, Y. H., Trouillet-Assant, S., Tafani, V., Kostoulias, X., Mouhtouris, E., et al. (2017). Vancomycin-intermediate *Staphylococcus aureus* isolates are attenuated for virulence when compared with susceptible progenitors. *Clin. Microbiol. Infect.* 23, 767–773. doi: 10.1016/j.cmi.2017.03.027

Campoy, E., and Colombo, M. I. (2009). Autophagy in intracellular bacterial infection. *Biochim. Biophys. Acta* 1793, 1465–1477. doi: 10.1016/j.bbamcr.2009.03.003

Castrejon-Jimenez, N. S., Leyva-Paredes, K., Hernandez-Gonzalez, J. C., Luna-Herrera, J., and Garcia-Perez, B. E. (2015). The role of autophagy in bacterial infections. *Biosci. Trends* 9, 149–159. doi: 10.5582/bst.2015.01035

Chang, W., Ding, D., Zhang, S., Dai, Y., Pan, Q., Lu, H., et al. (2015). Methicillin-resistant *Staphylococcus aureus* grown on vancomycin-supplemented screening agar displays enhanced biofilm formation. *Antimicrob. Agents Chemother.* 59, 7906–7910. doi: 10.1128/aac.00568-15

Dai, Y., Chang, W., Zhao, C., Peng, J., Xu, L., Lu, H., et al. (2017). VraR binding to the promoter region of agr inhibits its function in vancomycin-intermediate *Staphylococcus aureus* (VISA) and heterogeneous VISA. *Antimicrob. Agents Chemother.* 61:e02740-16. doi: 10.1128/aac.02740-16

Gardete, S., Kim, C., Hartmann, B. M., Mwangi, M., Roux, C. M., Dunman, P. M., et al. (2012). Genetic pathway in acquisition and loss of vancomycin resistance in a methicillin resistant *Staphylococcus aureus* (MRSA) strain of clonal type USA300. *PLoS Pathog.* 8:e1002505. doi: 10.1371/journal.ppat.1002505

Gomes, L. C., and Dikic, I. (2014). Autophagy in antimicrobial immunity. *Mol. Cell* 54, 224–233. doi: 10.1016/j.molcel.2014.03.009

Gong, L., Devenish, R. J., and Prescott, M. (2012). Autophagy as a macrophage response to bacterial infection. *IUBMB Life* 64, 740–747. doi: 10.1002/iub.1070

Irving, A. T., Mimuro, H., Kufer, T. A., Lo, C., Wheeler, R., Turner, L. J., et al. (2014). The immune receptor NOD1 and kinase RIP2 interact with bacterial peptidoglycan on early endosomes to promote autophagy and inflammatory signaling. *Cell Host Microbe* 15, 623–635. doi: 10.1016/j.chom.2014.04.001

Katayama, Y., Azechi, T., Miyazaki, M., Takata, T., Sekine, M., Matsui, H., et al. (2017). Prevalence of slow-growth vancomycin nonsusceptibility in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 61:e00452-17. doi: 10.1128/aac.00452-17

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., et al. (2008). A potential new pathway for Staphylococcus aureus dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS One* 3:e1409. doi: 10.1371/journal.pone.0001409

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., and Hiramatsu, K. (2003). Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol. Microbiol.* 49, 807–821. doi: 10.1046/j.1365-2958.2003.03599.x

Levine, B., Mizushima, N., and Virgin, H. W. (2011). Autophagy in immunity and inflammation. *Nature* 469, 323–335. doi: 10.1038/nature09782

Li, D., Guo, Y., Wang, S., Lv, J., Qi, X., Chen, Z., et al. (2017). capB2 expression is associated with *Staphylococcus aureus* pathogenicity. *Front. Microbiol.* 8:184. doi: 10.3389/fmicb.2017.00184

Lowy, F. D. (1998). *Staphylococcus aureus* infections. *N. Engl. J. Med.* 339, 520–532. doi: 10.1056/NEJM199808203390806

Mizushima, N., Yoshimori, T., and Levine, B. (2010). Methods in mammalian autophagy research. *Cell* 140, 313–326. doi: 10.1016/j.cell.2010.01.028

Monaco, M., Pimentel de Araujo, F., Cruciani, M., Coccia, E. M., and Pantosti, A. (2017). Worldwide epidemiology and antibiotic resistance of *Staphylococcus aureus*. *Curr. Top. Microbiol. Immunol.* 409, 21–56. doi: 10.1007/82_2016_3

Mostowy, S. (2013). Autophagy and bacterial clearance: a not so clear picture. *Cell Microbiol.* 15, 395–402. doi: 10.1111/cmi.12063

Mostowy, S. (2014). Multiple roles of the cytoskeleton in bacterial autophagy. *PLoS Pathog.* 10:e1004409. doi: 10.1371/journal.ppat.1004409

Munzenmayer, L., Geiger, T., Daiber, E., Schulte, B., Autenrieth, S. E., Fraunholz, M., et al. (2016). Influence of Sae-regulated and Agr-regulated factors on the escape of *Staphylococcus aureus* from human macrophages. *Cell Microbiol.* 18, 1172–1183. doi: 10.1111/cmi.12577

Neumann, Y., Bruns, S. A., Rohde, M., Prajsnar, T. K., Foster, S. J., and Schmitz, I. (2016). Intracellular *Staphylococcus aureus* eludes selective autophagy by activating a host cell kinase. *Autophagy* 12, 2069–2084. doi: 10.1080/15548627.2016.1226732

Ohsumi, Y. (2014). Historical landmarks of autophagy research. *Cell Res.* 24, 9–23. doi: 10.1038/cr.2013.169

O'Keeffe, K. M., Wilk, M. M., Leech, J. M., Murphy, A. G., Laabei, M., Monk, I. R., et al. (2015). Manipulation of autophagy in phagocytes facilitates *Staphylococcus aureus* bloodstream infection. *Infect. Immun.* 83, 3445–3457. doi: 10.1128/IAI.00358-15

Schnaith, A., Kashkar, H., Leggio, S. A., Addicks, K., Kronke, M., and Krut, O. (2007). *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death. *J. Biol. Chem.* 282, 2695–2706. doi: 10.1074/jbc.m609784200

Siqueira, M. D. S., Ribeiro, R. M., and Travassos, L. H. (2018). Autophagy and its interaction with intracellular bacterial pathogens. *Front. Immunol.* 9:935. doi: 10.3389/fimmu.2018.00935

Soong, G., Paulino, F., Wachtel, S., Parker, D., Wickersham, M., Zhang, D., et al. (2015). Methicillin-resistant *Staphylococcus aureus* adaptation to human keratinocytes. *MBio* 6:e00289-15. doi: 10.1128/mBio.00289-15

Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., Ueda, R., et al. (2008). Autophagic control of listeria through intracellular innate immune recognition in drosophila. *Nat. Immunol.* 9, 908–916. doi: 10.1038/ni.1634

Yla-Anttila, P., Vihinen, H., Jokitalo, E., and Eskelinen, E. L. (2009). Monitoring autophagy by electron microscopy in Mammalian cells. *Methods Enzymol.* 452, 143–164. doi: 10.1016/s0076-6879(08)03610-0

Zhang, Y., Yao, Y., Qiu, X., Wang, G., Hu, Z., Chen, S., et al. (2019). Listeria hijacks host mitophagy through a novel mitophagy receptor to evade killing. *Nat. Immunol.* 20, 433–446. doi: 10.1038/s41590-019-0324-2

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REVIEW
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The Commensal Microbiota and Viral Infection: A Comprehensive Review

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The human body is inhabited by a diverse microbial community that is collectively coined as commensal microbiota. Recent research has greatly advanced our understanding of how the commensal microbiota affects host health. Among the various kinds of pathogenic infections of the host, viral infections constitute one of the most serious public health problems worldwide. During the infection process, viruses may have substantial and intimate interactions with the commensal microbiota. A plethora of evidence suggests that the commensal microbiota regulates and is in turn regulated by invading viruses through diverse mechanisms, thereby having stimulatory or suppressive roles in viral infections. Furthermore, the integrity of the commensal microbiota can be disturbed by invading viruses, causing dysbiosis in the host and further influencing virus infectivity. In the present article, we discuss current insights into the regulation of viral infection by the commensal microbiota. We also draw attention to the disruption of microbiota homeostasis by several viruses.

Keywords: commensal microbiota, germ-free, antibiotics, virus, virus infectivity, antiviral immunity

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INTRODUCTION

Emerging data suggest that the human body is inhabited by a wide range of microorganisms that are collectively referred to as the commensal microbiota. A majority of the microbiota reside in the intestine, while distinct populations can also be found on the surfaces of the mouth, skin, and urinary tract (1–3). A wealth of evidence suggests that this incredibly diverse microbial community is regulated by host genetic factors, and more importantly, environmental and dietary factors (4–6). We now know that the coevolution of the commensal microbiota and their hosts has resulted in a mutually beneficial condition in which the host can benefit from physiological, metabolic, and immunological regulations provided by the microbiota, while the commensal microbiota depends absolutely on the host for nutrient acquisition and propagation sites (7). For example, the gut microbiota has a crucial role in shaping immune development and functionality in the host, as reflected by extensive defects in the development of gut-associated lymphoid tissues, significantly smaller and fewer mesenteric lymph nodes and Peyer's patches, reduced secretory immunoglobulin A (IgA) production, and abnormal intestinal T cell development in germ-free (GF) mice (8–10). In addition, the gut microbiota helps the host break down dietary substances that are too large to be digested, a process that produces critical nutrients and energy for the host and generates active products (i.e., short-chain fatty acids, lactic acid, choline, and bile acids) that are essential for host health (11, 12).

The recent awareness of the essential role of the commensal microbiota in host health has remarkably improved our understanding of the interactions between microbiota and invading pathogens. In fact, a healthy commensal microbiota, as well as its products, is essential for

protecting the host against a variety of pathogenic infections, through both direct elimination and indirect suppression, inside or outside of the gastrointestinal tract (13–16). Among the invading pathogens, viruses constitute one of the most common. During their infection processes, various viruses encounter the commensal microbiota of the hosts, making it possible that there are robust interactions between these viruses and the commensal microbiota. Indeed, a plethora of evidence has now shown that the commensal microbiota regulates and is inevitably regulated by invading viruses through a series of mechanisms, thereby yielding harmful or beneficial outcomes for the host (17–19). In the regulation of viral infection, commensal microbiota play varied and critical roles. They can promote viral infectivity through diverse mechanisms and can also exert substantial inhibitory effects on viral infection. On the other hand, a viral infection usually results in substantial perturbations in the commensal microbiota, causing dysbiosis in the host, which may in turn further affect viral infectivity. Although there have been several excellent reviews summarizing the modulation of viral infections by the commensal microbiota (17, 19–21), most of them focused only on infections by enteric viruses. In addition, none of these articles discussed commensal microbiota at sites other than the gut. Moreover, there have not been any literatures describing the effect of viral infections on the compositional and functional alterations of the commensal microbiota. In the present article, we systematically discuss the current progress concerning the modulation of various types of viral infections by the commensal microbiota. We also highlight the relevant mechanisms underlying these observations. In addition, we further describe the disruption of microbiota composition or homeostasis by viral infections and the relevant mechanisms.

PROMOTION OF VIRAL INFECTION BY THE GUT MICROBIOTA (SUMMARIZED IN TABLE 1)

Direct Promotion of Viral Infection

Facilitating Genetic Recombination

The commensal microbiota can facilitate genetic recombination of viruses to enhance their infectivity. This is true for the poliovirus infection. Several studies have demonstrated that RNA viruses such as poliovirus benefit from the delivery of various viral genomes into a single target cell, thereby allowing the recombination of multiple viral genomes, and this process potentiates the viral progeny with enhanced environmental fitness (36–38). Using polioviruses encoding either DsRed or GFP and HeLa cells as target cells, Erickson et al. found that preincubation of these viruses with certain commensal microbiota significantly increased the percentage of DsRed and GFP dual-positive cells compared with those preincubated with PBS (22). Mechanistically, bacterial adhesion to HeLa cells was the main promoting force for viral coinfection. Importantly, by employing two types of viruses that are either sensitive to the drug guanidine hydrochloride while resistant to high temperature ($Drug^{S}Temp^{R}$) or resistant to guanidine hydrochloride while sensitive to high temperature ($Drug^{R}Temp^{S}$), the authors found that preincubation of viruses with bacteria increased recombination yields significantly, as reflected by the generation of polioviruses with a $Drug^{R}Temp^{R}$ phenotype, and that the recombination frequencies were positively correlated with coinfection frequencies (22). Collectively, these data suggest that interactions of the commensal microbiota and poliovirus prior to infection increases the possibility that a cell will be infected by two or more viruses, which further facilitates genome recombination of the viruses, thereby generating progenies with more diverse populations and with increased resistance to otherwise restrictive conditions.

Enhancing Virion Stability

In addition to facilitating genetic recombination, bacterial surface polysaccharides, i.e., peptidoglycan and lipopolysaccharide (LPS), can enhance virion stability through several mechanisms, which have been demonstrated mostly for poliovirus and reovirus. For example, gut microbiota depletion with antibiotics prior to poliovirus infection results in less susceptibility of mice and minimal viral replication in the intestine (23). Notably, when orally inoculated poliovirus was isolated from the lumen contents of untreated, antibiotic-treated, and germ-free mice, significantly higher infectivity was identified for poliovirus isolated from untreated mice. In addition, at temperatures above 40°C, markedly increased poliovirus stability was identified when they were preincubated with untreated feces or feces from germ-free mice that had been supplemented with certain bacteria. Importantly, the enhancement of viral stability did not necessarily require live bacteria, as UV-inactivated bacteria, as well as bacterial surface polysaccharides (LPS and peptidoglycan), significantly increased the viral yield over PBS when incubated with poliovirus. Furthermore, using a poliovirus mutant with reduced LPS-binding capacity, which was generated by a single amino acid substitution in the viral capsid protein VP1-T99K, the same group of authors found that while the mutant viruses showed similar replication, attachment, shedding, and pathogenesis with wild-type viruses following peroral inoculation, they displayed poorer environmental stability compared to their wild-type counterparts, as highlighted by the findings that mutant viruses were more unstable in feces and that an additional cycle of infection in mice aggravated this instability (24).

Consistent with the findings shown in poliovirus infection, another enteric virus, reovirus, also uses commensal microbiota or bacterial components to enhance thermostability (25). Similar to poliovirus, the pathogenesis of reovirus is also negatively affected by antibiotic treatment prior to infection (23). Mechanistically, the direct interaction of reovirus virions with Gram-negative and Gram-positive bacteria promotes the attachment to and infection of target cells at a variety of temperatures (23). It should be noted that commensal bacteria do not affect the overall number of viral capsid proteins, indicating that the bacterial effect on the reovirus is not exerted through regulating the overall number of viral capsid proteins (23). Collectively, these findings highlight the notion that interactions with commensal microbiota can increase the infectivity of viruses by enhancing virion stability.

TABLE 1 | Promotion of viral infections by the commensal microbiota and the relevant mechanisms.

	Mechanisms	Virus types	References
Facilitating genetic recombination	Increasing the possibility that a cell will be infected by more than one virus	Poliovirus	(22)
Enhancing virion stability	Bacterial surface polysaccharides enhance the environmental stability of the virus	Poliovirus, reovirus	(23–25)
Stimulating lytic reactivation	SCFAs reactivate the lytic stage	Herpesvirus, Epstein-Barr virus	(26)
Driving the proliferation of target cells	Promoting the proliferation of CD300lf-expressing tuft cells in the colon	Norovirus	(27, 28)
Stimulating attachment to permissive cells	Increasing the binding of virus to PVR-expressing target cells	Poliovirus	(23, 24)
Contributing to viral replication	HBGA-expressing bacteria control viral replication	Norovirus	(29)
Inducing the production of immunoregulatory cytokines	LPS stimulates the production of IL-6, inducing IL-10 secretion	MMTV	(30–32)
Suppressing local antiviral immune responses	Inhibiting IFN-λ production and virus-specific immunoglobulin production.	Norovirus, rotavirus, retrovirus	(33–35)

Stimulating Lytic Reactivation

The lytic stages during viral infection involve viral gene expression, viral DNA replication and the production of new virions, making this stage indispensable for transmission and persistence of viruses (39). The direct promotion of viral infectivity by the commensal microbiota is also reflected by the stimulation of lytic reactivation by the commensal microbiota. Asai et al. found that short-chain fatty acids (SCFA) present in the culture fluids of oral bacteria induced the synthesis of early antigens in Epstein-Barr viruses (40). In addition, Gorres et al. used several short-chain fatty acids (SCFAs) and their inhibitors to explore the effect of SCFAs on lytic reactivation of Epstein-Barr virus and herpesvirus. Their results showed that all SCFAs that are histone deacetylase inhibitors can reactivate herpesvirus, whereas only several of these SCFAs reactivated the Epstein-Barr virus (26). As is widely reported, the production of SCFAs is the result of a complex interaction between the gut microbiota and diet (41, 42). These results demonstrated that there is likely a link between commensal microbiota and the lytic reactivation of viruses.

Driving the Proliferation of Target Cells

Tuft cells are a rare type of intestinal epithelial cells that are the reservoir for fecal shedding and persistence of murine norovirus (43). Similar to certain commensal bacteria that express receptors for human norovirus, tuft cells also express a functional receptor for norovirus, CD300lf, the expression of which dictates norovirus tropism and the efficient establishment of enteric norovirus infections (44). Elegant work from Virgin et al. revealed that both type-2 cytokines and the commensal microbiota are critical in governing the proliferation of tuft cells. In antibiotic-treated mice, a marked decrease in tuft cell-specific gene expression in the colon was observed, accompanied by a reduced number of tuft cells in the colon, a phenomenon that can be rescued by adding the type-2 cytokines interleukin (IL)-4 and IL-25 (27, 28).

Stimulating Attachment to Permissive Cells

The elegant work of Kuss et al. revealed that both Gram-negative and Gram-positive bacteria are potent enhancers of poliovirus infectivity (23). The authors used ^{35}S-labeled poliovirus and HeLa cells and established an *in vitro* infection model. In this system, when poliovirus was incubated with *Bacillus cereus* before incubation with HeLa cells, the virus displayed dramatically increased infectivity and enhanced adherence to HeLa cells (23). Further work by the same group revealed that increased viral attachment to target cells was mainly mediated by the direct facilitation of viral binding to the poliovirus receptor (PVR) by bacterial surface polysaccharides (24). Consistent with this observation, pretreatment of HeLa cells with anti-PVR antibody significantly reduced the binding of poliovirus to HeLa cells, regardless of whether the virus was preincubated with LPS. Mechanistically, LPS treatment directly enhanced the PVR-binding ability of poliovirus, thereby stimulating attachment of the virus to target cells.

Contributing to Viral Replication

Certain types of viruses have evolved to interact with and use members of the host microbiota or their components to achieve optimal replication. Histo-blood group antigens (HBGAs) have been identified as receptors or coreceptors for human noroviruses. As reported, certain species of enteric bacteria express HBGAs (45). Jones et al. found that the binding of norovirus and HBGA-expressing bacteria determines the transmission and infection process of these viruses in their hosts, as infection of B cells by human norovirus can only be achieved with the presence of HBGA-positive enteric bacteria. Notably, the antibiotic depletion of normal enteric flora resulted in dramatically decreased virus titers, the mechanism of which presumably lay in the control of viral replication by the commensal microbiota (29). However, direct evidence for the control of viral replication by the commensal microbiota is lacking in this study.

Indirect Promotion of Viral Infection

Inducing an Immunoregulatory Microenvironment

Emerging evidence suggests that a rich and diverse commensal microbiota plays an essential role in modulating the development of the host immune system, both inside and outside of the gut (46–49). This is true not only for the eliciting of effector

immune responses by stimulating the production of various proinflammatory cytokines such as interferon (IFN)-γ during infection, but also for the establishment of an immunotolerant microenvironment by contributing to the generation of immunoregulatory cells such as Treg cells to maintain homeostasis (50–52). In fact, the commensal microbiota profoundly dictates the development, differentiation, and activation of colonic regulatory T (Treg) cells, which contribute to the maintenance of homeostasis against components of the commensal microbiota and innocuous food antigens (51). Therefore, it is possible that commensal microbiota-induced Treg cells and Treg cell-related cytokines limit the degrees of antiviral immune responses.

Several lines of evidence add to this idea. In a model of mouse mammary tumor virus (MMTV) infection, interactions between the intestinal microbiota and the invading MMTV led to an immune evasion pathway for the virus, as intestinal microbiota-derived LPS can be utilized by MMTV to generate an IL-6-dependent induction of the immunoregulatory cytokine IL-10, a key cytokine mediating the immunoregulatory functions of Treg cells (30). However, MMTV was rapidly lost in toll-like receptor 4 (TLR4) mutant mice, which exhibited robust antiviral cytotoxic immune responses (31). The same group further found that the interactions between MMTV and LPS could not be achieved without the expression of LPS-binding protein (LBP), as reflected by the fact that MMTV isolated from mice lacking LBPs cannot capture LPS and stimulate TLR4, thereby showing a remarkable transmission defect (32). Interestingly, binding to MMTV would dramatically potentiate the LPS stimulation of TLR4 expression and induction of IL-6 production compared to those with virus-free LPS, indicating that virus incorporation guarantees a greater immunostimulatory ability of LPS (32). Collectively, these data indicate that interactions between the commensal microbiota and MMTV foster the establishment of an immunotolerant microenvironment in the host and lead to persistent viral infection.

Suppressing Local Antiviral Immune Responses

In addition to fostering the generation of immunoregulatory Treg cells, the commensal microbiota also directly skews antiviral immunity by suppressing the activation of effector immune cells and by inhibiting the production of various inflammatory cytokines that are pivotal for virus elimination, thus creating a more favoring environment for viral infection. This is true for norovirus. In a murine norovirus infection model, the authors found that antibiotic treatment prevented persistent viral infection, a phenomenon that was reversed by replenishment of the commensal microbiota (33). Interestingly, antibiotics did not directly affect viral replication or prevent tissue infection but acted specifically to trigger the expression of receptors for antiviral cytokine IFN-λ and to stimulate the expression of *Stat1* and *Irf3*. In another murine model of norovirus infection, while IL-$10^{-/-}$ SPF mice showed dramatically aggravated intestinal inflammation and mucosa damage, IL-$10^{-/-}$ GF mice were free of epithelial barrier disruption, and transplantation of defined flora to these mice was sufficient to restore inflammatory lesions in the intestine (53).

In addition, mounting research has shown that the commensal microbiota also hinders the activation of antiviral humoral responses, mainly through regulating the production of virus-specific antibodies. In a murine rotavirus infection model, commensal microbiota elimination via antibiotic treatment or germ-free housing reduced the level of rotavirus antigen, delayed infection and decreased infectivity significantly (34). Notably, this phenotype was accompanied by a stronger antiviral humoral response, as more enhanced serum IgA, serum IgG and fecal IgA levels were observed. Consistent with these findings, antibiotic treatment results in greater maintenance of virus-specific antibody-secreting cells in the intestine. In contrast, when mice were treated with a low dose of dextran sodium sulfate to generate enhanced exposure to the microbiota, impaired production of rotavirus-specific antibodies following virus infection was identified (34). This finding is further supported by an independent study, which showed that although wild-type mice efficiently controlled endogenous retrovirus to a baseline level, mice with a defective antibody-secreting ability could not prevent viral activation and propagation (35). Importantly, this conclusion was true only when the intestinal microbiota was intact, as viral replication was clearly prevented in the host mice when these mice were kept in a germ-free condition, regardless of whether they had antibody-producing abilities or not, further supporting that the commensal microbiota promotes viral infectivity through suppressing the antiviral humoral immune response (35).

SUPPRESSION OF VIRAL INFECTION BY THE GUT MICROBIOTA (SUMMARIZED IN TABLE 2)

Direct Suppression of Viral Infection

Because the commensal microbiota is present at sites that are used by certain viruses to gain entry into the host, it is likely that there are substantial interactions between the invading viruses and commensal microbiota that could have suppressive outcomes for viral infection. Supporting this notion, it was shown that lactic bacteria are able to reduce the infectivity of vesicular stomatitis virus through direct binding to the viruses, thereby blocking the cell internalization process of these viruses (54). In addition, *Enterococcus faecium* can prevent infection by influenza viruses upon direct adsorptive trapping of these viruses (55). Organisms of the commensal microbiota also produce various metabolites with antimicrobial effects to prevent virus infection. This is true for the inhibition of infections by influenza virus. First, it was found that commensal microbiota-derived LPS can bind to and destabilize the morphology of influenza virions, thereby decreasing the overall stability of the virus (56). Second, an extracellular matrix-binding protein produced by *Staphylococcus epidermidis*, a Gram-positive bacterium that lives in the human nasal cavity as a commensal, can stably bind to influenza virus and thus block further viral infection (57). In addition to influenza virus, the replication of herpes simplex virus (HSV)-2 can also be suppressed by commensal microbiota metabolites. For example, lactic acid, a major end product of

TABLE 2 | Suppression of viral infections by the commensal microbiota and the relevant mechanisms.

	Mechanisms	Virus types	References
Direct suppression	Blocking cell internalization process	Vesicular stomatitis virus	(54)
	Adsorptive trapping of viruses	Influenza viruses	(55)
	Binding to and destabilizing virion morphology	Influenza viruses	(56)
	Binding to and blocking further infections	Influenza viruses	(57)
	Suppressing virus replication	HSV-2	(58–60)
Indirect suppression	Enhancing type I IFN signaling	Influenza virus	(61, 62)
	Promoting Th17 and Th22 responses	SIV	(63)
	Increasing antiviral activities of macrophages	Systemic lymphocytic choriomeningitis virus and influenza virus	(64)
	Promoting APC migration and T cell activation	Influenza virus	(65)
	Inhibiting IL-33-mediated immune suppression	HSV	(66)
	Stimulating TLR-mediated cellular and humoral antiviral immune responses	Influenza virus and vaccinia virus	(65, 67, 68)
	Enhancing $CD8^+$ T cell activation of the infant by maternal microbiota	Vaccinia virus	(69)
	Preventing excessive inflammation and inflammation-associated pathology	Influenza virus, Sendai virus and SIV	(63, 70, 71)

the carbohydrate fermentation of all *Lactobacillus* species, can strongly inactivate HSV-2 in the vaginal mucosa by maintaining an acidic pH in the local environment (58). Consistent with this finding, in an *in vitro* study, it was shown that metabolites of vaginal *Lactobacillus* strains (i.e., lactic acid and hydrogen peroxide) exhibited potent virucidal activity, as highlighted by the dramatic suppression of virus replication by these substances (59). Commensal microbiota also exert their antiviral activity through bacterial components. For example, a vaginal strain of *Lactobacillus brevis*-extracted cell wall-associated component, which was resistant to high temperatures and protease digestion, potently inhibited the replication of HSV-2 in an *in vitro* model (60).

Indirect Suppression of Viral Infection

The commensal microbiota plays a critical role in shaping the host immune response, which essentially guarantees effective elimination of invading viruses. Supporting this notion, mounting studies have shown that intact healthy commensal microbiota help maintain robust antiviral immunity, while microbiota disruption increases viral infectivity due to the impaired capacity of the immune system to limit viral infection. For example, *Clostridium orbiscindens*, a specific human-associated gut microbe, produces desaminotyrosine to prime the amplification loop of type I IFN signaling, thereby mediating protection against influenza infection (61). In another influenza virus-infected chicken model, antibiotic treatment resulted in significantly higher oropharyngeal and cloacal virus shedding, which was also presumably mediated by reduced type I IFN responses after microbiota depletion, while the antibody-mediated antiviral immunity remained unaffected (**Figure 1**) (62). In simian immunodeficiency virus (SIV)-infected rhesus macaques, fecal microbiota transplantation (FMT) treatment after commensal microbiota depletion induced greater antiviral immunity, as reflected by dramatically increased peripheral Th17 and Th22 cells post-FMT (63). In contrast, when antibiotic-treated mice are infected by systemic lymphocytic choriomeningitis virus or influenza virus, their macrophages show decreased expression of genes associated with viral suppression, impaired responses to type I and type II IFNs and defective ability to control viral replication (**Figure 1**) (64). Consistent with this finding, it has been shown that during respiratory influenza virus infection, antibiotic exposure led to a defective generation of virus-specific CD4 and CD8 T cells and antibodies due to an impaired inflammasome-dependent migration of antigen-presenting cells (APC) from the lung to the draining lymph nodes (65) (**Figure 1**). This finding was further supported by another study, which showed that while oral antibiotic treatment had little effect on innate immune responses after HSV infection of the vaginal mucosa, a dramatic increase in the level of IL-33, an alarmin produced in response to epithelial cell damage, was observed after antibiotic treatment. Mechanistically, IL-33 acted as an immune-regulatory factor that suppressed local antiviral immunity by hindering the recruitment of effector T cells to the infection site and thus blocking the secretion of IFN-γ in vaginal mucosa (66).

It seems that insufficient TLR ligand stimulation after antibiotic exposure was partly responsible for the compromised immune cell function. When TLR agonists were applied during virus challenge in antibiotic-treated mice, both cellular and humoral antiviral responses could be largely restored (**Figures 1**, **2**) (65, 67). Moreover, TLR2 activation by bacterial products produced by the gut microbiota is necessary for the recruitment of mast cells to sites of viral infection and the further release of cathelicidin, a mast cell-derived antiviral protein (**Figure 2**) (68). However, this situation seems different in young mice, whose gut microbiota has not been completely established. In a hepatitis B virus infection model, TLR4-intact young mice failed to resolve viruses and developed chronic infections, while their TLR4 mutant counterparts exhibited

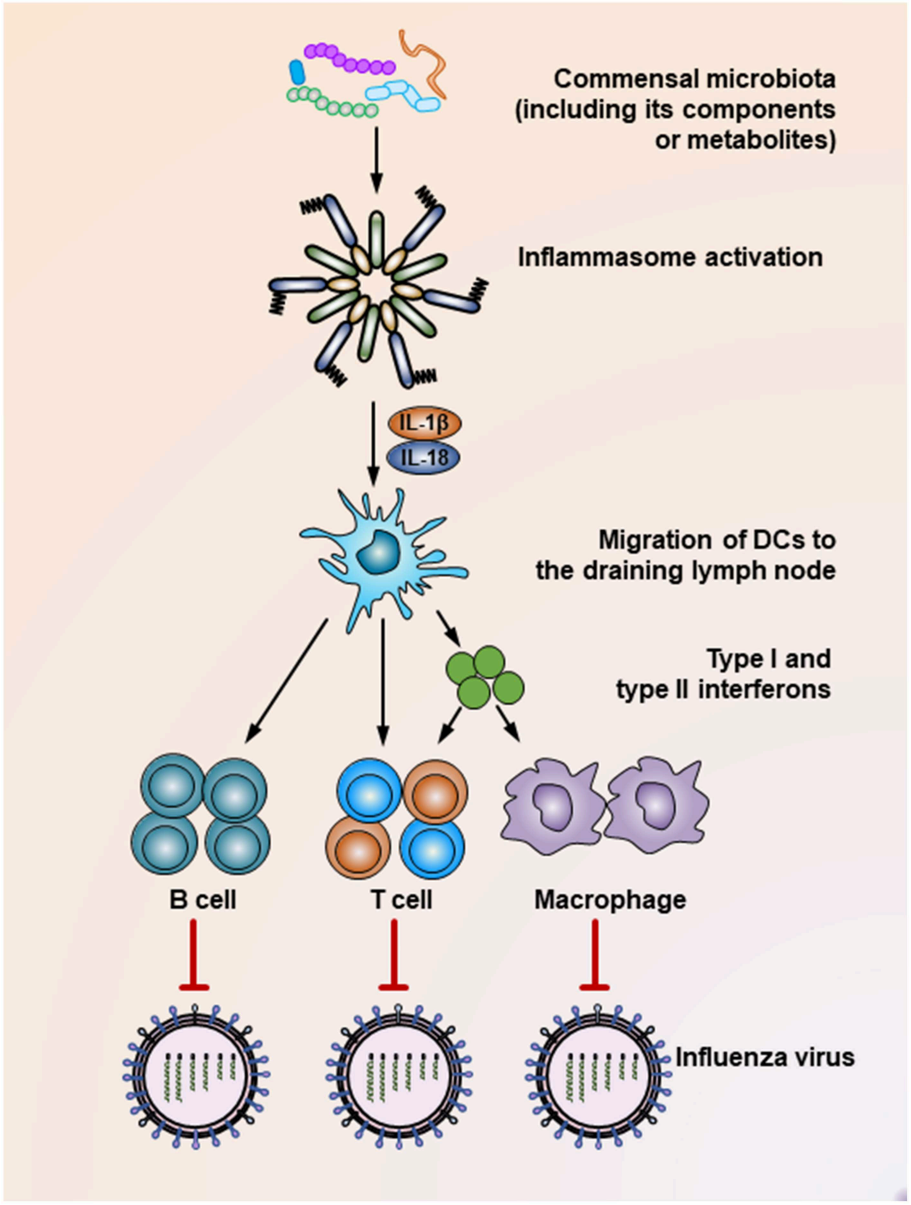

FIGURE 1 | Mechanisms underlying the suppression of influenza virus infection by the commensal microbiota. During the influenza virus infection, organisms of the commensal microbiota, as well as their components (i.e., various TLR ligands) or metabolites (i.e., desaminotyrosine) activate the inflammasome, resulting in IL-1β and IL-18 production. The production of these two cytokines induces the migration of dendritic cells from the lung to the draining lymph nodes, where they act as antigen-presenting cells to prime virus-specific B cells, $CD4^+$ T cells, $CD8^+$ T cells, and macrophages. In addition, dendritic cells also secrete type I and type II interferons to stimulate the activation of T cells or macrophages. As a result, these effector cells secrete virus-specific antibodies or inflammatory cytokines or exert direct virus-killing effects to suppress the infection process of the influenza virus.

rapid viral clearance, suggesting that an immune-tolerant pathway mediated by TLR4 signaling was predominant in young mice (72). Intriguingly, it seems that antibiotic treatment-induced gut microbiota alteration is transient and recoverable, as a more exacerbated disease condition only appears when antibiotics are used during influenza A virus infections; when such treatment ceases before the infection, neither an antiviral immunity defect, nor enhanced viral susceptibility are observed (73).

It should be noted that maternal antibiotic treatment confers an altered commensal microbiota to their offspring, thereby profoundly impacting their antiviral immunity. This idea is supported by a recent study that investigated the effect of antibiotic treatment of pregnant mice on the antiviral immunity of their neonatal offspring following vaccinia virus infection (69). In this study, maternal antibiotic treatment during pregnancy and lactation resulted in remarkable alterations in the composition of the gut microbiota of the infant mice,

with *Enterococcus faecalis* predominating within the infant enteric flora. Notably, maternal antibiotic treatment resulted in an accelerated and increased mortality following vaccinia virus infection of the offspring, which was partly mediated by a defective IFN-γ-secreting ability of virus-specific $CD8^+$ T cells (**Figure 2**).

In the case of the infection of several viruses, higher levels of immune activation may persist, associated with inflammation-induced comorbidities of the host (74). In these cases, immune recognition of the gut microbiota is necessary for the generation and activation of immunoregulatory cells to diminish local or systemic immune activation. Indeed, Rosshart et al. found that reconstitution of the gut microbiota from wild mice confers potent protective effects to laboratory GF mice during lethal influenza virus infections, an effect mainly mediated through the prevention of excessive inflammation via IL-10 and IL-13 production in the virus-affected mice by the natural gut microbiota (70). In addition, Grayson et al. found that antibiotic treatment before or during murine Sendai virus infection resulted in greatly increased morbidity and mortality, accompanied by an abnormal immune response characterized by increased proinflammatory cytokines (i.e., IFN-γ, IL-6, and monocyte chemoattractant protein 1) and decreased Tregs in the lung (71). Notably, the neutralization of IFN-γ or the adoptive transfer of Treg cells abrogated tissue inflammation and prevented increased mortality (71). In cases of human immunodeficiency virus (HIV) infection, the gut microbiota is intimately associated with activation of the immune system in HIV-infected individuals (75). Importantly, decreased activation of $CD4^+$ T cells was observed post-FMT in SIV-infected rhesus macaques (63). This conclusion is reinforced by another independent study, which showed that the capacity of NKT cells to produce IL-4 and IL-10 in gastrointestinal-associated lymphoid tissues was associated with fewer markers of microbial transmission and less immune activation, a process dependent on the recognition of *Bacteroides* species by these cells (76).

Intriguingly, a recent study by Stewart et al. revealed that the interrelations between nasopharyngeal microbiota and host systemic inflammatory responses (reflected by serum metabolomic signatures) likely contribute to bronchiolitis in infants (77). Of note, the relative abundance of *Streptococcus*, which is specifically pathological in respiratory health, was positively correlated with metabolites associated with more severe disease (77). In comparison, the abundance of *Moraxella*, another important component of the nasopharyngeal microbiota, showed the opposite correlation patterns (77). Similar findings were reported in an independent study. In this study, Piters et al. showed that severity of respiratory syncytial virus (RSV) bronchiolitis induced by RSV infection was positively associated with abundance of *Streptococcus* and *Haemophilus influenzae* and negatively associated with abundance of *Staphylococcus aureus* in the nasal mucus (78). Interestingly, transcriptome profiles of whole blood from children with RSV infection and *Streptococcus*- and *Haemophilus influenza*-dominated microbiota revealed greater overexpression of several proinflammatory genes linked to macrophage and neutrophil activation (78). Thus, although the underlying mechanism is still unclear, these data clearly suggest that airway microbiota play an important role in regulating the systemic immune responses, thereby controlling the outcome of viral infections in the respiratory tract.

Suppression of Viral Infection With Unclear Mechanisms

Several clinical cases suggest a definite suppression of viral infectivity by the commensal gut microbiota, although the detailed mechanism is unclear. For example, hepatitis B virus (HBV) e-antigen (HBeAg), which may persist in patients for years after HBV infection, is commonly used as a sensitive indicator of remission activity and improved long-term outcome (79). In a trial of FMT for the treatment of HBV, the authors found a significant decrease in the HBeAg titer in patients after FMT treatment, and the HBeAg titer decreased gradually following each FMT treatment, suggesting the efficacy of modulating the gut microbiota for chronic hepatitis B treatment (80). In another trial investigating the underlying mechanism of the development of lower respiratory tract infection (LRTI) after viral infection, the authors found that patients with a higher abundance of butyrate-producing bacteria in their fecal samples showed a 5-fold lower possibility of developing viral LRTI (81).

VIRAL INFECTION NOT AFFECTED BY COMMENSAL MICROBIOTA

Although a wealth of evidence has shown that the commensal microbiota regulates viral infectivity, there is also evidence showing that these microbial communities may have no effects on modulating antiviral responses. For example, Gopinath et al. recently found that vaginal application of the aminoglycoside antibiotic neomycin enhanced the host resistance to a broad range of viral infections, i.e., HSVs, Zika virus and influenza A virus. However, the antiviral activity of antibiotics was independent of the commensal microbiota, as the protection was also applicable to germ-free mice and *in vitro* cultured primary cells. Instead, neomycin increased the expression of IFN-stimulated genes in the host, a process mediated by TLR3 expressed by a specific subset of dendritic cells (82). Consistent with this finding, Zhu et al. found that B cells mediate the early control of murine norovirus infections and that this effect can also be achieved even in the absence of commensal microbiota via antibiotic treatment (83). In this study, the authors found that B cells mainly function as antigen-presenting cells but not as antibody-secreting cells to exert their virus-elimination effects. In murine leukemia virus (MuLV)-infected mice, Wilks et al. found that during retrovirus infection, both the production of virus-specific antibodies and the antibody-mediated virus-neutralizing responses were independent of the commensal microbiota, as both GF and specific pathogen-free (SPF) mice produced similar levels of virus-specific antibodies, and the antibody-mediated virus-neutralizing effects were similar in both mice, suggesting that antibody-mediated immune control of MuLV does not require commensal microbiota (84). It should be noted that several earlier studies found the overall pathogenicity of murine

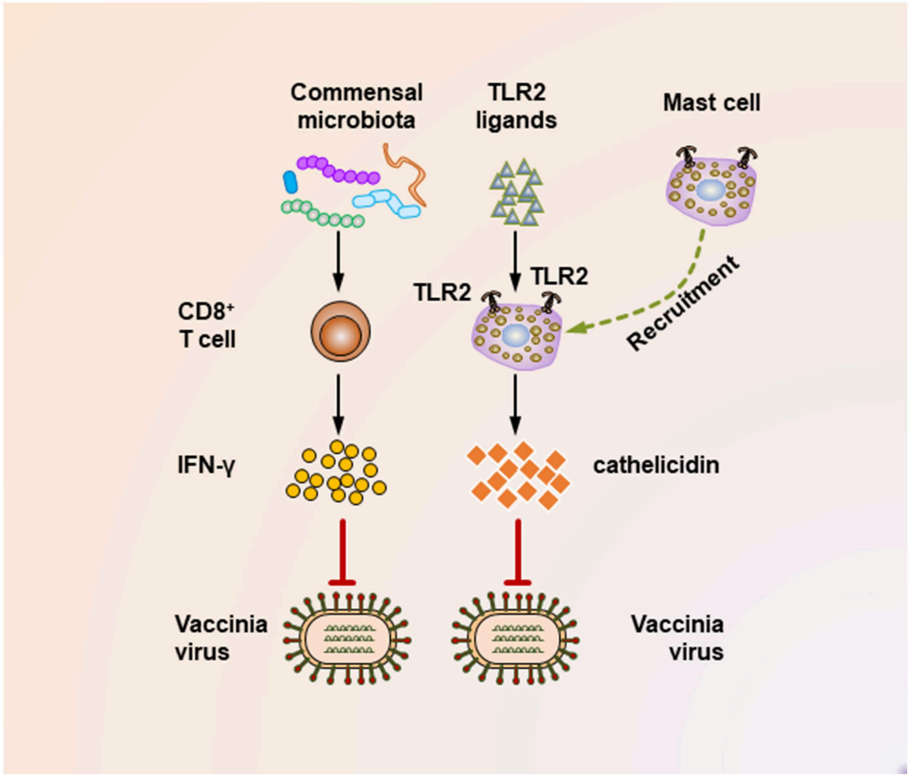

FIGURE 2 | Mechanisms underlying the suppression of vaccinia virus infection by the commensal microbiota. During the vaccinia virus infection, the commensal microbiota primes virus-specific CD8+ T cells to secrete large amounts of IFN-γ, which critically mediates the corresponding antiviral immunity. In addition, during vaccinia virus infections, the activation of TLR2 by bacterial products is essential for recruiting mast cells to sites of viral infection. These mast cells also contribute to suppressing the viral infection by secreting an antiviral cathelicidin.

leukemia virus were affected by the commensal microbiota. For example, compared to GF mice, conventionally reared mice developed higher levels of virus expression and longer latency period following infection of MuLV-Moloney (85, 86). However, when GF mice were stimulated with sheep erythrocytes, a significant increase in leukemia development was observed (86). The authors hypothesized that GF mice may not possess certain lymphoid cells that were required by MuLV replication stimulated by the commensal microbiota (86). Conflicting results were obtained in other studies showing that GF mice were more sensitive than conventional housed mice to MuLV infection (87). One potential explanation for this discrepancy is that the likely contamination of MuLV isolates by lactate dehydrogenase-elevating virus that can potently induce systemic lymphocyte activation (88), as suggested by Wilks et al. (89).

MODULATION OF THE COMMENSAL MICROBIOTA BY VIRUSES

While modulation of the commensal microbiota by viruses is still poorly understood, studies to date do suggest an important role of virus infection in inducing microbiota dysbiosis. This is true for HIV/SIV infection, influenza virus infection, HBV or hepatitis C virus (HCV) infection and norovirus infection, as discussed in detail below. In addition to these four types of viral infections, alteration of the gut microbiota following infection has also been described in cases of rotavirus infection in pigs or calves (90, 91), avian leukosis viruses in chickens (92), canine distemper virus infection in giant pandas (93), and white spot syndrome virus infection in crabs (94), although the relevant reports are sporadic and the corresponding mechanistic evidence is very limited.

HIV/SIV

A plethora of studies have emphasized that in SIV-infected non-human primates and HIV-infected patients, the commensal microbiota composition is disrupted with the enrichment of potentially pathogenic bacterial families. For example, microbial diversity in saliva of HIV patients was significantly reduced than healthy controls, accompanied by increased abundance of potentially pathogenic *Megasphaera*, *Campylobacter*, *Veillonella* and *Prevotella* species, and decreased commensal *Veillonella* and *Streptococcus* species (95, 96). In a recent study, Mukherjee et al. found that fungal communities differed significantly between HIV-infected and uninfected individuals, with *Epicoccum*, *Candida* and *Alternaria* being the most abundant fungi in HIV-infected individuals, while *Pichia*, *Candida* and *Fusarium* being the most common genera in healthy controls (97). Intriguingly, *Pichia* can efficiently inhibited *Candida* colonization (97). In

bronchoalveolar lavage fluid, although there were no significant differences among the microbial composition in HIV-infected and uninfected subjects, specific metabolic profiles were associated with bacterial organisms that potentially play a role in the pathogenesis of pneumonia (i.e., Bacteria from families *Nocardioidaceae*, *Staphylococcaceae*, *Caulobacteraceae*, and genus *Streptococcus*) in HIV-infected patients (98). In a long-term monitoring of chimpanzees following SIV infection, Moeller et al. observed a marked increase in the genera *Selenomonas*, *Staphylococcus*, and *Sarcina*, all containing opportunistic pathogens that were never detected at high abundances in SIV-negative chimpanzees (99, 100). However, SIV infection had little effect on the frequencies of *Enterobacteriales*, *Bacteroidales*, or *Pseudomonas*, nor did the authors find any differences in alpha-diversity between SIV-positive and SIV-negative chimpanzees (99). In fecal samples, HIV infection was associated with consistently reduced overall microbiota richness but selective enrichment of the phyla *Firmicutes* and *Proteobacteria*, with the most prominent increase in *Bacteroides* and *arabacteroides* at the genus level (101–103). In addition, the alpha-diversity of species in the fecal microbiota is negatively associated with the severity of immunodeficiency in patients (104). Notably, combined antiretroviral therapy can effectively restore the alpha-diversity of the fecal microbiota. In addition to compositional alteration of the commensal microbiota, HIV infection also robustly alters the metabolic activity of gut microbiota (105). In contrast to healthy controls and patients with systemic lupus erythematosus and bacterium-induced diarrhea, HIV infection results in a defective metabolic capacity of gut bacteria to produce three amino acids, namely, proline, phenylalanine and lysine (105). In comparison, 3-hydroxyanthranilate, one of the major metabolites of the kynurenine pathway during the oxidative catabolism of tryptophan, was found to be significantly accumulated in the gut microbiota of all HIV-infected patients (105), which is in agreement with the finding of a previous study showing that gut microbiota with the ability to catabolize tryptophan through the kynurenine pathway are enriched in these patients (102).

Mechanistically, specific immune suppression by HIV is partly responsible for the enrichment of certain potentially pathogenic bacteria. For example, *Salmonella typhimurium*, a member of the *Proteobacteria* phylum, is tightly controlled by Th17 cells (106). In SIV-infected rhesus macaques, Th17 cells are markedly depleted, resulting in blunted Th17 responses to *Salmonella typhimurium* and finally leading to systemic dissemination of *S. typhimurium* (107), a phenomenon also observed in clinical cases of HIV-infected patients (108). In contrast, it seems that neither HIV-induced B cell dysfunction nor enteropathy affect overall systemic antibody responses to the commensal microbiota (109).

Influenza Viruses

Influenza viruses enter the host through the upper respiratory tract (URT) and can alter the microbial composition of the URT significantly following infection. Several studies have demonstrated that influenza virus infection can result in decreased colonization by healthy bacteria and increased abundance of potentially pathogenic microbiota. For example, a case-control study using next-generation sequencing of the 16S rRNA gene to analyze specific bacteria in patients with influenza infection and healthy controls showed that the healthy core microbiota, specially *Prevotella* spp. and anaerobes, were significantly decreased in influenza virus-infected patients (110). In comparison, eight potentially pathogenic bacteria were significantly enriched in these patients, including *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Corynebacterium propinquum/pseudodiphtheriticum,* and *Dol osigranulum pigrum* (110). Consistent with this study, Li et al. found that *Prevotella* was decreased after H1N1 virus infection (111). It should be noted that nasopharyngeal and oropharyngeal microbiota show distinct alteration profiles following influenza infection. For example, while nasopharyngeal *Streptococcus* showed higher abundance after infection (112, 113), oropharyngeal *Streptococcus* was significantly decreased following influenza infection (112). By contrast, Ramos-Sevillano et al. found that the throat microbiota was resilient to influenza infection, with remarkably stable bacterial communities following influenza infection, which was consistent with a recent murine model-based study (114, 115). The discrepancy may result from different infection doses, sample collection method, subtypes of influenza virus and environmental factors (i.e., pH, CO_2, and O_2 concentrations).

In contrast to the commensal microbiota of the URT, studies have only recently begun to evaluate how influenza virus infections affect gut microbiota. Consensus has been reached that influenza virus infection alters the commensal microbiota of the host, causing corresponding disruptions of the microbiota-host homeostasis, which largely accounts for the mechanisms by which infections are established. However, this general conclusion is based on several contradictory findings. For example, while infection of influenza virus was shown to lead to an increase in *Bacteroidetes* phyla abundance in one mouse-based study (116), a remarkable drop of *Bacteroidetes* (mainly *S24-7*) was observed in influenza A virus-infected birds and mice (115, 117). Interestingly, a murine study even found unchanged *Bacteroidetes* levels after respiratory influenza virus infection (118). In addition, in avian influenza A-infected migrating whooper swans, fewer *Proteobacteria* were detected in the fecal sample (117), while this was not recapitulated in another two studies showing that selective enrichment of *Proteobacteria* (mostly *Bdellovibrionaceae*) in the gut was the result of influenza virus infection (119, 120). These contradictory findings may result from the differences in virus subtypes and doses, experimental animal types, and the age, diet, as well as the lifestyle of the same animals. However, it seems that alterations in *Firmicutes* after influenza virus infection are more uniform, as decreases in the richness of *Firmicutes* (represented by *Lactobacillus*) were observed by most of the abovementioned studies (115–117). There is also interest in whether vaccination affects commensal microbiota. However, after live attenuated influenza virus vaccination, no changes in gut microbiota composition were discovered, indicating that only live viruses can drive an altered commensal microbiota diversity (116).

Investigating the underlying mechanism, several lines of data have highlighted that the modulation of immune responses

by influenza contributes to the dysbiosis of the gut. Deriu et al. found that pulmonary infection of influenza virus induced the production of type I IFNs in the lung, which acted as a central player in upregulating *Proteobacteria* and depleting obligate anaerobic bacteria (120). Moreover, IFN-mediated dysbiosis inhibited the antimicrobial inflammatory immune responses in the gut during *Salmonella* infection, further promoting *Salmonella* colonization and systemic dissemination (120). Consistent with the findings of this study, commensal microbiota dysregulation following influenza virus infection could also be the result of overproduction of a type II IFN, IFN-γ, which was secreted by a subset of lung-derived CC chemokine receptor 9 $(CCR9)^{+}CD4^{+}$ T cells in the small intestine (118). The disturbed gut microbiota further stimulated IL-15 production from intestinal epithelial cells, which subsequently facilitated the polarization of Th17 cells *in situ*, finally leading to intestinal injury (118).

HBV/HCV

Quite a few studies have implicated that dysbiosis of the commensal microbiota occurs following infection with HBV/HCV and is relevant to the progression of liver disease. In patients with HBV, a profound alteration in the composition of gut microbiota is reflected by the significantly enriched *Actinomyces*, *Clostridium sensu stricto*, *Megamonas* and *Lachnospiraceae*, and a concomitant decrease in *Alistipes*, *Bacteroides*, *Asaccharobacter*, *Parabacteroides*, *Butyricimonas*, *Clostridium IV*, *Coriobacteriaceae*, *Escherichia/Shigella*, *Ruminococcus*, *Clostridiales*, *Enterobacteriaceae*, *Lachnospiraceae,* and *Ruminococcaceae* (121). In a species-level study, bacterial species with an opportunistically pathogenic nature were significantly elevated, while species with potential beneficial effects were downregulated in the fecal samples of HBV-infected patients (122). In HCV, a reduced bacterial diversity, with an increase in the order *Streptococcus* and *Lactobacillus*, and a decrease in *Clostridiales* was observed (123, 124). In another study, a lower abundance of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* and a higher abundance of *Bacteroidetes* were detected in stool samples of HCV patients (124).

The course of liver disease progression in HBV/HCV patients can also be reflected by profiles of the commensal microbiota. In a study comparing the diversity of gut fungal microbiota in patients with HBV infection, the authors found that the diversity of intestinal fungi was positively associated with disease progression, as reflected by the higher richness of enteric fungal species in patients with HBV-related cirrhosis than in those with chronic infection. Moreover, patients with chronic hepatitis B exhibited higher richness of fungal species compared with asymptomatic HBV carriers and healthy controls (125). In a cross-sectional investigation, it was shown that alpha-diversity of the fecal microbiota decreased significantly from healthy controls to HCV patients without cirrhosis to those with cirrhosis (126). In addition, the ratio of *Bifidobacterium*/*Enterobacteriaceae* was suggested to be a sensitive biomarker for the clinical course of HBV, as a gradual decrease in this ratio was observed in asymptomatic HBV carriers, patients with chronic hepatitis B, and patients with HBV-associated cirrhosis (127).

In addition to the gut microbiota, an alteration of the oral microbiota in HBV-affected individuals has also been reported in a recent study, which showed that the ratio of *Firmicutes*/*Bacteroidetes* was increased significantly (128). Interestingly, HBV infection resulted in a marked increase in bacteria capable of producing H_2S and CH_3SH, implicating the potential contribution of the altered microbiota to the oral malodor in these patients (128). Compositional and metabolic changes in the tongue-coating microbiota have also been documented in HBV-infected individuals. As reported, yellow tongue was associated with higher HBV titers compared with those in patients with a white tongue. Moreover, a significant decrease in *Bacteroidetes* and an increase in *Proteobacteria* was found in HBV-associated yellow tongues, which also showed a selective enrichment of the metagenomic pathways involved in amino acid metabolism, consistent with the metabolic disorder of these patients (129).

In summary, HBV/HCV infection indeed caused profound changes in the composition and metabolism of the commensal microbiota. However, it should be noted that most of these studies are based on observational data. Thus, further studies exploring the underlying mechanism of HBV/HCV-induced microbiota alterations are clearly needed.

Norovirus

Norovirus represents one of the most important causes of acute viral gastroenteritis worldwide (20), usually causing severe diarrhea and occasionally causing chronic infections in immunocompromised individuals (20). In a human-based study investigating the effect of norovirus infection on the gut microbiota, the authors found that while the fecal microbiota in most infected individuals exhibited a similar composition to that of uninfected controls, a significant loss of diversity and richness of the gut microbiota characterized by a clear increase in the relative numbers of *Proteobacteria* and a corresponding decrease in *Bacteroidetes* was observed in a small proportion of norovirus-infected patients (130). Further analysis revealed that a single operational taxonomic unit of *Escherichia coli* was partially responsible for the increase in *Proteobacteria* in these patients (130). Consistent with this observation, human norovirus can bind to certain human stool-isolated bacteria, including those in the phylum *Proteobacteria* (i.e., *Hafnia alvei*, *Citrobacter* spp., *Klebsiella* spp., and *Enterobacter cloacae*), with high efficiency, implicating a direct modulation of the gut microbiota by norovirus (131). Intriguingly, murine norovirus even has the capacity to maintain gut homeostasis and shape intestinal immunity, similar to the functions of the gut microbiota. Kernbauer et al. found that norovirus infection of antibiotic-treated or germ-free mice restored the aberrant lymphocyte compartment and the abnormal intestinal morphology without inducing overt inflammation and disease (132). Importantly, norovirus infection protected antibiotic-treated mice from dextran sulfate sodium-induced intestinal injury and *C. rodentium* superinfection, suggesting that

norovirus has the potential to replace the beneficial functions of commensal microbiota in the intestine (132).

Theiler's Murine Encephalomyelitis Virus (TMEV)

Emerging evidence supports the intriguing concept of the brain-gut microbiome axis and has shown bidirectional interactions within it (133, 134). Several systems including the central, autonomic and enteric nervous systems, the neuroendocrine and the neuroimmune systems are at work to guarantee proper functioning of this axis (135). Current data have suggested that this complex communication axis is essentially linked to the regulation of multiple aspects of host physiology ranging from gastrointestinal homeostasis to psychiatric, motivational and cognitive functions (136–139). As a result, perturbation of the brain-gut microbiome axis is involved in several disorders including metabolic dysregulation and psychiatric and non-psychiatric diseases (140, 141). Thus, it is not surprising that viral infection-associated brain abnormalities can result in gut dysbiosis, which may in turn affect the development and severity of virus-associated tissue pathology. This was evidenced by a recent study investigating the effect of intracerebral TMEV infection on commensal microbiota (142). In this study, Carrillo-Salinas et al. found that TMEV infection was associated with significantly altered gut microbiota, reflected by a reduction in the relative abundance of *Alloprevotela* (phylum *Bacteroidetes*) at 14 days post infection and a decrease in *Anaerotruncus* (phylum *Firmicutes*) and *Akkermansia* (phylum *Verrucomicrobia*) while an increase in *Clostridium XIVa* (phylum *Firmicutes*) at 28 days post infection (142). Intriguingly, the effect of TMEV infection on gut microbiota is profound and lasting, as alterations in *Firmicutes* and *Bacteroidetes* of the gut microbiota still exist at 85 days post infection (142). In addition, oral administration of antibiotics dampened TMEV infection by enhancing antiviral immune responses during acute phase of infection (142).

CONCLUSIONS AND FUTURE PERSPECTIVES

We have discussed the current understanding of the modulation of virus infectivity by the commensal microbiota of the host and the underlying mechanisms in this regulation. We have also described the contribution of viral infection to the disturbances of microbiota homeostasis in the host. We do not yet fully understand the extent to which commensal microbiota may determine the efficiency of viral replication, transmission, and persistence, and in most cases reported, the relevant mechanisms underlying the influence of the host microbiota by invading viruses are unclear. However, the data presented do support an intimate interaction between the commensal microbiota and invading viruses, an interaction that always dictates the outcome of an infection. Thus, it is tempting to speculate that antiviral drugs aimed at modulating virus-microbiota interactions may be particularly effective in controlling the activity of many viral diseases. In fact, the pharmaceutical application of FMT and probiotic supplements have already been proven useful in reducing the severity of several diseases in human- and non-human primate-based studies, although these efforts may turn out to be ineffective in certain circumstances and may even result in unwanted complications (63, 143–146). Therefore, there are still major gaps in our understanding of the interactions between the commensal microbiota and viruses, and constant optimization of these potential treatment methods is clearly needed to better control viral infections via the modulation of commensal microbiota.

Recent works have shed light on the role of the commensal microbiota in health and many diseases. However, considering the immense diversity of the commensal microbiota, most studies investigating the functional attributes of these microbial communities are based on population-level analyses, and the majority of species in the commensal microbiota have never been isolated and cultured in the laboratory, thus greatly hindering progress in identifying the unique phenotypes and functions of each species of the commensal microbiota to minimize the risk of complications of FMT brought about by unwanted microbiota. Therefore, further efforts into developing more effective approaches for commensal microbiota culture *in vitro* are urgently needed. In addition, an important gap in microbiota research is that most studies discuss only bacterial microbiota and often overlook fungi or viruses, which are also important components of the commensal microbiota of the host (19, 147). Thus, future studies on components of the commensal microbiota other than bacteria are also needed.

As discussed above, several studies have suggested that the commensal microbiota may potently promote viral infections, and commensal microbiota depletion with antibiotics could conceivably be used as a strategy to treat viral infections. However, one must recognize that all the antibiotic-induced inhibitions of viral infection have been designed only in mouse studies, and usually only the effect of such treatment on viral infection is evaluated, regardless of the potentially negative consequences on a broader scale of infections. For example, when the majority of commensal microorganisms are depleted by broad-spectrum antibiotic treatments, the beneficial effects of the commensal microbiota on host health, the maintenance of host physiological homeostasis and the promotion of host immune functions, will concomitantly disappear, making these adverse consequences far outweigh the benefits of blocking a particular viral infection. In addition, it is now well-recognized that antibiotic overuse leads to the emergence of antibiotic-resistant bacteria or even superbacteria that may bring about severe or even life-threatening infections. Thus, we do not advocate the use of antibiotics to treat or prevent viral diseases in humans. However, understanding how the commensal microbiota enhances viral infection, especially the molecular requirements for the microbiota-mediated promotion of viral infections, may lead to the development of novel, feasible antiviral strategies.

Emerging data suggest that the newly discovered cyclic-GMP-AMP (cGAMP) synthase (cGAS)-cGAMP-stimulator of interferon genes (STING) pathway as the major pathway in sensing cytosolic DNA following viral infections (148–150). In fact, the cGAS-cGAMP-STING axis has been

shown to be involved in restricting both DNA and RNA virus infections (150, 151). Notably, a recent work has shown that guanylate cyclase C, which is expressed on intestinal epithelial cells and is crucial for the generation of cGMP, critically regulates microbiome composition of the intestine through maintaining barrier integrity by cGMP production (152). However, during viral infections, the effect of cGAMP on the commensal microbiota is unclear. Considering that the mechanism underlying the modulation of commensal microbiota by viral infections has not been fully clarified, this intriguing question undoubtedly warrants further investigations.

AUTHOR CONTRIBUTIONS

W-TM and J-LH designed the structure of this review. NL and W-TM wrote the manuscript. NL, MP, Q-LF, and J-LH revised the manuscript. All authors have reviewed the final version of the manuscript.

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REFERENCES

1. Proctor DM, Relman DA. The landscape ecology and microbiota of the human nose, mouth, and throat. *Cell Host Microbe.* (2017) 21:421–32. doi: 10.1016/j.chom.2017.03.011
2. Gimblet C, Meisel JS, Loesche MA, Cole SD, Horwinski J, Novais FO, et al. Cutaneous leishmaniasis induces a transmissible dysbiotic skin microbiota that promotes skin inflammation. *Cell Host Microbe.* (2017) 22:13–24 e4. doi: 10.1016/j.chom.2017.06.006
3. Whiteside SA, Razvi H, Dave S, Reid G, Burton JP. The microbiome of the urinary tract–a role beyond infection. *Nat Rev Urol.* (2015) 12:81–90. doi: 10.1038/nrurol.2014.361
4. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, et al. Environment dominates over host genetics in shaping human gut microbiota. *Nature.* (2018) 555:210–5. doi: 10.1038/nature25973
5. Borgo F, Garbossa S, Riva A, Severgnini M, Luigiano C, Benetti A, et al. Body mass index and sex affect diverse microbial niches within the gut. *Front Microbiol.* (2018) 9:213. doi: 10.3389/fmicb.2018.00213
6. Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. Genetic determinants of the gut microbiome in UK twins. *Cell Host Microbe.* (2016) 19:731–43. doi: 10.1016/j.chom.2016.04.017
7. Faust K, Raes J. Host-microbe interaction: Rules of the game for microbiota. *Nature.* (2016) 534:182–3. doi: 10.1038/534182a
8. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell.* (2005) 122:107–18. doi: 10.1016/j.cell.2005.05.007
9. Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut immune maturation depends on colonization with a host-specific microbiota. *Cell.* (2012) 149:1578–93. doi: 10.1016/j.cell.2012.04.037
10. Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG, et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature.* (2008) 456:507–10. doi: 10.1038/nature07450
11. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. *Nature.* (2012) 489:242–9. doi: 10.1038/nature11552
12. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. *Science.* (2012) 336:1262–7. doi: 10.1126/science.1223813
13. Piewngam P, Zheng Y, Nguyen TH, Dickey SW, Joo HS, Villaruz AE, et al. Pathogen elimination by probiotic Bacillus via signalling interference. *Nature.* (2018) 562:532–7. doi: 10.1038/s41586-018-0616-y
14. Kim YG, Sakamoto K, Seo SU, Pickard JM, Gillilland MG, Pudlo NA, et al. Neonatal acquisition of Clostridia species protects against colonization by bacterial pathogens. *Science.* (2017) 356:312–5. doi: 10.1126/science.aag2029
15. Sassone-Corsi M, Nuccio SP, Liu H, Hernandez D, Vu CT, Takahashi AA, et al. Microcins mediate competition among Enterobacteriaceae in the inflamed gut. *Nature.* (2016) 540:280–283. doi: 10.1038/nature20557
16. Schuijt TJ, Lankelma JM, Scicluna BP, Melo FD, Roelofs TH, de Boer JD, et al. The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. *Gut.* (2016) 65:575–83. doi: 10.1136/gutjnl-2015-309728
17. Karst SM, The influence of commensal bacteria on infection with enteric viruses. *Nat Rev Microbiol.* (2016) 14:197–204. doi: 10.1038/nrmicro.2015.25
18. Berger AK, Mainou BA. Interactions between enteric bacteria and eukaryotic viruses impact the outcome of infection. *Viruses.* (2018) 10:19. doi: 10.3390/v10010019
19. Pfeiffer JK, Virgin HW. Viral immunity. Transkingdom control of viral infection and immunity in the mammalian intestine. *Science.* (2016) 351:5872. doi: 10.1126/science.aad5872
20. Sullender ME, Baldridge MT. Norovirus interactions with the commensal microbiota. *PLoS Pathog.* (2018) 14:e1007183. doi: 10.1371/journal.ppat.1007183
21. Robinson CM, Pfeiffer JK. Viruses and the microbiota. *Ann Rev Virol.* (2014) 1:55–69. doi: 10.1146/annurev-virology-031413-085550
22. Erickson AK, Jesudhasan PR, Mayer MJ, Narbad A, Winter SE, Pfeiffer JK. Bacteria facilitate enteric virus co-infection of mammalian cells and promote genetic recombination. *Cell Host Microbe.* (2018) 23:77–88 e5. doi: 10.1016/j.chom.2017.11.007
23. Kuss SK, Best GT, Etheredge CA, Pruijssers AJ, Frierson JM, Hooper LV, et al. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science.* (2011) 334:249–52. doi: 10.1126/science.1211057
24. Robinson CM, Jesudhasan PR, Pfeiffer JK. Bacterial lipopolysaccharide binding enhances virion stability and promotes environmental fitness of an enteric virus. *Cell Host Microbe.* (2014) 15:36–46. doi: 10.1016/j.chom.2013.12.004
25. Berger AK, Yi H, Kearns DB, Mainou BA. Bacteria and bacterial envelope components enhance mammalian reovirus thermostability. *PLoS Pathog.* (2017) 13:e1006768. doi: 10.1371/journal.ppat.1006768
26. Gorres KL, Daigle D, Mohanram S, Miller G. Activation and repression of Epstein-Barr Virus and Kaposi's sarcoma-associated herpesvirus lytic cycles by short- and medium-chain fatty acids. *J Virol.* (2014) 88:8028–44. doi: 10.1128/JVI.00722-14
27. Wilen CB, Lee S, Hsieh LL, Orchard RC, Desai C, Hykes BL Jr, et al. Tropism for tuft cells determines immune promotion of norovirus pathogenesis. *Science.* (2018) 360:204–8. doi: 10.1126/science.aar3799
28. von Moltke J, Ji M, Liang HE, Locksley RM. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature.* (2016) 529:221–5. doi: 10.1038/nature16161
29. Jones MK, Watanabe M, Zhu S, Graves CL, Keyes LR, Grau KR, et al. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science.* (2014) 346:755–9. doi: 10.1126/science.1257147
30. Kane M, Case LK, Kopaskie K, Kozlova A, MacDearmid C, Chervonsky AV, et al. Successful transmission of a retrovirus depends on the commensal microbiota. *Science.* (2011) 334:245–9. doi: 10.1126/science.1210718
31. Jude BA, Pobezinskaya Y, Bishop J, Parke S, Medzhitov RM, Chervonsky AV, et al. Subversion of the innate immune system by a retrovirus. *Nat Immunol.* (2003) 4:573–8. doi: 10.1038/ni926
32. Wilks J, Lien E, Jacobson AN, Fischbach MA, Qureshi N, Chervonsky AV, et al. Mammalian lipopolysaccharide receptors incorporated into the retroviral envelope augment virus transmission. *Cell Host Microbe.* (2015) 18:456–62. doi: 10.1016/j.chom.2015.09.005

33. Baldridge MT, Nice TJ, McCune BT, Yokoyama CC, Kambal A, Wheadon M, et al. Commensal microbes and interferon-lambda determine persistence of enteric murine norovirus infection. *Science.* (2015) 347:266–9. doi: 10.1126/science.1258025
34. Uchiyama R, Chassaing B, Zhang BY, Gewirtz AT. Antibiotic treatment suppresses rotavirus infection and enhances specific humoral immunity. *J Infect Dis.* (2014) 210:171–82. doi: 10.1093/infdis/jiu037
35. Young GR, Eksmond U, Salcedo R, Alexopoulou L, Stoye JP, Kassiotis G. Resurrection of endogenous retroviruses in antibody-deficient mice. *Nature.* (2012) 491:774–48. doi: 10.1038/nature11599
36. Aguilera ER, Erickson AK, Jesudhasan PR, Robinson CM, Pfeiffer JK. Plaques formed by mutagenized viral populations have elevated coinfection frequencies. *MBio*. (2017) 8:16. doi: 10.1128/mBio.02020-16
37. Chen YH, Du W, Hagemeijer MC, Takvorian PM, Pau C, Cali A, et al. Phosphatidylserine vesicles enable efficient en bloc transmission of enteroviruses. *Cell.* (2015) 160:619–30. doi: 10.1016/j.cell.2015.01.032
38. Combe M, Garijo R, Geller R, Cuevas JM, Sanjuan R. Single-cell analysis of RNA virus infection identifies multiple genetically diverse viral genomes within single infectious units. *Cell Host Microbe.* (2015) 18:424–32. doi: 10.1016/j.chom.2015.09.009
39. Ma SD, Hegde S, Young KH, Sullivan R, Rajesh D, Zhou Y, et al. A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. *J Virol.* (2011) 85:165–77. doi: 10.1128/JVI.01512-10
40. Asai S, Nakamura Y, Yamamura M, Ikezawa H, Namikawa I. Quantitative analysis of the Epstein-Barr virus-inducing properties of short-chain fatty acids present in the culture fluids of oral bacteria. *Arch Virol.* (1991) 119:291–6. doi: 10.1007/BF01310678
41. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell.* (2016) 165:1332–45. doi: 10.1016/j.cell.2016.05.041
42. Sawicki CM, Livingston KA, Obin M, Roberts SB, Chung M, McKeown NM. Dietary fiber and the human gut microbiota: application of evidence mapping methodology. *Nutrients.* (2017) 9:125. doi: 10.3390/nu9020125
43. Lee S, Wilen CB, Orvedahl A, McCune BT, Kim KW, Orchard RC, et al. Norovirus cell tropism is determined by combinatorial action of a viral non-structural protein and host cytokine. *Cell Host Microbe*. (2017) 22:449–59 e4. doi: 10.1016/j.chom.2017.08.021
44. Haga K, Fujimoto A, Takai-Todaka R, Miki M, Doan YH, Murakami K, et al. Functional receptor molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect cells. *Proc Natl Acad Sci USA*. (2016) 113:E6248–55. doi: 10.1073/pnas.1605575113
45. Miura T, Sano D, Suenaga A, Yoshimura T, Fuzawa M, Nakagomi T, et al. Histo-blood group antigen-like substances of human enteric bacteria as specific adsorbents for human noroviruses. *J Virol.* (2013) 87:9441–51. doi: 10.1128/JVI.01060-13
46. Tedesco D, Thapa M, Chin CY, Ge Y, Gong M, Li J, et al. Alterations in intestinal microbiota lead to production of interleukin 17 by intrahepatic gammadelta T-cell receptor-positive cells and pathogenesis of cholestatic liver disease. *Gastroenterology*. (2018) 154:2178–93. doi: 10.1053/j.gastro.2018.02.019
47. Yu H, Gagliani N, Ishigame H, Huber S, Zhu S, Esplugues E, et al. Intestinal type 1 regulatory T cells migrate to periphery to suppress diabetogenic T cells and prevent diabetes development. *Proc Natl Acad Sci USA*. (2017) 114:10443–8. doi: 10.1073/pnas.1705599114
48. Zhao Q, Elson CO. Adaptive immune education by gut microbiota antigens. *Immunology*. (2018) 154:28–37. doi: 10.1111/imm.12896
49. Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, et al. Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. *Science.* (2015) 349:993–7. doi: 10.1126/science.aaa9420
50. Jakobsson HE, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C, et al. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut*. (2014) 63:559–66. doi: 10.1136/gutjnl-2012-303249
51. Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. *Nat Rev Immunol.* (2016) 16:295–309. doi: 10.1038/nri.2016.36
52. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci USA*. (2010) 107:12204–9. doi: 10.1073/pnas.0909122107
53. Basic M, Keubler LM, Buettner M, Achard M, Breves G, Schroder B, et al. Norovirus triggered microbiota-driven mucosal inflammation in interleukin 10-deficient mice. *Inflamm Bowel Dis*. (2014) 20:431–43. doi: 10.1097/01.MIB.0000441346.86827.ed
54. Botic T, Klingberg TD, Weingartl H, Cencic A. A novel eukaryotic cell culture model to study antiviral activity of potential probiotic bacteria. *Int J Food Microbiol*. (2007) 115:227–34. doi: 10.1016/j.ijfoodmicro.2006.10.044
55. Wang ZY, Chai WD, Burwinkel M, Twardziok S, Wrede P, Palissa C, et al. Inhibitory influence of *Enterococcus faecium* on the propagation of swine influenza A virus *in vitro*. *PLoS ONE*. (2013) 8:e53043. doi: 10.1371/journal.pone.0053043
56. Bandoro C, Runstadler JA. Bacterial lipopolysaccharide destabilizes influenza viruses. *mSphere.* (2017) 2:17. doi: 10.1128/mSphere.00267-17
57. Chen HW, Liu PF, Liu YT, Kuo S, Zhang XQ, Schooley RT, et al. Nasal commensal Staphylococcus epidermidis counteracts influenza virus. *Sci Rep*. (2016) 6:27870. doi: 10.1038/srep27870
58. Tuyama CG, Cheshenko N, Carlucci MJ, Li JH, Li H, Goldberg CL, et al. ACIDFORM inactivates herpes simplex virus and prevents genital herpes in a mouse model: optimal candidate for microbicide combinations. *J Infect Dis.* (2006) 194:795–803. doi: 10.1086/506948
59. Conti C, Malacrino C, Mastromarino P. Inhibition of herpes simplex virus type 2 by vaginal lactobacilli. *J Physiol Pharmacol*. (2009) 60:19–26.
60. Mastromarino P, Cacciotti F, Masci A, Mosca L. Antiviral activity of *Lactobacillus brevis* towards herpes simplex virus type 2: Role of cell wall associated components. *Anaerobe*. (2011) 17:334–6. doi: 10.1016/j.anaerobe.2011.04.022
61. Steed AL, Christophi GP, Kaiko GE, Sun LL, Goodwin VM, Jain U, et al. The microbial metabolite desaminotyrosine protects from influenza through type I interferon. *Science.* (2017) 357:498–502. doi: 10.1126/science.aam5336
62. Yitbarek A, Alkie T, Taha-Abdelaziz K, Astill J, Rodriguez-Lecompte JC, Parkinson J, et al. Gut microbiota modulates type I interferon and antibody-mediated immune responses in chickens infected with influenza virus subtype H9N2. *Benef Microbes.* (2018) 9:417–27. doi: 10.3920/BM2017.0088
63. Hensley-McBain T, Zevin AS, Manuzak J, Smith E, Gile J, Miller C, et al. Effects of fecal microbial transplantation on microbiome and immunity in simian immunodeficiency virus-infected macaques. *J Virol.* (2016) 90:4981–9. doi: 10.1128/JVI.00099-16
64. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity*. (2012) 37:158–70. doi: 10.1016/j.immuni.2012.04.011
65. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci USA*. (2011) 108:5354–9. doi: 10.1073/pnas.1019378108
66. Oh JE, Kim BC, Chang DH, Kwon M, Lee SY, Kang D, et al. Dysbiosis-induced IL-33 contributes to impaired antiviral immunity in the genital mucosa. *Proc Natl Acad Sci USA*. (2016) 113:E762–71. doi: 10.1073/pnas.1518589113
67. Gonzalez-Perez G, Lamouse-Smith ES, Gastrointestinal microbiome dysbiosis in infant mice alters peripheral $CD8^+$ T cell receptor signaling. *Front Immunol.* (2017) 8:265. doi: 10.3389/fimmu.2017.00265
68. Wang Z, MacLeod DT, Di Nardo A. Commensal bacteria lipoteichoic acid increases skin mast cell antimicrobial activity against vaccinia viruses. *J Immunol.* (2012) 189:1551–8. doi: 10.4049/jimmunol.1200471
69. Gonzalez-Perez G, Hicks AL, Tekieli TM, Radens CM, Williams BL, Lamouse-Smith ES. Maternal antibiotic treatment impacts development of the neonatal intestinal microbiome and antiviral immunity. *J Immunol.* (2016) 196:3768–79. doi: 10.4049/jimmunol.1502322
70. Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al. Wild mouse gut microbiota promotes host fitness and improves disease resistance. *Cell.* (2017) 171:1015–28 e13. doi: 10.1016/j.cell.2017.09.016

71. Grayson MH, Camarda LE, Hussain SA, Zemple SJ, Hayward M, Lam V, et al. Intestinal microbiota disruption reduces regulatory T cells and increases respiratory viral infection mortality through increased IFNgamma production. *Front Immunol.* (2018) 9:1587. doi: 10.3389/fimmu.2018.01587
72. Chou HH, Chien WH, Wu LL, Cheng CH, Chung CH, Horng JH, et al. Age-related immune clearance of hepatitis B virus infection requires the establishment of gut microbiota. *Proc Natl Acad Sci USA.* (2015) 112:2175–80. doi: 10.1073/pnas.1424775112
73. Fuglsang E, Pizzolla A, Krych L, Nielsen DS, Brooks AG, Frokiaer H, et al. Changes in gut microbiota prior to influenza A virus infection do not affect immune responses in pups or juvenile mice. *Front Cell Infect Microbiol.* (2018) 8:319. doi: 10.3389/fcimb.2018.00319
74. Miedema F, Hazenberg MD, Tesselaar K, van Baarle D, de Boer RJ, Borghans JA. Immune activation and collateral damage in AIDS pathogenesis. *Front Immunol.* (2013) 4:298. doi: 10.3389/fimmu.2013.00298
75. Lu W, Feng Y, Jing F, Han Y, Lyu N, Liu F, et al. Association between gut microbiota and CD4 recovery in HIV-1 infected patients. *Front Microbiol.* (2018) 9:1451. doi: 10.3389/fmicb.2018.01451
76. Paquin-Proulx D, Ching C, Vujkovic-Cvijin I, Fadrosh D, Loh L, Huang Y, et al. Bacteroides are associated with GALT iNKT cell function and reduction of microbial translocation in HIV-1 infection. *Mucosal Immunol.* (2017) 10:69–78. doi: 10.1038/mi.2016.34
77. Stewart CJ, Mansbach JM, Ajami NJ, Petrosino JF, Zhu Z, Liang L, et al. Serum metabolome is associated with nasopharyngeal microbiota and disease severity among infants with bronchiolitis. *J Infect Dis.* (2019) 219:2005–14. doi: 10.1093/infdis/jiz021
78. de Steenhuijsen Piters WA, Heinonen S, Hasrat R, Bunsow E, Smith B, Suarez-Arrabal MC, et al. Nasopharyngeal microbiota, host transcriptome, and disease severity in children with respiratory syncytial virus infection. *Am J Res Critic Care Med.* (2016) 194:1104–15. doi: 10.1164/rccm.201602-0220OC
79. Terrault NA, Bzowej NH, Chang KM, Hwang JP, Jonas MM, Murad MH, et al. AASLD guidelines for treatment of chronic hepatitis B. *Hepatology.* (2016) 63:261–83. doi: 10.1002/hep.28156
80. Ren YD, Ye ZS, Yang LZ, Jin LX, Wei WJ, Deng YY, et al. Fecal microbiota transplantation induces hepatitis B virus e-antigen (HBeAg) clearance in patients with positive HBeAg after long-term antiviral therapy. *Hepatology.* (2017) 65:1765–8. doi: 10.1002/hep.29008
81. Haak BW, Littmann ER, Chaubard JL, Pickard AJ, Fontana E, Adhi F, et al. Impact of gut colonization with butyrate-producing microbiota on respiratory viral infection following allo-HCT. *Blood.* (2018) 131:2978–86. doi: 10.1182/blood-2018-01-828996
82. Gopinath S, Kim MV, Rakib T, Wong PW, van Zandt M, Barry NA, et al. Topical application of aminoglycoside antibiotics enhances host resistance to viral infections in a microbiota-independent manner. *Nat Microbiol.* (2018) 3:611–21. doi: 10.1038/s41564-018-0138-2
83. Zhu S, Jones MK, Hickman D, Han S, Reeves W, Karst SM. Norovirus antagonism of B-cell antigen presentation results in impaired control of acute infection. *Mucosal Immunol.* (2016) 9:1559–70. doi: 10.1038/mi.2016.15
84. Wilks J, Beilinson H, Theriault B, Chervonsky A, Golovkina T. Antibody-mediated immune control of a retrovirus does not require the microbiota. *J Virol.* (2014) 88:6524–7. doi: 10.1128/JVI.00251-14
85. Isaak DD, Bartizal KF, Caulfield MJ. Decreased pathogenicity of murine leukemia virus-Moloney in gnotobiotic mice. *Leukemia.* (1988) 2:540–4.
86. Kouttab NM, Jutila JW. Friend leukemia virus infection in germfree mice following antigen stimulation. *J Immunol.* (1972) 108:591–5.
87. Mirand EA, Grace JT Jr. Responses of germ-free mice to friend virus. *Nature.* (1963) 200:92–3. doi: 10.1038/200092a0
88. Ammann CG, Messer RJ, Peterson KE, Hasenkrug KJ. Lactate dehydrogenase-elevating virus induces systemic lymphocyte activation via TLR7-dependent IFNalpha responses by plasmacytoid dendritic cells. *PLoS ONE.* (2009) 4:e6105. doi: 10.1371/journal.pone.0006105
89. Wilks J, Golovkina T. Influence of microbiota on viral infections. *PLoS Pathog.* (2012) 8:e1002681. doi: 10.1371/journal.ppat.1002681
90. Kumar A, Vlasova AN, Deblais L, Huang HC, Wijeratne A, Kandasamy S, et al. Impact of nutrition and rotavirus infection on the infant gut microbiota in a humanized pig model. *BMC Gastroenterol.* (2018) 18:93. doi: 10.1186/s12876-018-0810-2
91. Jang JY, Kim S, Kwon MS, Lee J, Yu DH, Song RH, et al. Rotavirus-mediated alteration of gut microbiota and its correlation with physiological characteristics in neonatal calves. *J Microbiol.* (2018) 57:113–21. doi: 10.1007/s12275-019-8549-1
92. Ma X, Wang Q, Li H, Xu C, Cui N, Zhao X. 16S rRNA genes Illumina sequencing revealed differential cecal microbiome in specific pathogen free chickens infected with different subgroup of avian leukosis viruses. *Vet Microbiol.* (2017) 207:195–204. doi: 10.1016/j.vetmic.2017.05.016
93. Zhao N, Li M, Luo J, Wang S, Liu S, Wang S, et al. Impacts of canine distemper virus infection on the giant panda population from the perspective of gut microbiota. *Sci Rep.* (2017) 7:39954. doi: 10.1038/srep39954
94. Ding ZF, Cao MJ, Zhu XS, Xu GH, Wang RL. Changes in the gut microbiome of the Chinese mitten crab (*Eriocheir sinensis*) in response to White spot syndrome virus (WSSV) infection. *J Fish Dis.* (2017) 40:1561–71. doi: 10.1111/jfd.12624
95. Li Y, Saxena D, Chen Z, Liu G, Abrams WR, Phelan JA, et al. HIV infection and microbial diversity in saliva. *J Clin Microbiol.* (2014) 52:1400–11. doi: 10.1128/JCM.02954-13
96. Dang AT, Cotton S, Sankaran-Walters S, Li CS, Lee CY, Dandekar S, et al. Evidence of an increased pathogenic footprint in the lingual microbiome of untreated HIV infected patients. *BMC Microbiol.* (2012) 12:153. doi: 10.1186/1471-2180-12-153
97. Mukherjee PK, Chandra J, Retuerto M, Sikaroodi M, Brown RE, Jurevic R, et al. Oral mycobiome analysis of HIV-infected patients: identification of Pichia as an antagonist of opportunistic fungi. *PLoS Pathog.* (2014) 10:e1003996. doi: 10.1371/journal.ppat.1003996
98. Cribbs SK, Uppal K, Li S, Jones DP, Huang L, Tipton L, et al. Correlation of the lung microbiota with metabolic profiles in bronchoalveolar lavage fluid in HIV infection. *Microbiome.* (2016) 4:3. doi: 10.1186/s40168-016-0147-4
99. Moeller AH, Shilts M, Li Y, Rudicell RS, Lonsdorf EV, Pusey AE, et al. SIV-induced instability of the chimpanzee gut microbiome. *Cell Host Microbe.* (2013) 14:340–5. doi: 10.1016/j.chom.2013.08.005
100. Degnan PH, Pusey AE, Lonsdorf EV, Goodall J, Wroblewski EE, Wilson ML, et al. Factors associated with the diversification of the gut microbial communities within chimpanzees from Gombe National Park. *Proc Natl Acad Sci USA.* (2012) 109:13034–9. doi: 10.1073/pnas.1110994109
101. Noguera-Julian M, Rocafort M, Guillen Y, Rivera J, Casadella M, Nowak P, et al. Gut microbiota linked to sexual preference and HIV infection. *EBioMed.* (2016) 5:135–46. doi: 10.1016/j.ebiom.2016.01.032
102. Vujkovic-Cvijin I, Dunham RM, Iwai S, Maher MC, Albright RG, Broadhurst MJ, et al. Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Sci Transl Med.* (2013) 5:193ra91. doi: 10.1126/scitranslmed.3006438
103. Sun Y, Ma Y, Lin P, Tang YW, Yang L, Shen Y, et al. Fecal bacterial microbiome diversity in chronic HIV-infected patients in China. *Emerg Microbes Infect.* (2016) 5:e31. doi: 10.1038/emi.2016.25
104. Ji Y, Zhang F, Zhang R, Shen Y, Liu L, Wang J, et al. Changes in intestinal microbiota in HIV-1-infected subjects following cART initiation: influence of $CD4^+$ T cell count. *Emerg Microbes Infect.* (2018) 7:113. doi: 10.1038/s41426-018-0117-y
105. Serrano-Villar S, Rojo D, Martinez-Martinez M, Deusch S, Vazquez-Castellanos JF, Sainz T, et al. HIV infection results in metabolic alterations in the gut microbiota different from those induced by other diseases. *Sci Rep.* (2016) 6:26192. doi: 10.1038/srep26192
106. Mayuzumi H, Inagaki-Ohara K, Uyttenhove C, Okamoto Y, Matsuzaki G. Interleukin-17A is required to suppress invasion of Salmonella enterica serovar Typhimurium to enteric mucosa. *Immunology.* (2010) 131:377–85. doi: 10.1111/j.1365-2567.2010.03310.x
107. Raffatellu M, Santos RL, Verhoeven DE, George MD, Wilson RP, Winter SE, et al. Simian immunodeficiency virus-induced mucosal interleukin-17 deficiency promotes Salmonella dissemination from the gut. *Nat Med.* (2008) 14:421–8. doi: 10.1038/nm1743
108. Manfredi R, Chiodo F. Salmonella typhi disease in HIV-infected patients: case reports and literature review. *Infez Med.* (1999) 7:49–53.
109. Haas A, Zimmermann K, Graw F, Slack E, Rusert P, Ledergerber B, et al. Systemic antibody responses to gut commensal bacteria during chronic HIV-1 infection. *Gut.* (2011) 60:1506–19. doi: 10.1136/gut.2010.224774

110. Edouard S, Million M, Bachar D, Dubourg G, Michelle C, Ninove L, et al. The nasopharyngeal microbiota in patients with viral respiratory tract infections is enriched in bacterial pathogens. *Eur J Clin Microbiol Infect Dis*. (2018) 37:1725–33. doi: 10.1007/s10096-018-3305-8
111. Li Y, Ding J, Xiao Y, Xu B, He W, Yang Y, et al. 16S rDNA sequencing analysis of upper respiratory tract flora in patients with influenza H1N1 virus infection. *Front Lab Med*. (2017) 1:16–26. doi: 10.1016/j.flm.2017.02.005
112. Wen Z, Xie G, Zhou Q, Qiu C, Li J, Hu Q, et al. Distinct nasopharyngeal and oropharyngeal microbiota of children with influenza a virus compared with healthy children. *BioMed Res Intl*. (2018) 2018:6362716. doi: 10.1155/2018/6362716
113. Borges L, Giongo A, Pereira LM, Trindade FJ, Gregianini TS, Campos FS, et al. Comparison of the nasopharynx microbiome between influenza and non-influenza cases of severe acute respiratory infections: a pilot study. *Health Science Rep*. (2018) 1:e47. doi: 10.1002/hsr2.47
114. Ramos-Sevillano E, Wade WG, Mann A, Gilbert A, Lambkin-Williams R, Killingley B, et al. The effect of influenza virus on the human oropharyngeal microbiome. *Clin Infect Dis*. (2019) 68:1993–2002. doi: 10.1093/cid/ciy821
115. Yildiz S, Mazel-Sanchez B, Kandasamy M, Manicassamy B, Schmolke M. Influenza A virus infection impacts systemic microbiota dynamics and causes quantitative enteric dysbiosis. *Microbiome*. (2018) 6:9. doi: 10.1186/s40168-017-0386-z
116. Groves HT, Cuthbertson L, James P, Moffatt MF, Cox MJ, Tregoning JS. Respiratory disease following viral lung infection alters the murine gut microbiota. *Front Immunol*. (2018) 9:182. doi: 10.3389/fimmu.2018.00182
117. Zhao N, Wang SP, Li HY, Liu SL, Li M, Luo J, et al. Influence of novel highly pathogenic avian influenza A(H5N1) virus infection on migrating whooper swans fecal microbiota. *Front Cell Infect Microbiol*. (2018) 8:46. doi: 10.3389/fcimb.2018.00046
118. Wang J, Li FQ, Wei HM, Lian ZX, Sun R, Tian ZG. Respiratory influenza virus infection induces intestinal immune injury via microbiota-mediated Th17 cell-dependent inflammation. *J Exp Med*. (2014) 211:2397–410. doi: 10.1084/jem.20140625
119. Yitbarek A, Weese JS, Alkie TN, Parkinson J, Sharif S. Influenza A virus subtype H9N2 infection disrupts the composition of intestinal microbiota of chickens. *Fems Microbiol Ecol*. (2018) 94:165. doi: 10.1093/femsec/fix165
120. Deriu E, Boxx GM, He XS, Pan C, Benavidez SD, Cen LJ, et al. Influenza virus affects intestinal microbiota and secondary salmonella infection in the gut through type I interferons. *Plos Pathog*. (2016) 12:e1005572. doi: 10.1371/journal.ppat.1005572
121. Wang J, Wang Y, Zhang X, Liu J, Zhang Q, Zhao Y, et al. Gut microbial dysbiosis is associated with altered hepatic functions and serum metabolites in chronic hepatitis B patients. *Front Microbiol*. (2017) 8:2222. doi: 10.3389/fmicb.2017.02222
122. Xu M, Wang B, Fu Y, Chen Y, Yang F, Lu H, et al. Changes of fecal Bifidobacterium species in adult patients with hepatitis B virus-induced chronic liver disease. *Microb Ecol*. (2012) 63:304–13. doi: 10.1007/s00248-011-9925-5
123. Inoue T, Nakayama J, Moriya K, Kawaratani H, Momoda R, Ito K, et al. Gut dysbiosis associated with hepatitis C virus infection. *Clin Infect Dis*. (2018) 67:869–77. doi: 10.1093/cid/ciy205
124. Aly AM, Adel A, El-Gendy AO, Essam TM, Aziz RK. Gut microbiome alterations in patients with stage 4 hepatitis C. *Gut Pathog*. (2016) 8:42. doi: 10.1186/s13099-016-0124-2
125. Chen Y, Chen ZJ, Guo RY, Chen N, Lu HF, Huang SA, et al. Correlation between gastrointestinal fungi and varying degrees of chronic hepatitis B virus infection. *Diagn Micr Infec Dis*. (2011) 70:492–8. doi: 10.1016/j.diagmicrobio.2010.04.005
126. Heidrich B, Vital M, Plumeier I, Doscher N, Kahl S, Kirschner J, et al. Intestinal microbiota in patients with chronic hepatitis C with and without cirrhosis compared with healthy controls. *Liver Int*. (2018) 38:50–8. doi: 10.1111/liv.13485
127. Lu H, Wu Z, Xu W, Yang J, Chen Y, Li L. Intestinal microbiota was assessed in cirrhotic patients with hepatitis B virus infection. Intestinal microbiota of HBV cirrhotic patients. *Microb Ecol*. (2011) 61:693–703. doi: 10.1007/s00248-010-9801-8
128. Ling Z, Liu X, Cheng Y, Jiang X, Jiang H, Wang Y, et al. decreased diversity of the oral microbiota of patients with hepatitis B virus-induced chronic liver disease: a pilot project. *Sci Rep*. (2015) 5:17098. doi: 10.1038/srep 17098
129. Zhao Y, Mao YF, Tang YS, Ni MZ, Liu QH, Wang Y, et al. Altered oral microbiota in chronic hepatitis B patients with different tongue coatings. *World J Gastroenterol*. (2018) 24:3448–61. doi: 10.3748/wjg.v24.i30.3448
130. Nelson AM, Walk ST, Taube S, Taniuchi M, Houpt ER, Wobus CE, et al. Disruption of the human gut microbiota following Norovirus infection. *PLoS ONE*. (2012) 7:e48224. doi: 10.1371/journal.pone.00 48224
131. Almand EA, Moore MD, Outlaw J, Jaykus LA. Human norovirus binding to select bacteria representative of the human gut microbiota. *PLoS ONE*. (2017) 12:e0173124. doi: 10.1371/journal.pone.0173124
132. Kernbauer E, Ding Y, Cadwell K. An enteric virus can replace the beneficial function of commensal bacteria. *Nature*. (2014) 516:94–8. doi: 10.1038/nature13960
133. Martin CR, Osadchiy V, Kalani A, Mayer EA. The brain-gut-microbiome axis. *Cell Mol Gastroenterol Hepatol*. (2018) 6:133–48. doi: 10.1016/j.jcmgh.2018.04.003
134. O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav Brain Res*. (2015) 277:32–48. doi: 10.1016/j.bbr.2014.07.027
135. Dinan TG, Cryan JF. Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. *J Physiol*. (2017) 595:489–503. doi: 10.1113/JP273106
136. de Lartigue G, de La Serre CB, Raybould HE. Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. *Physiol Behav*. (2011) 105:100–5. doi: 10.1016/j.physbeh.2011.02.040
137. Park AJ, Collins J, Blennerhassett PA, Ghia JE, Verdu EF, Bercik P, et al. Altered colonic function and microbiota profile in a mouse model of chronic depression. *Neurogastroent Motil*. (2013) 25:733–E575. doi: 10.1111/nmo.12153
138. Pulikkan J, Mazumder A, Grace T. Role of the gut microbiome in autism spectrum disorders. *Adv Exp Med Biol*. (2019) 1118:253–69. doi: 10.1007/978-3-030-05542-4_13
139. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature*. (2011) 479:538–41. doi: 10.1038/nature10554
140. Mohajeri MH, La Fata G, Steinert RE, Weber P. Relationship between the gut microbiome and brain function. *Nutr Rev*. (2018) 76:481–96. doi: 10.1093/nutrit/nuy009
141. Osadchiy V, Martin CR, Mayer EA. The gut-brain axis and the microbiome: mechanisms and clinical implications. *Clin Gastroenterol*. (2019) 17:322–32. doi: 10.1016/j.cgh.2018.10.002
142. Carrillo-Salinas FJ, Mestre L, Mecha M, Feliu A, Del Campo R, Villarrubia N, et al. Gut dysbiosis and neuroimmune responses to brain infection with Theiler's murine encephalomyelitis virus. *Sci Rep*. (2017) 7:44377. doi: 10.1038/srep44377
143. O'Toole PW, Marchesi JR, Hill C. Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nat Microbiol*. (2017) 2:17057. doi: 10.1038/nmicrobiol.2017.57
144. Zuo T, Wong SH, Lam K, Lui R, Cheung K, Tang W, et al. Bacteriophage transfer during faecal microbiota transplantation in Clostridium difficile infection is associated with treatment outcome. *Gut*. (2018) 67:634–43. doi: 10.1136/gutjnl-2017-313952
145. Suez J, Zmora N, Zilberman-Schapira G, Mor U, Dori-Bachash M, Bashiardes S, et al. Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell*. (2018) 174:1406–23 e16. doi: 10.1016/j.cell.2018.08.047
146. Zmora N, Zilberman-Schapira G, Suez J, Mor U, Dori-Bachash M, Bashiardes S, et al. Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell*. (2018) 174:1388–405 e21. doi: 10.1016/j.cell.2018. 08.041
147. Limon JJ, Skalski JH, Underhill DM. Commensal fungi in health and disease. *Cell Host Microbe*. (2017) 22:156–65. doi: 10.1016/j.chom.2017. 07.002

148. Barnett KC, Coronas-Serna JM, Zhou W, Ernandes MJ, Cao A, Kranzusch PJ, etal. Phosphoinositide interactions position cGAS at the plasma membrane to ensure efficient distinction between self- and viral DNA cell. (2019) 176:1432–1446.e11. doi: 10.1016/j.cell.2019.01.049
149. Lian H, Wei J, Zang R, Ye W, Yang Q, Zhang XN, et al. ZCCHC3 is a co-sensor of cGAS for dsDNA recognition in innate immune response. *Nat Commun.* (2018) 9:3349. doi: 10.1038/s41467-018-05559-w
150. Seo GJ, Kim C, Shin WJ, Sklan EH, Eoh H, Jung JU. TRIM56-mediated monoubiquitination of cGAS for cytosolic DNA sensing. *Nat Commun.* (2018) 9:613. doi: 10.1038/s41467-018-02936-3
151. Schoggins JW, MacDuff DA, Imanaka N, Gainey MD, Shrestha B, Eitson JL, et al. Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature.* (2014) 505:691–5. doi: 10.1038/nature12862
152. Mann EA, Harmel-Laws E, Cohen MB, Steinbrecher KA. Guanylate cyclase C limits systemic dissemination of a murine enteric pathogen. *BMC Gastroenterol.* (2013) 13:135. doi: 10.1186/1471-230X-13-135

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REVIEW
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Diet-Microbe-Host Interactions That Affect Gut Mucosal Integrity and Infection Resistance

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The gastrointestinal tract microbiome plays a critical role in regulating host innate and adaptive immune responses against pathogenic bacteria. Disease associated dysbiosis and environmental induced insults, such as antibiotic treatments can lead to increased susceptibility to infection, particularly in a hospital setting. Dietary intervention is the greatest tool available to modify the microbiome and support pathogen resistance. Some dietary components can maintain a healthy disease resistant microbiome, whereas others can contribute to an imbalanced microbial population, impairing intestinal barrier function and immunity. Characterizing the effects of dietary components through the host-microbe axis as it relates to gastrointestinal health is vital to provide evidence-based dietary interventions to mitigate infections. This review will cover the effect of dietary components (carbohydrates, fiber, proteins, fats, polyphenolic compounds, vitamins, and minerals) on intestinal integrity and highlight their ability to modulate host-microbe interactions as to improve pathogen resistance.

Keywords: microbiota, diet, infection resistance, gastrointestinal integrity, disease susceptibility

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INTRODUCTION

Infectious enteric diseases are a major cause of morbidity and mortality worldwide and are of particular concern in hospital settings and developing countries. According to the World Health Organization, infectious enteric diseases are one of the top 10 causes of death leading to over two billion cases and one million deaths worldwide in 2010 (1). Host resistance toward invading pathogens requires tight regulation of the gastrointestinal environment, maintained through a synergistic relationship between the host immune system and microbiome. Disruption to a host's intestinal homeostasis, including insults from diet, stress, antibiotic and drug treatment, allergies, cancer, and related illnesses can leave the host vulnerable to enteric pathogens (2). It is well-understood that diet can play a major role on health by positively and negatively shaping gastrointestinal ecology (3, 4), and therefore should be a major focus in mitigating the severity of infection.

Although humans have successfully reduced pathogen exposure through effective sanitation practices, the adoption of a "Western diet," over-sanitation and lack of physical exercise are hypothesized to have contributed to the rise in autoimmune disorders (5). The "Western diet" is characterized by the excessive consumption of fats, proteins, refined sugar, and low intake of dietary fiber. Other dietary patterns such as the Mediterranean, Vegetarian-based, Japanese-based, and Ketogenic type diets can positively regulate immune responsiveness to reduce immune activity and support health (6). However, human epidemiology studies on diet tend to exclude important interindividual variations that govern the gastrointestinal microbiota and may explain the diverse

claims to which foods are known as "protective" and "harmful" (7). Establishing a mechanistic link between individual diet components using microbe-host interactions will aid to provide evidence driven recommendations to help control an overactive immune response.

An overactive immune system is associated with autoimmune disorders such as irritable bowel disease (IBD) that affects host immune activity and leads to increased incidence of infection (8, 9). Likewise, "westernized diets" have shown to enhance *Escherichia coli* colonization and associated inflammation in mice by altering the host mucus layer, increasing intestinal permeability, and impairing immune function (10). Dietary fiber and other microbiota-accessible carbohydrates (MACs) are a key component missing from the "westernized diet" that when re-introduced provides a beneficial balance to host health and microbiome (11). Fiber is exhaustively studied as a microbial fermentation substrate that produces short chain fatty acids (SCFAs) with known benefits to host intestinal homeostasis and health (12). However, we fear that this focus on the beneficial effects of fiber-associated SCFA production has led researchers to overlook other common dietary components that may positively or negatively influence the host gastrointestinal environment and health.

Diet intervention should be considered a valuable tool to manipulate the host-microbe axis to help sustain intestinal homeostasis and infection resistance. Dietary components such as carbohydrates, lipids, proteins, phytochemicals, minerals, and vitamins all have unique structural and chemical (physicochemical) properties that influence host pathogen resistance directly and indirectly through the microbiome. Bridging the gap between diet, host, and microbiome as they relate to immunity and disease resistance is a multifaceted field that requires an understanding of their combined effects on intestinal homeostasis (**Figure 1**). This review explores the role of common dietary components on host-microbe interactions that modulate host resistance and tolerance toward common infectious diseases. We highlight the opportunity to improve outcomes, yet recognize the current knowledge limits the ability to provide concrete dietary advice. This is partially limited by the fact that diet focused infection resistance research is scarce and difficult to translate to humans.

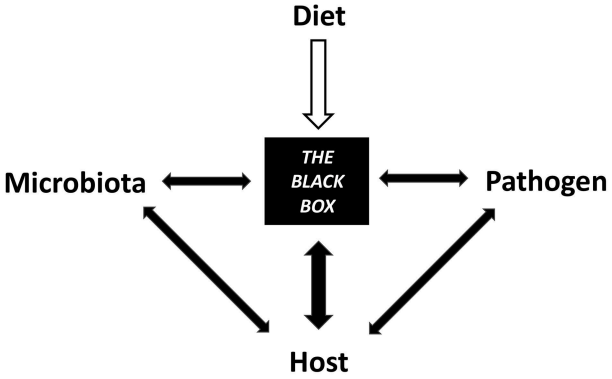

FIGURE 1 | Diet contributes to a black box of intertwined mechanisms between the microbiota, host, and pathogen that have yet to be elucidated.

GALT AND MICROBIOME REGULATE HOST DEFENSES

The gut associated lymphoid tissue (GALT) plays a crucial role in regulating intestinal homeostasis and is composed of lymph nodes, lamina propria, and epithelial cells that together provide the host with a protective barrier and immune defense against invading pathogens (13). On the other hand, the microbiota provides a physical presence that can directly prevent pathogen colonization by competing for attachment sites or nutrient resources. Indirectly, the microbiota helps to improve host resistance by modulating intestinal integrity through the mucus layer, tight junction proteins, and antimicrobial peptides (AMPs: cathelicidins, C-type lectins, and defensins) (14, 15). Mucins secreted by goblet cells provide the first line of defense by forming a physical barrier composed of highly glycosylated and interlinked proteins between luminal bacteria and host epithelial cells (16). The mucus layer provides lubricant and is metabolized by mucin-degrading (mucolytic) bacteria forming the loosely attached layer (17), whereas the adherent layer, when properly formed, secures a balance of host AMPs and immune factors that maintain intestinal homeostasis (18).

Disruptions to the balanced microbial ecosystem greatly increase a host's vulnerability to infection (19). In particular, antibiotic exposure can cause major shifts in microbial communities leading to mucus layer thinning, predisposing, and exacerbating infections, as shown with antibiotic accompanied *Citrobacter rodentium* infections in mice (20). Antibiotic-induced microbiota imbalances are well-documented to alter the production of AMPs, tight junction proteins, and immune factors that normally contribute to intestinal homeostasis and infection resistance (21, 22). Secretory immunoglobulin A (SIgA) antibodies are abundant immune factors of the intestinal lumen that protect epithelial cells from enteric pathogens and toxins by blocking their access to epithelial receptors and entrapping them in mucus to promote clearance (23). Although SIgA targets and disrupts pathogens and antigens, commensal microbes such as *Bacteroides fragilis* alter their surface proteins to attract SIgA to enhance mucosal colonization (24). Intestinal epithelial cells (IECs) produce reactive oxygen species (ROS) (25) and Resistin-like molecules (e.g., RELMβ) (26) that hinder commensal and pathogenic bacteria colonization, further maintaining intestinal hemostasis. IECs apical surface fucosylation is another useful host strategy that controls commensal microbes and inhibits pathogens. Secreted fucose is metabolized by bacteria to produce bioactive metabolites, reduce virulence factors, and enrich beneficial gut microbes to strengthen colonization exclusion (27). Alternatively, fucose can be fermented by commensal microbes into 1,2-propanediol and utilized by *Salmonella* during inflammation to drive their fitness in the colon (28).

The host has significant control over microbial communities of the small and large intestine; however, this relationship is complex and is managed in part through gastric acid secretions, intestinal motility, bile secretions, oxygen gradients,

and regulation of pattern recognition receptors (PPRs), such as Toll-like receptors (TLRs) (4). The host recognizes commensal bacteria through activation of TLRs and relays an appropriate response in accordance to the specific microbial derived ligands [e.g., peptidoglycan, lipoprotein, lipopolysaccharide (LPS), and flagellin] (29). Innate lymphoid cells (ILCs) have been identified as key immune regulatory cells of the GALT controlling pathogen resistance, inflammation, and metabolic homeostasis (30). ILCs concentrate within mucosal surfaces and relay signals sent between the microbiota, epithelia, immune cells, and metabolites in the intestine to maintain epithelial barrier function. Transcriptomic analysis of 15 ILC subtypes revealed their regulatory functions depend on the presence of the microbiome, nutrients, and xenobiotics (31). Ultimately, it is the combined relationship between the gut microbiota, host, and diet that help improve or worsen a host's ability to tolerate and resist pathogenic bacteria (**Figure 2**). The remainder of this review will focus on specific dietary components and how they stimulate some of these and other host-microbe interactions resulting in impaired or improved host disease resistance.

CARBOHYDRATES

Dietary carbohydrates are often classified by their degree of polymerization into mono-, di-, oligo-, or poly-saccharides and composition of their monosaccharides: glucose, fructose, galactose, and xylose. Typically, carbohydrates are categorized as either digestible or indigestible (fiber). Binding and structural properties of carbohydrates dictate the glucosidase enzymes required to break bonds into their basic units for absorption (32). The digestible carbohydrates escaping host small intestinal digestion, along with dietary fiber, become available as microbial energy substrates and are able to substantially alter the intestinal ecosystem and community structure (33).

Increasing intake of digestible carbohydrates has been scrutinized for contributing to the worldwide obesity and diabetes epidemics. However, carbohydrates are essential energy substrates for the central nervous system and red blood cells, are required to maintain cellular energy balance after sustained increases in metabolic activity, and to restore energy levels and glycogen stores (34). Humans and animals are able to regulate blood glucose levels; however, excessive dietary carbohydrate consumption can worsen acute hyperglycemia, particularly during times of an illness (35, 36) and stress (37, 38). A medical illness can enhance the negative effects of acute hyperglycemia, which include inhibition of neutrophil migration, phagocytosis, superoxide production, and microbial killing, compromising host innate immunity against bacterial and fungal infections (39). Diets high in simple and refined carbohydrates are shown to negatively impact gastrointestinal microbial communities leading to intestinal barrier dysfunction and greater risk for enteric infection (36). Whereas, balanced diets containing resistant starch and fiber stimulate microbial fermentation leading to a stable diverse microbiome and production of beneficial SCFAs (40). Understanding both negative and positive effects of carbohydrate consumption on gastrointestinal immunity and microbial populations will provide vital insight toward dietary strategies to help maintain pathogen resistance.

Dietary trehalose, a food component used to improve a product's texture, flavor, glycemic index and shelf life, was introduced in the early 2000's and has since been proposed to have contributed to the global *Clostridioides difficile* epidemic (41). Trehalose is a disaccharide composed of two glucose molecules linked by a resistant α,α−1,1-glucosidic bond found in plants, algae, fungi, yeast, bacteria, insects, and other invertebrates (42). Mammals and other vertebrates lack the ability to synthesize trehalose, and the dietary fate of trehalose depends on the capacity of the small intestinal trehalase enzyme to hydrolyze it into glucose (43). Trehalase deficiency is rare in humans but excessive consumption of trehalose can lead to negative intestinal imbalances similar to those associated with lactose and fructose intolerances. Researchers believe the increased use of trehalose in food production has naturally selected for *C. difficile* with the capacity to metabolize trehalose more efficiently, thus increasing pathogen fitness and contributing to their hypervirulent outbreaks in the human population (41). To combat reoccurring *C. difficile* infections a fecal microbial transplant (FMT) from a healthy donor has become a helpful treatment option, however the mechanism of remission remains unclear (44). The success of FMTs to treat *C. difficile* infections highlights the importance of a "healthy" gut microbiome to promote infection resistance. Additional research is needed to confirm the impact of specific carbohydrates and their malabsorption on immune and microbial networks in the gut as it relates to pathogen fitness. Interestingly, studies in mice comparing fiber-rich and fiber-deprived diets support the detrimental effect of a simple carbohydrate dominated diet and the importance of fiber on infection resistance (11, 33).

DIETARY FIBER

Health benefits associated with foods rich in non-digestible dietary fiber depend on their type, source, and proportion of water soluble and insoluble carbohydrate components (45). Fruits, vegetables, and grains are excellent sources of numerous fiber types, however, not all fiber sources and types are created equal. The food source, glycosylated chain structures, and their fermentability, along with other inherent components are key parameters for their functional quality within the gastrointestinal tract (12). Non-digestible carbohydrates are composed of monosaccharide units (glucose, fructose, galactose, xylose, fucose, and sialic acid) found naturally in plants, algae, fungi, bacteria, and mammalian milk, or produced by chemical or enzymatic processes (46, 47). Short chain fructo-oligosaccharides (FOS) have received a great deal of attention due to their prebiotic effects (48) and fact that they occur naturally (mostly as inulin) with different degrees of polymerization in foods (47). The consumption of prebiotic fibers have helped with diarrhea and constipation (49–51), however, not everyone benefits from their consumption, and can even lead to excessive gas production, bloating, and discomfort (50, 52). In cases of gastrointestinal

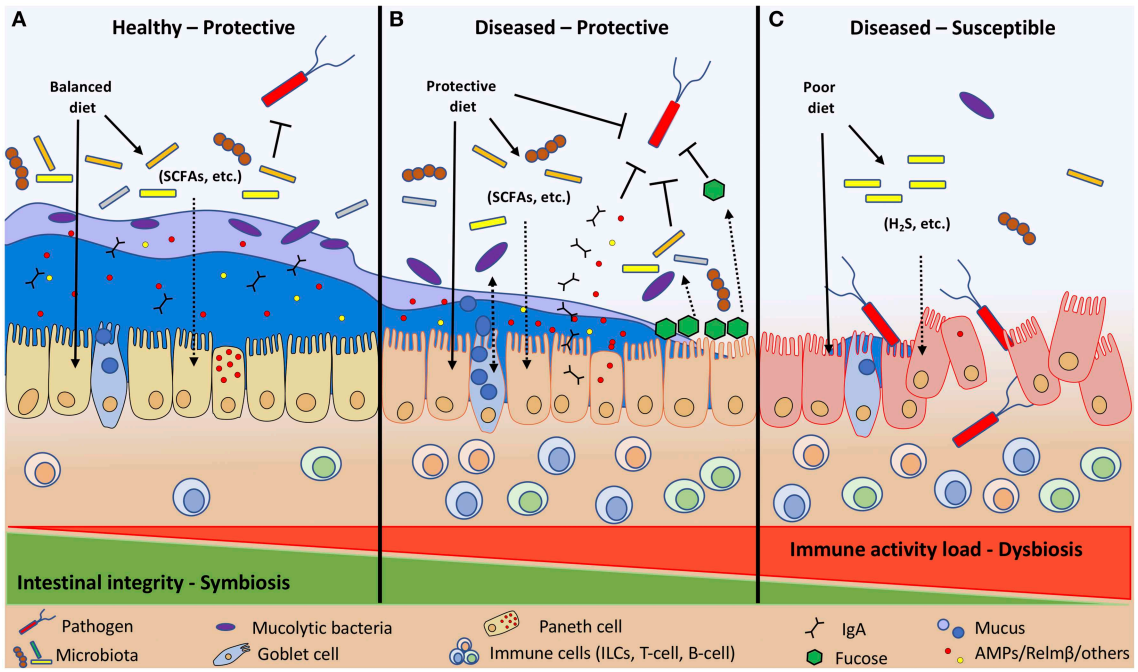

FIGURE 2 | Diet and immune activity load (allergies, cancer, other illness, etc.) determine host intestinal integrity toward invading pathogens. Diet affects intestinal integrity directly by stimulating IECs, ILCs, and microbial communities, and indirectly through microbial fermentation by-products (SCFAs, H_2S, etc.). A healthy individual following a balanced diet to maintain symbiosis between host and microbial populations has enhanced intestinal integrity with a thick inner and outer mucus layer that retains AMPs and other compounds to protect the host against pathogen colonization **(A)**. A diseased host with heightened immune activity maintains symbiosis by consuming dietary components that protect and boost host innate defenses (IgA, AMPs, mucus, fucosylation) and adaptive immune responses to prevent pathogen colonization **(B)**. Whereas, diseased individuals with heightened immune activity consuming a poor diet are more susceptible to enteric infections due to impaired host defenses that cannot control the dysbiotic intestinal environment **(C)**.

discomfort, a diet low in fructans (FODMAP-restricted diet) or reducing dietary fiber is often effective but remains controversial, and individualized (53–55).

The effects of various non-digestible fiber on health and microbiota is thoroughly reviewed (12, 45, 46). In general, dietary fiber can modify gastrointestinal function directly through fecal bulking and indirectly through the modification of microbial community structure, and by increasing microbial biomass and fermentation products (45). Fiber fermentation leads to beneficial SCFAs (mainly acetate, propionate, and butyrate) but also undesired gases such as carbon dioxide, hydrogen, and methane (56). Increased gas production, fecal bulking and delayed gastric emptying can lead to discomfort, bloating, and flatus in many individuals (45). Microbial fermentation products such as SCFAs interact with the intestinal epithelium to promote certain defense mechanisms. In particular, microbial production of butyrate provides an energy substrate to epithelial cells (57), maintains the hypoxic environment (58), and promotes improved barrier function through hypoxia inducible factor (HIF) (59). Induction of HIF transcription factor subsequently stimulates downstream signaling to increase mucus production (60) and expression of AMPs (61) ultimately helping to minimize facultative pathogen growth.

According to the Global Burden of Diseases, Injuries, and Risk Factors Study of 2015 infectious diarrhea is a leading cause of death globally among all ages (1.3 million deaths); with a large proportion of those occurring in infants under 5 years of age (499,000 deaths) (62). Providing children with MACs is an important strategy to mitigate infection burden by stabilizing the microbiota and by bolstering intestinal immunity. Infants that are exclusively breast fed have reduced risk of developing diarrheal disease (63), partially due to the naturally occurring human milk oligosaccharides (HMOs) present in breast milk. HMOs are soluble complex carbohydrates that act as prebiotics, providing a substrate for the intestinal microbiota and can prevent pathogenic bacterial adhesion through a variety of mechanisms (64). *In vitro* studies determined that HMOs act as pathogen decoy receptors to prevent infections and their activities depend on the location and degree of fucosylation (65). Human breast milk contains a multitude of other bioactive factors, immunoglobulins, cytokines, chemokines, growth factors, hormones, and lactoferrin which all likely contribute to the improved disease resistance of breast fed infants and is reviewed elsewhere (66). Human milk has shown the ability to directly inhibit the adherence of *Streptococcus pneumonia* and *Haemophilus influenza* to human mucosal cells *ex vivo* (67). When HMOs were fractioned, it was found that the acidic fraction had greater anti-adhesive properties toward enteropathogenic *E. coli* (EPEC), *Vibrio cholera,* and *Salmonella fyris* compared to the neutral high and low molecular weight

fractions (68). Similarly, HMOs blocked EPEC adherence to epithelial cells *in vitro* and reduced EPEC colonization in newborn mice, further implying the essential role HMO play in the prevention of infectious disease in human infants (69). Experimentally, it was shown that supplementing formula with HMO reduced the duration of diarrhea in rotavirus-infected pigs and promoted IFNγ and IL-10 expression in the ileum, suggesting HMOs may also protect infants against rotavirus infection (70). Therefore, research efforts have focused on HMO substitutes that can be added to formula fed to infants that are unable to breastfeed. Human and animal studies suggest supplementing formula with fermentable fiber (e.g., soy polysaccharides, fructo- & galacto- oligosaccharide) reduces infection-associated diarrhea burden by improving intestinal homeostasis (71) and increasing beneficial *Bifidobacterium* species (72–74).

Minimizing infectious diarrheal disease with dietary tools has become the focus of recent research efforts. The importance of non-digestible fermentable fiber or MACs intake in adults has clearly been shown where a greater intake (comparing top vs. bottom quartiles) reduced risk of death from cardiovascular, infectious, and respiratory disease by 24–56% in men and 34–59% in women (75). Galacto-oligosaccharides (GOS) have shown to increase bifidobacteria and beneficially modulate immune function when supplemented to elderly volunteers. Along with improving phagocytosis and natural killer cell activity, the GOS supplemented volunteers had an anti-inflammatory cytokine profiles with increased IL-10 and reduced IL-1B, IL-6, and TNFα (76). In a double blind placebo controlled trial, those supplemented with GOS had reduced diarrhea incidence, duration, and severity (77). *Clostridioides difficile* is the leading cause of health care-associated diarrheal infections, commonly affecting the elderly and antibiotic treated hospitalized patients (78). Significant evidence suggests that the inclusion of soluble fiber to the diet, specifically MACs that increase SCFA production, may be a useful strategy to enhance infection resistance (79). In a mouse model, dietary inclusion of MACs or inulin alone was shown to suppress *C. difficile* infection; whereas diets devoid of MACs exacerbated the infection (11). The mechanisms by which MACs help to mitigate *C. difficile* infection is through the expansion of fiber fermenting microbiota (via competitive exclusion) and subsequent increases in their immune-stimulatory metabolites (promote host defenses), which limit a pathogen's fitness (11).

β-glucans are one type of fermentable fiber that is frequently studied due to its common occurrence in the cell walls of yeast, fungi, and cereals such as barley and oats. Aside from acting as a microbial fermentation substrate, β-glucans are also of great interest for their direct effect on host immune activities and functions that alter immunity toward infections. In humans, the immune modulating property is due to the binding of β-glucans with host receptor dectin-1 (80), which contributes to macrophages activation, and induce phagocytosis (81). Studies in mice found that oat derived β-glucans supplemented at 3 mg every other day stimulated a systemic immune response that reduced fecal oocyst shedding of *Eimeria vermiformis* by 39.6% post-challenge by increasing specific antibodies against the parasite (81). Oral administration of β-glucan from a fungal source (*Sclerotinia sclerotiorum* at 80 mg/kg every 2 or 3 days) was shown to directly stimulate proliferative responses of Peyer's patches to both T and B-cell mitogens, suggesting β-glucans may also stimulate a mucosal immune activation (82). Intraperitoneal injection of β-glucans has also shown to work as a potent adjuvant to enhance host resistance to both bacterial (81) and parasitic (Leishmania) infections (83). The use of immunostimulants derived from naturally occurring polysaccharides (e.g., β-glucan or chitosan) has become somewhat commonplace in the aquaculture industry as an alternative strategy for disease prevention. Inclusion of oligo-β-glucans (100–200 mg/kg) to striped catfish has shown to improve growth performance and reduce mortality post *Edwardsiella ictaluri* challenge via heightened phagocytic and lysozyme activity (84). The inclusion of dietary β-glucans (200 mg/kg) in poultry has also been used effectively to reduce the severity of necrotic enteritis when challenged with *Eimeria* and *C. perfringens* (85) and inhibited growth depression when challenged with *Salmonella enteritidis* (86) by increasing specific antibody levels. In both cases, inclusion of dietary β-glucans reduced pathogen colonization (*C. perfringens* and *S. enteritidis*).

Generally, increasing fiber will change the microbiome and improve gastrointestinal heath. As stated previously, the benefits associated from consuming food sources or supplements high in fiber is individualized and should be carefully monitored for side-effects.

FATS

Fats are an essential dietary macronutrient that have been criticized and are commonly avoided in developed countries with the objective of reducing weight, cholesterol levels, and cardiovascular disease risk. Fat avoidance and subsequent reliance on simple carbohydrates for caloric intake with reduced energy expenditure is believed to have contributed to the unintended rise of obesity worldwide (87). In healthy individuals most fats are emulsified and absorbed in the small intestine; however, in excess and during intestinal stress fats can travel toward the colon as a substrate for the microbiota (88). Human and animal studies have shown that intestinal microbes have the capacity to alter host homeostasis through a variety of metabolites, including carcinogenic and cytotoxic secondary bile acids (89). The effects of the microbiota on host homeostasis is through alteration to hepatic lipid and bile metabolism, reverse cholesterol transport, energy expenditure, and insulin sensitivity in peripheral tissue (90). In this respect, dietary lipids are capable of directly affecting the host and microbiome, while indirectly altering host homeostasis through the microbiome and their metabolites.

The direct effect of microbial fat metabolism on intestinal health has yet to be established but studies have shown that dietary lipid profiles can alter the outcome of enteric infections. Fat consumption with regards to infection have been thoroughly reviewed elsewhere (91), and provides a bases to establish the connection between microbe and host

on enteric pathogen resistance. A study comparing dietary saturated (SFA, milk), monounsaturated (MUFA, olive oil), and polyunsaturated (PUFA, omega-6 corn oil) fatty acids uncovered distinct lipid mediated immune responses in mice after an acute *C. rodentium* challenge (92). SFA and MUFA dominated diets induced protective T-regulatory cells, interleukin (IL)-10, IL-33, and SCFAs that helped mitigate inflammation during enteric infection (92). Interestingly, in a dextran sodium sulfate (DSS) model, IL-10 knockout mice fed a diet containing milk SFAs, but not lard fat SFAs, resulted in a pro-inflammatory T_H1 immune response associated with a bloom of *Bilophila wadsworthia* and its metabolites, hydrogen sulfide and secondary bile acids (93). Diets high in medium-chain SFAs like coconut oil have antifungal action toward *Candida albicans* (94) and antibacterial properties against enteric pathogens (95). Moreover, the addition of fish oil, high in omega-3 (n-3) fatty acids to a SFA dominated diet activated intestinal alkaline phosphatase (IAP), an enzyme that detoxifies proinflammatory lipopolysaccharide (LPS) endotoxins from gram-negative bacteria that accumulates during infection; whereas supplementing n-3 to an n-6 rich diet did not enhance IAP activity (92). Previously it has been observed that high levels of dietary n-6 PUFAs in fact reduce IAP activity leading to LPS endotoxemia in mice (96). Transgenic *Fat-1* mice, which genetically retain a higher concentration of n-3 in their tissues, demonstrated elevated serum IL-10 and IAP activity (96). In mice, safflower and canola oil based diets (high in n-6) heighten mucosal T_H1/T_H17 responses and inflammation, whereas a fish oil based diet has shown to have a protective anti-inflammatory effect following a *C. rodentium* infection (97). Diets rich in n-3 PUFAs have proven protective against many extracellular pathogens (*Mycobacterium tuberculosis*, *Salmonella typhimurium*, *S. pneumoniae*, *Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus*, *C. rodentium*, *Helicobacter hepaticus*, *H. pylori*, and *Listeria monocytogene*); however, potentially damaging effects were observed during intracellular viral infections (98, 99). Dose and timing of n-3 PUFAs is critical for intestinal immune homeostasis. Sustained high doses alter microbial communities and host immune system toward an anti-inflammatory state that could exacerbate infections, especially when proinflammatory responses are essential for infection clearance (98). Interestingly, lipid composition affects host-microbial interactions even when administered via a non-enteral route. The inclusion of mixed lipids containing soybean oil, medium-chain triglycerides, olive oil, and fish oil in parenteral formula was shown to reduce intestinal inflammation and alter microbial composition in a piglet model of infant total parenteral nutrition as compared to soybean oil alone (100).

PROTEIN

Protein homeostasis is crucial for host health, physiology, and immune development that together foster a fast-acting immune response toward pathogens. The role of dietary protein and amino acids on host immune function related to diet malnutrition and pathogen interactions has been thoroughly reviewed (101, 102). Amino acids play a major role in regulating immune cell activation, cellular redox homeostasis, lymphocyte proliferation, and production of cytokines, cytotoxins, and antibodies (101). Protein deficiency is well-known to impair immunity and infection resistance, especially during stress and illness due to protein malabsorption and protein consuming processes such as tissue repair (103). Protein deficits have been shown to exacerbate parasitic *Cryptosporidium* infections in mice through disruption of baseline (primary) Th1-type mucosal immunity (104). Furthermore, protein-deprived diets decreased small intestinal macrophage proliferation and IL-10 production independently of the microbiota (105).

In contrast, researchers propose that protein-rich diets can be just as harmful since they lead to an increase in undigested proteins that encourage protein-fermenting bacteria and disease susceptibility (106). Resistant and undigested proteins can interfere with host functions directly as biologically active proteins (BAP) like trypsin and chymotrypsin inhibitors, and indirectly through microbial proteolytic fermentation by-products [H_2, CO_2, CH_4, H_2S, SCFA, branched chain amino acids (BCAA), nitrogenous compounds, phenols, and indoles] with poorly understood health outcomes (107). It is important to note that dietary crude protein can contain a high concentration of BAPs whose activities can be reduced upon hydrolysis digestion (heating, chemical, or enzymatic). A study replacing crude protein (wheat and casein) with purified amino acids to diets fed to weaned pigs reduced proteolytic fermentation before and after an enterotoxigenic *E. coli* (ETEC) K88 challenge (108). Three days post-infection, ETEC K88 colonized the small intestine of pigs fed the crude protein diet whereas no colonization was observed in the small intestine of pigs receiving the purified amino acid diet. In this context, undigested protein or other components associated with crude protein diets promoted ETEC growth and colonization in the small intestine.

Furthermore, the source of proteins can impact microbial communities depending on the digestibility and total amino acids in the diet (106). For instance, animal proteins tend to be highly digestible in the proximal intestine compared to plant-based proteins (109). Processing proteins with heat can impact their digestibility, for example, rats fed thermolyzed (heated to 180°C for 1–2 h) casein, soy, or egg white protein had reduced proximal intestinal digestibility, leading to a greater degree of protein fermentation in the cecum (110). The number of aberrant crypts were measured after azoxymethane challenge to assess the carcinogenic promoting properties of casein, soy, and egg proteins. For the heat-treated proteins, the number of aberrant crypts increased with casein, remained unchanged with soy, and decreased with egg white compared to untreated protein diets. In agreement, a DSS mouse model study using multiple custom diets demonstrated that casein and soy proteins worsened DSS associated weight loss, whereas no effect was seen in mice fed the egg white protein diets (111). In contrast, a human trial compared high- and low-fat diets with non-meat protein (legumes, nuts, grains, soy), red meat protein (beef) or white meat protein (chicken and turkey) on the gut microbiome and found only a modest impact of protein source on the microbiome (112). For cardiovascular health, the plant-based proteins outperformed meat protein diets but white meat was no better than red

meat for reducing disease risk (113). However, animal protein dominated diets tend to include higher amounts of fats, which ultimately may be more impactful on health than the proteins themselves. Plant-based protein diets may inherently contain detrimental components. For example, soybean isoflavones are suggested to contribute to greater parasitic oocyst fecal output and reduce immune responsiveness in mice fed a soy-based diet compared to casein and whey protein fed groups (114). For this reason, crude protein diet studies make it difficult to identify the bioactive component responsible for the observed phenotype. A study in rats comparing protein from soy, casein, pork, beef, chicken, and fish indicates that protein source alters microbial composition (115). Specifically, white meat (chicken and fish) increased beneficial *Lactobacillus* species. Blood levels of lipopolysaccharide-binding protein (LBP), a marker for lipopolysaccharide (LPS) endotoxemia, was found to be significantly higher in the soy protein diet group compared to fish, chicken, pork, beef, and casein protein fed groups. Further research is needed in controlled animal models to investigate isolated protein types and processing techniques on host digestion, microbiome, and fermentation products to mechanistically link the impact of protein on infection resistance.

Dietary glutamine supplementation has proven to be an effective therapy to help restore intestinal integrity in patients with post-infectious associated irritable bowel syndrome (116). Although glutamine significantly improved IBS scores compared to a placebo supplemented group, a larger cohort and mechanistic studies are warranted. The effect of glutamine supplementation may be associated with glutamines ability to enhance intestinal cell proliferation (117), decrease the Firmicute population, and activate innate immunity through NF-κB, MAPK, and PI3K-Akt signaling pathways (118). Similar effects have been observed with arginine supplementation (119). Over a 14-day study, daily supplementation of 30 g of L-glutamine to overweight individuals led to a significant decrease in Firmicute populations, including species from the genus *Dialister*, *Dorea*, *Pseudobutyrivibrio*, and *Veillonella* (120). Since overweight individuals typically have a higher Firmicute/Bacteroidetes ratio than lean individuals (121), a decrease in Firmicutes with glutamine supplementation suggests that dietary glutamine may play a beneficial role in restoring microbiota balance. In accordance, glutamine and arginine supplementation promoted the activation of innate immunity and lowered intestinal pathogen load in ETEC-infected mice (122). In humans, enteral glutamine administration in critically ill patients with severe trauma, burns, and sepsis significantly reduced the number of isolated enteric bacteria such as *Pseudomonas* sp., *Klebsiella* sp., *E. coli*, and *Acinetobacter* sp., all of which can contribute to pneumonia if transmitted to the lungs (123, 124). Enteral glutamine administration reduced bacterial overgrowth within the gastrointestinal tract, which may have reduced the chance of bacterial exposure to the lungs and explain the reduced incidence of pneumonia in patients. Moreover, a systematic review and meta-analysis concluded that glutamine-enriched enteral formulae can significantly reduce gut permeability in critically ill patients (125). The requirement and importance of enteric glutamine has been extensively reviewed (126), but requires further research in healthy subjects and animals models to understand the impact on the microbiome and enteric infection resistance.

Further emerging evidence suggests that numerous microbially-derived indoles from tryptophan catabolism can promote intestinal homeostasis by activating regulatory T cells (Tregs) through their interaction with the aryl hydrocarbon receptor (AhR) (127). Roager and Licht summarize known microbes responsible for producing tryptophan-derivatives that positively act on tight junctions, gastrointestinal motility, host metabolism, AhR to activate IL-22, along with their systemic anti-oxidative and anti-inflammatory properties (128). In this respect, dietary tryptophan likely contributes to infection resistance by priming host defense strategies. The importance of tryptophan is further supported by the ability of host dendritic cells to metabolize tryptophan into kynurenine using indoleamine 2,3-dioxygenase-1 (IDO1) in order to control host inflammation during a *C. difficile* infection (129). Kynurenine production during *C. difficile* infection is proposed to be beneficial as it reduces excessive interferon-γ (IFNγ) cytokine production by limiting neutrophil populations in the lamina propria (129). Clinically, these findings provide important insight into the use of IDO1 inhibitors for cancer treatment which would prevent kynurenine production, and increase the severity of *C. difficile* infection (129). Like tryptophan, threonine is another essential amino acid that must be obtained from diet with deficiencies leading to immune and barrier dysfunctions (130). Dietary threonine is essential for the production of mucin with deficient diets leading to altered mucosal integrity and persistent diarrhea in neonatal piglets (131). The importance of dietary threonine for mucus production and structure may not only provide protection for host IECs but also could stimulate mucolytic bacteria with unknown functions (**Figure 3**).

Dietary protein source, amount, and processing can alter their impact and effects within gastrointestinal environment. Clearly host protein digestion shares an intimate relationship with the gut microbiome and their fermentation products (132). A balanced macronutrient or low indigestible protein diet is recommended to discourage proteolytic bacteria from overproducing cytotoxic, genotoxic, and carcinogenic by-products that disrupt intestinal integrity and increase the risk of infection (106).

PHYTOCHEMICALS

Plants synthesize a large pool of compounds known as phytochemicals to protect themselves from stress, predation, and infection. Complex mixtures of phytochemicals are found in the roots, seeds, leaves, bark, flowers, and fruit of plants and have been intensively studied for their antimicrobial, anti-inflammatory, and antioxidants activities (133). The physicochemical properties of phytochemicals give plants their unique color, smell, and flavor profiles, and dictates their bioactivities and bioavailability within the gastrointestinal tract (134). Condensed tannins, mainly polymeric flavanols can act as antinutritional factors that reduce host digestion

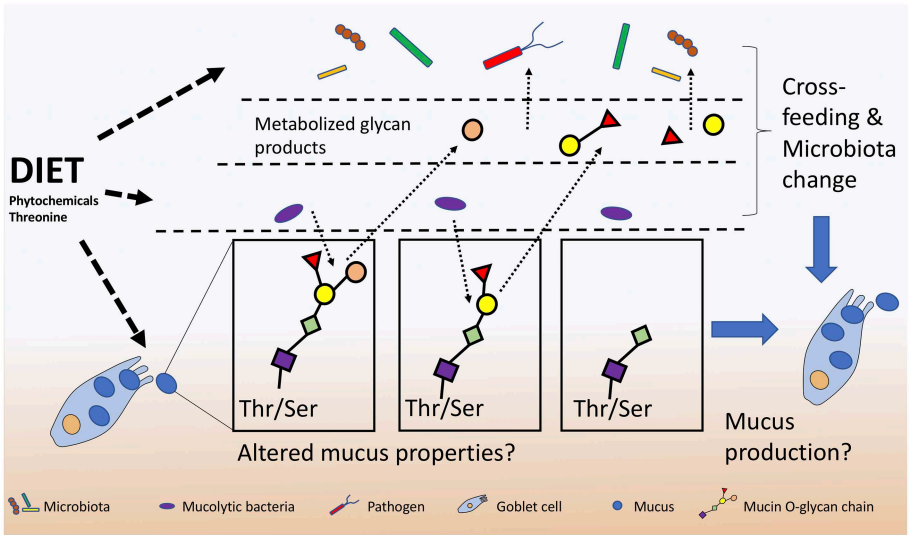

FIGURE 3 | Diet alters host-microbiota-pathogen mechanisms of mucus production and consumption. Mucolytic specialists that digest the mucus O-glycans and subsequently cross-feed with other bacteria and pathogens can lead to further microbiota changes and alterations to mucosal integrity.

through enzyme inhibition and protein precipitation (135). However, the consumption of phytochemicals is typically associated with beneficial health outcomes from their activities on the resident microbial population and host metabolism (14, 136, 137). Phytochemicals are treated as xenobiotics by the host and because of this, the liver can reintroduce phytochemical derivatives to microbes through enterohepatic circulation, further complicating their effects on host health. Many studies fail to demonstrate and characterize absorbed phytochemical derivatives to investigate whether their impact on host are direct or indirect through the microbiota.

Research has focused on the use of phytochemicals as an alternative to antibiotics and as a dietary supplement to strengthen host pathogen resistance (138). For instance, chickens fed a mixture of pepper (*Capsicum*) and turmeric oleoresin had less weight loss and reduced intestinal lesions scores in a necrotic enteritis disease model (139). The phytochemical mixture lowered intestinal but increased splenic proinflammatory cytokines/chemokines (IL-8, lipopolysaccharide-induced TNF-a factor, IL-17) levels altering host immunity through immune cell differentiation, proliferation, apoptosis and NO production (139). Reactive nitrogen and oxygen species produced by peripheral leucocytes is an essential defense strategy against pathogens. In fish, dietary supplementation of a grass extract (*Cynodon dactylon*) to infected *Catla catla* carp stimulated reactive oxygen and nitrogen species production and decreased mortality in a dose depend manner (140). Screening multiple phytonutrients revealed that the dietary flavonoid naringenin can act as an agonist on the AhR to induce regulatory T cells (Treg) that suppress allergy and autoimmune disease (141). Interestingly, phytochemicals such as indole-3-carbinol (I3C) present in cruciferous vegetables (e.g., broccoli, cabbage) act as ligands for AhR leading to the expansion of the anti-inflammatory IL-22 producing ILCs (142). Functioning AhR has proven to be crucial for immunity because AhR-deficient mice failed to control *C. rodentium* infections (143). Moreover, mice fed a phytochemical-free diet had a reduced formation of lymphocyte aggregates and follicles, a similar phenotype as seen in AhR-deficient mice (142). Dietary I3C supplementation protected against *C. difficile* infection through activation of AhR but also through unknown AhR-independent mechanisms likely caused by changes to microbial populations (144).

Anti-adhesion properties are well sought after when studying the direct effects of phytochemicals on pathogen fitness. Cranberry extracts are documented to inhibit pathogenic *E. coli* adhesins (e.g., fimbriae) limiting their ability to attach to host cells (145, 146). The anti-adhesion activity of cranberry extract is attributed to the polyphenolic flavan-3-ol compounds known as A-type proanthocyanidins (PACs) (147). Cranberry A-type PACs reduced adherence of multiple strains uropathogenic *E. coli* and *Proteus mirabilis in vitro* (145). However, *in vivo*, intestinal and microbial PACs metabolites are found at higher concentrations in urine than the intact PACs and thus may be the bioactive metabolites responsible for the anti-adhesive properties (148). Interestingly, an analysis of urine phytochemical metabolites indicated that they change over-time due to multiple rounds of enterohepatic circulation modifications (148) with poorly understood activities (149). Moreover, cranberry PACs are thought to inhibit host and microbial enzymes (e.g., lipase, glycosidases) protecting against diet-induced obesity (150). PACs are associated with increased *Akkermansia* sp. abundance; however, it is unknown whether microbiota changes are a direct action of PACs or an indirect result of their effects on host metabolism (151). B-type PACs are known to be less inhibitory to both bacteria and host metabolism (150). Work from our group demonstrates that pea seed coats rich in B-type PACs lead to a

significant decrease in the Firmicutes population, increased fecal mucin content, and caused greater pathogen colonization in mice compared to a PAC-poor diet (152). B-type PACs may have led to improper mucus formation leading to a greater concentration of mucin excreted in feces. Phytonutrient supplementation is associated with increases in beneficial Clostridia species and can strengthen mucosal barrier function by increasing mucus production and thickness (153), protecting epithelial cells from invading pathogens and disease. Interestingly, a positive feedback loop may be established between mucolytic bacteria such as *Akkermansia* sp. that can degrade mucus O-linked glycans, thereby producing SCFAs that could stimulate goblet cells to secrete more mucus (14). Polyphenolic compounds may stimulate the microbiota directly or indirectly through modulation of mucus production, however further research is needed to establish direct links between diet and infection resistance (**Figure 3**).

VITAMINS AND MINERALS

Micronutrients are essential for proper metabolic and immune function. Nutrient and mineral deficiencies, typical in those that are critically-ill and in developing countries, can lead to metabolic changes, oxidative damage, immunological defects, weakness, and death (154). The effects of essential minerals, including iron, zinc, copper, selenium, silver, sulfur, calcium, phosphorus, and magnesium have been shown to affect resident microbial populations and health outcomes in both animal and human studies (155). Phagocytes have been shown to utilize the bactericidal actions of copper and zinc to enhance intracellular killing of pathogens (156). For instance, mice fed a zinc-deficient diet and challenged with Enteroaggregative *E. coli* (EAEC) had reduced leukocyte infiltration and increased virulence factors in luminal content, indicating an impaired immune response and increased infection severity (157). Regular supplementation of vitamin C (1–2 g/day) and zinc (<100 mg/day) reduced the duration of the common cold by 8–14 and 33%, respectively (158, 159). For vitamin C, prophylactic doses >0.2 g/day alleviated respiratory associated problems, particularly in physically strained and stressed individuals, however, its use as a therapy to treat the common cold remains controversial (160). In contrast, zinc supplementation studies support its use as a treatment option to reduce the duration and severity of the common cold (159). Vitamin D had the best overall protection against the common cold, however baseline vitamin D levels and dose must be considered since lower doses and deficient individuals experienced the most benefit (158). More mechanistic research is required to understand the impact of vitamins on immune responsiveness, especially with respect to the microbe-host gut axis in deficient and in excess conditions. Experiments in germ-free, conventionalized and infectious *C. rodentium* mice models confirm that the microbiota influences vitamin D metabolism by lowering fibroblast growth factor (FGF) 23 through increased activation of TNF-α in the colon (161). The fact that the presence of the microbial community or mono-colonization with *C. rodentium* increases serum vitamin D levels highlights their role on host homeostasis, especially since vitamin D levels control calcium homeostasis and bone formation (161). Research suggests that proper regulation of vitamins and minerals is key for establishing a proper immune response and intestinal barrier function. Similar to vitamin and mineral deficiencies, excessive supplementation can impair a host ability to resist enteric infections by altering intestinal integrity or enhancing pathogen fitness.

Recently, oral iron and vitamin B12 supplementation are suggested to impair microbiota dependent infection resistance. A systematic review and meta-analysis comprising 6831 adult participants concluded that oral ferrous sulfate (iron) supplementation is associated with a significant increase in gastrointestinal side-effects compared to placebo and intravenous iron delivery (162). This reveals that the effects of iron supplementation are possibly initiated through the microbe-gut axis with unknown consequences and should be used cautiously. For instance, excessive luminal iron affects intestinal integrity through oxygen radical production, encourages pathogen virulence, and alters microbial populations leading to pathogen overgrowth (163, 164). In a dose dependent manner, iron increased epithelial invasion and translocation of *S. typhimurium* in Caco-2 cells *in vitro* and reduced the survival of the nematode *Caenorhabditis elegans* infected with *S. typhimuriumi* (163, 165). Regulation of luminal iron is extremely important for maintaining intestinal integrity and controlling pathogen expansion (166). Furthermore, lipocalin-2 is a protein produced by neutrophils and epithelial cells during inflammation that directly limits bacterial iron uptake, reducing pathogen overgrowth and severity (167). Unlike iron, vitamin B12 is directly regulated in the gut by intrinsic factors for absorption and in excess, it can escape host absorption and affect microbial competition. The gut commensal bacteria *Bacteroides thetaiotaomicron* may compete against enterohemorrhagic *E. coli* (EHEC) to sequester dietary vitamin B12 (168). *In vitro* competition assays show that *B. thetaiotaomicron* reduced EHEC shiga toxins but when co-cultured with a mutant *B. thetaiotaomicron* lacking a vitamin B12 transporter, EHEC had normal shiga toxin production (168). Microbial vitamin B12 transporters have different affinities toward vitamin B12 allowing them to compete with host cells and other microbes to take up exogenous vitamin B12 (169, 170). More research is needed into micronutrient supplementation on host-microbe interactions toward pathogens, especially in the context of over-supplementation, which may be detrimental depending on the micronutrient balance and host intestinal homeostasis. Limiting the expansion of enteric pathogens can be accomplished by reducing their access to vitamin or minerals either through diet or stimulation of gut commensals to compete with pathogen for vital nutrients.

CONCLUSION

Pathogen resistance and tolerance requires tight host regulation of dietary components and subsequent microbial actions that together influence each other and host immunity. Undigested

and unabsorbed dietary components are able to influence microbial populations and their fermentation by-products can indirectly contribute to infection resistance by modulating host intestinal integrity. Dietary intervention studies are difficult to control and compare due to seasonal variations in diets sources. We suggest that dietary intervention studies should include diet backgrounds designed with macro- and micro- nutrients that stress and protect the gastrointestinal environment, as to give a proper assessment of that dietary component on host. In general, a balanced diet of SFA, MUFA, MACs, protein, phytochemicals, vitamins, and minerals with limited sources of n-6 PUFAs, simple carbohydrates, BAPs, and iron may help restore intestinal homeostasis in compromised individuals. Dietary individuality makes it difficult to make general diet recommendations as each individual may have genetic, microbiota, and unforeseen environmental factors that influence diet digestibility and utilization. Together, these factors ultimately provide the context to which dietary components may influence intestinal integrity and homeostasis.

AUTHOR CONTRIBUTIONS

The concept of this review was developed by BW and AF. This review was written by AF and JF, and was edited by BW, AF, and JF.

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REFERENCES

1. Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, et al. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* (2015) 12:e1001921. doi: 10.1371/journal.pmed.1001921
2. Stecher B. The roles of inflammation, nutrient availability and the commensal microbiota in enteric pathogen infection. *Microbiol Spectr.* (2015) 3:297–320. doi: 10.1128/microbiolspec.MBP-0008-2014
3. Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. *J Transl Med.* (2017) 15:73. doi: 10.1186/s12967-017-1175-y
4. Byndloss MX, Pernitzsch SR, Bäumler AJ. Healthy hosts rule within: ecological forces shaping the gut microbiota. *Mucosal Immunol.* (2018) 11:1299–305. doi: 10.1038/s41385-018-0010-y
5. Manzel A, Muller DN, Hafler DA, Erdman SE, Linker RA, Kleinewietfeld M. Role of "Western diet" in inflammatory autoimmune diseases. *Curr Allergy Asthma Rep.* (2014) 14:404. doi: 10.1007/s11882-013-0404-6
6. Soldati L, Di Renzo L, Jirillo E, Ascierto PA, Marincola FM, De Lorenzo A. The influence of diet on anti-cancer immune responsiveness. *J Transl Med.* (2018) 16:75. doi: 10.1186/s12967-018-1448-0
7. Ananthakrishnan AN. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol.* (2015) 12:205–17. doi: 10.1038/nrgastro.2015.34
8. Singh H, Nugent Z, Yu BN, Lix LM, Targownik LE, Bernstein CN. Higher incidence of *Clostridium difficile* infection among individuals with inflammatory bowel disease. *Gastroenterology.* (2017) 153:430–8.e2. doi: 10.1053/j.gastro.2017.04.044
9. Hong SN, Kim HJ, Kim KH, Han SJ, Ahn IM, Ahn HS. Risk of incident *Mycobacterium tuberculosis* infection in patients with inflammatory bowel disease: a nationwide population-based study in South Korea. *Aliment Pharmacol Ther.* (2017) 45:253–63. doi: 10.1111/apt.13851
10. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, et al. Western diet induces dysbiosis with increased e coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut.* (2014) 63:116–24. doi: 10.1136/gutjnl-2012-304119
11. Hryckowian AJ, Van Treuren W, Smits SA, Davis NM, Gardner JO, Bouley DM, et al. Microbiota-accessible carbohydrates suppress *Clostridium difficile* infection in a murine model. *Nat Microbiol.* (2018) 3:662–9. doi: 10.1038/s41564-018-0150-6
12. Makki K, Deehan EC, Walter J, Bäckhed F. The impact of dietary fiber on gut microbiota in host health and disease. *Cell Host Microbe.* (2018) 23:705–15. doi: 10.1016/j.chom.2018.05.012
13. Forchielli ML, Walker WA. The role of gut-associated lymphoid tissues and mucosal defence. *Br J Nutr.* (2005) 93:S41. doi: 10.1079/BJN20041356
14. Anhê FF, Varin TV, Le Barz M, Desjardins Y, Levy E, Roy D, et al. Gut microbiota dysbiosis in obesity-linked metabolic diseases and prebiotic potential of polyphenol-rich extracts. *Curr Obes Rep.* (2015) 4:389–400. doi: 10.1007/s13679-015-0172-9
15. Dudek-Wicher RK, Junka A, Bartoszewicz M. The influence of antibiotics and dietary components on gut microbiota. *Prz Gastroenterol.* (2018) 13:85–92. doi: 10.5114/pg.2018.76005
16. Dupont A, Heinbockel L, Brandenburg K, Hornef MW. Antimicrobial peptides and the enteric mucus layer act in concert to protect the intestinal mucosa. *Gut Microbes.* (2014) 5:761–5. doi: 10.4161/19490976.2014.972238
17. Sicard J-F, Le Bihan G, Vogeleer P, Jacques M, Harel J. Interactions of intestinal bacteria with components of the intestinal mucus. *Front Cell Infect Microbiol.* (2017) 7:387. doi: 10.3389/fcimb.2017.00387
18. Antoni L, Nuding S, Weller D, Gersemann M, Ott G, Wehkamp J, et al. Human colonic mucus is a reservoir for antimicrobial peptides. *J Crohn's Colitis.* (2013) 7:e652–64. doi: 10.1016/j.crohns.2013.05.006
19. Willing BP, Russell SL, Finlay BB. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nat Rev Microbiol.* (2011) 9:233–43. doi: 10.1038/nrmicro2536
20. Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, et al. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated *Citrobacter rodentium*-induced colitis. *Infect Immun.* (2011) 79:1536–45. doi: 10.1128/IAI.01104-10
21. Menendez A, Willing BP, Montero M, Wlodarska M, So CC, Bhinder G, et al. Bacterial stimulation of the TLR-MyD88 pathway modulates the homeostatic expression of ileal paneth cell α-defensins. *J Innate Immun.* (2013) 5:39–49. doi: 10.1159/000341630
22. Cash HL. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science.* (2006) 313:1126–30. doi: 10.1126/science.1127119
23. Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol.* (2011) 4:603–11. doi: 10.1038/mi.2011.41
24. Donaldson GP, Ladinsky MS, Yu KB, Sanders JG, Yoo BB, Chou WC, et al. Gut microbiota utilize immunoglobulin a for mucosal colonization. *Science.* (2018) 360:795–800. doi: 10.1126/science.aaq0926
25. Knaus UG, Hertzberger R, Pircalabioru GG, Yousefi SPM, Branco Dos Santos F. Pathogen control at the intestinal mucosa - H2O2 to the rescue. *Gut Microbes.* (2017) 8:67–74. doi: 10.1080/19490976.2017.1279378
26. Pine GM, Batugedara HM, Nair MG. Here, there and everywhere: resistin-like molecules in infection, inflammation, and metabolic disorders. *Cytokine.* (2018) 110:442–51. doi: 10.1016/j.cyto.2018.05.014
27. Pickard JM, Chervonsky AV. Intestinal fucose as a mediator of host-microbe symbiosis. *J Immunol.* (2015) 194:5588–93. doi: 10.4049/jimmunol.1500395
28. Faber F, Thiennimitr P, Spiga L, Byndloss MX, Litvak Y, Lawhon S, et al. Respiration of microbiota-derived 1,2-propanediol

drives salmonella expansion during colitis. *PLoS Pathog.* (2017) 13:e1006129. doi: 10.1371/journal.ppat.1006129
29. Hug H, Mohajeri MH, La Fata G. Toll-like receptors: regulators of the immune response in the human gut. *Nutrients.* (2018) 10:11–3. doi: 10.3390/nu10020203
30. Klose CSN, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol.* (2016) 17:765–74. doi: 10.1038/ni.3489
31. Gury-BenAri M, Thaiss CA, Serafini N, Winter DR, Giladi A, Lara-Astiaso D, et al. The spectrum and regulatory landscape of intestinal innate lymphoid cells are shaped by the microbiome. *Cell.* (2016) 166:1231–46.e13. doi: 10.1016/j.cell.2016.07.043
32. Goodman BE. Insights into digestion and absorption of major nutrients in humans. *Adv Physiol Educ.* (2010) 34:44–53. doi: 10.1152/advan.00094.2009
33. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, et al. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell.* (2016) 167:1339–53.e21. doi: 10.1016/j.cell.2016.10.043
34. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: the role of glucose in physiological and pathological brain function. *Trends Neurosci.* (2013) 36:587–97. doi: 10.1016/j.tins.2013.07.001
35. Ingels C, Vanhorebeek I, Van den Berghe G. Glucose homeostasis, nutrition and infections during critical illness. *Clin Microbiol Infect.* (2018) 24:10–5. doi: 10.1016/j.cmi.2016.12.033
36. Thaiss CA, Levy M, Grosheva I, Zheng D, Soffer E, Blacher E, et al. Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection. *Science.* (2018) 1383:eaar3318. doi: 10.1126/science.aar3318
37. Marfella R, Quagliaro L, Nappo F, Ceriello A, Giugliano D, Pennathur S, et al. Acute hyperglycemia induces an oxidative stress in healthy subjects. *J Clin Invest.* (2001) 108:635–6. doi: 10.1172/JCI13727
38. van der Kooij MA, Jene T, Treccani G, Miederer I, Hasch A, Voelxen N, et al. Chronic social stress-induced hyperglycemia in mice couples individual stress susceptibility to impaired spatial memory. *Proc Natl Acad Sci USA.* (2018) 115:E10187–96. doi: 10.1073/pnas.1804412115
39. Jafar N, Edriss H, Nugent K. The effect of short-term hyperglycemia on the innate immune system. *Am J Med Sci.* (2016) 351:201–11. doi: 10.1016/j.amjms.2015.11.011
40. Martens EC, Neumann M, Desai MS. Interactions of commensal and pathogenic microorganisms with the intestinal mucosal barrier. *Nat Rev Microbiol.* (2018) 16:457–70. doi: 10.1038/s41579-018-0036-x
41. Collins J, Robinson C, Danhof H, Knetsch CW, Van Leeuwen HC, Lawley TD, et al. Dietary trehalose enhances virulence of epidemic *Clostridium difficile*. *Nature.* (2018) 553:291–4. doi: 10.1038/nature25178
42. Richards AB, Krakowka S, Dexter LB, Schmid H, Wolterbeek APM, Waalkens-Berendsen DH, et al. Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies. *Food Chem Toxicol.* (2002) 40:871–98. doi: 10.1016/S0278-6915(02)00011-X
43. Argüelles JC. Why can't vertebrates synthesize trehalose? *J Mol Evol.* (2014) 79:111–6. doi: 10.1007/s00239-014-9645-9
44. Baktash A, Terveer EM, Zwittink RD, Hornung BVH, Corver J, Kuijper EJ, et al. Mechanistic insights in the success of fecal microbiota transplants for the treatment of *Clostridium difficile* infections. *Front Microbiol.* (2018) 9:1242. doi: 10.3389/fmicb.2018.01242
45. Eswaran S, Muir J, Chey WD. Fiber and functional gastrointestinal disorders. *Am J Gastroenterol.* (2013) 108:718–27. doi: 10.1038/ajg.2013.63
46. Mussatto SI, Mancilha IM. Non-digestible oligosaccharides: a review. *Carbohydr Polym.* (2007) 68:587–97. doi: 10.1016/j.carbpol.2006.12.011
47. Belorkar SA, Gupta AK. Oligosaccharides: a boon from nature's desk. *AMB Express.* (2016) 6:82 doi: 10.1186/s13568-016-0253-5
48. Singh SP, Jadaun JS, Narnoliya LK, Pandey A. Prebiotic oligosaccharides: special focus on fructooligosaccharides, its biosynthesis and bioactivity. *Appl Biochem Biotechnol.* (2017) 183:613–35. doi: 10.1007/s12010-017-2605-2
49. Suares NC, Ford AC. Systematic review: the effects of fibre in the management of chronic idiopathic constipation. *Aliment Pharmacol Ther.* (2011) 33:895–901. doi: 10.1111/j.1365-2036.2011.04602.x
50. Souza D da S, Tahan S, Weber TK, de Araujo-Filho HB, de Morais MB. Randomized, double-blind, placebo-controlled parallel clinical trial assessing the effect of fructooligosaccharides in infants with constipation. *Nutrients.* (2018) 10:E1602. doi: 10.3390/nu10111602
51. Beleli CAV, Antonio MARGM, Dos Santos R, Pastore GM, Lomazi EA. Effect of 4′galactooligosaccharide on constipation symptoms. *J Pediatr.* (2015) 91:567–73. doi: 10.1016/j.jped.2015.01.010
52. Yang J, Wang HP, Zhou L, Xu CF. Effect of dietary fiber on constipation: a meta-analysis. *World J Gastroenterol.* (2012) 18:7378–83. doi: 10.3748/wjg.v18.i48.7378
53. Ho KS, Tan CYM, Daud MAM, Seow-Choen F. Stopping or reducing dietary fiber intake reduces constipation and its associated symptoms. *World J Gastroenterol.* (2012) 18:4593–6. doi: 10.3748/wjg.v18.i33.4593
54. Eswaran S, Farida JP, Green J, Miller JD, Chey WD. Nutrition in the management of gastrointestinal diseases and disorders: the evidence for the low FODMAP diet. *Curr Opin Pharmacol.* (2017) 37:151–7. doi: 10.1016/j.coph.2017.10.008
55. Rao SSC, Yu S, Fedewa A. Systematic review: dietary fibre and FODMAP-restricted diet in the management of constipation and irritable bowel syndrome. *Aliment Pharmacol Ther.* (2015) 41:1256–70. doi: 10.1111/apt.13167
56. Krompiewski S. Graphene nanoribbons with end- and side-contacted electrodes. *Acta Phys Pol A.* (2012) 121:1216–8. doi: 10.12693/APhysPolA.121.1216
57. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther.* (2008) 27:104–9. doi: 10.1111/j.1365-2036.2007.03562.x
58. Litvak Y, Byndloss MX, Bäumler AJ. Colonocyte metabolism shapes the gut microbiota. *Science.* (2018) 362:eaat9076. doi: 10.1126/science.aat9076
59. Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, et al. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe.* (2015) 17:662–71. doi: 10.1016/j.chom.2015.03.005
60. Louis NA, Hamilton KE, Canny G, Shekels LL, Ho SB, Colgan SP. Selective induction of mucin-3 by hypoxia in intestinal epithelia. *J Cell Biochem.* (2006) 99:1616–27. doi: 10.1002/jcb.20947
61. Kelly CJ, Glover LE, Campbell EL, Kominsky DJ, Ehrentraut SF, Bowers BE, et al. Fundamental role for HIF-1α in constitutive expression of human β defensin-1. *Mucosal Immunol.* (2013) 6:1110–8. doi: 10.1038/mi.2013.6
62. Troeger C, Forouzanfar M, Rao PC, Khalil I, Brown A, Reiner RC, et al. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect Dis.* (2017) 17:909–48. doi: 10.1016/S1473-3099(17)30276-1
63. Popkin BM, Adair L, Akin JS, Black R, Briscoe J, Flieger W. Breast-feeding and diarrheal morbidity. *Pediatrics.* (1990) 86:874–82.
64. Le Doare K, Holder B, Bassett A, Pannaraj PS. Mother's milk: a purposeful contribution to the development of the infant microbiota and immunity. *Front Immunol.* (2018) 9:361. doi: 10.3389/fimmu.2018.00361
65. Craft KM, Thomas HC, Townsend SD. Interrogation of human milk oligosaccharide fucosylation patterns for antimicrobial and antibiofilm trends in group B streptococcus. *ACS Infect Dis.* (2018) 4:1755–65. doi: 10.1021/acsinfecdis.8b00234
66. Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: a review on its composition and bioactivity. *Early Hum Dev.* (2015) 91:629–35. doi: 10.1016/j.earlhumdev.2015.08.013
67. Andersson B, Porras O, Hanson L, Lagergård T, Svanborg-Eden C. Inhibition of attachment of streptococcus pneumoniae and haemophilus influenza by human milk and receptor oligosaccharides. *J Infect Dis.* (1986) 153:368–70. doi: 10.1093/infdis/153.2.232
68. Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, et al. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. *Pediatr Res.* (2006) 59:377–82. doi: 10.1203/01.pdr.0000200805.45593.17
69. Manthey CF, Autran CA, Eckmann L, Bode L. Human milk oligosaccharides reduce EPEC attachment *in vitro* and EPEC colonization in mice. *J Pediatr Gastroenterol Nutr.* (2014) 58:165–8. doi: 10.1097/MPG.0000000000000172
70. Li M, Monaco MH, Wang M, Comstock SS, Kuhlenschmidt TB, Fahey GC, et al. Human milk oligosaccharides shorten rotavirus-induced diarrhea and

modulate piglet mucosal immunity and colonic microbiota. *ISME J.* (2014) 8:1609–20. doi: 10.1038/ismej.2014.10
71. Correa-Matos NJ, Donovan SM, Isaacson RE, Gaskins HR, White BA, Tappenden KA. Fermentable fiber reduces recovery time and improves intestinal function in piglets following *Salmonella typhimurium* infection. *J Nutr.* (2003) 133:1845–52. doi: 10.1093/jn/133.6.1845
72. Giovannini M, Verduci E, Gregori D, Ballali S, Soldi S, Ghisleni D, et al. Prebiotic effect of an infant formula supplemented with galacto-oligosaccharides: randomized multicenter trial. *J Am Coll Nutr.* (2014) 33:385–93. doi: 10.1080/07315724.2013.878232
73. Brown KH, Perez F, Peerson JM, Fadel J, Brunsgaard G, Ostrom KM, et al. Effect of dietary fiber (soy polysaccharide) on the severity, duration, and nutritional outcome of acute, watery diarrhea in children. *Pediatrics.* (1993) 92:241–7.
74. Vanderhoof JA, Murray ND, Paule CL, Ostrom KM. Use of soy fiber in acute diarrhea in infants and toddlers. *Clin Pediatr.* (1997) 36:135–9. doi: 10.1177/000992289703600303
75. Park Y, Subar AF, Hollenbeck A, Schatzkin A. Dietary fiber intake and mortality in the NIH-AARP diet and health study. *Arch Intern Med.* (2011) 171:1061–8. doi: 10.1001/archinternmed.2011.18
76. Vulevic J, Drakoularakou A, Yaqoob P, Tzortzis G, Gibson GR. Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. *Am J Clin Nutr.* (2008) 88:1438–46. doi: 10.3945/ajcn.2008.26242
77. Drakoularakou A, Tzortzis G, Rastall RA, Gibson GR. A double-blind, placebo-controlled, randomized human study assessing the capacity of a novel galacto-oligosaccharide mixture in reducing travellers' diarrhoea. *Eur J Clin Nutr.* (2010) 64:146–52. doi: 10.1038/ejcn.2009.120
78. Leffler DA, Lamont JT. *Clostridium difficile* infection. *N Engl J Med.* (2015) 372:1539–48. doi: 10.1056/NEJMra1403772
79. Verspreet J, Damen B, Broekaert WF, Verbeke K, Delcour JA, Courtin CM. A critical look at prebiotics within the dietary fiber concept. *Annu Rev Food Sci Technol.* (2016) 7:167–90. doi: 10.1146/annurev-food-081315-032749
80. Brown GD, Gordon S. Immune recognition: a new receptor for β-glucans. *Nature.* (2001) 413:36–7. doi: 10.1038/35092620
81. Yun CH, Estrada A, Van Kessel A, Park BC, Laarveld B. β-Glucan, extracted from oat, enhances disease resistance against bacterial and parasitic infections. *FEMS Immunol Med Microbiol.* (2003) 35:67–75. doi: 10.1016/S0928-8244(02)00460-1
82. Hashimoto K, Suzuki I, Yadomae T. Oral administration of SSG, a β -glucan obtained from *Sclerotinia sclerotiorum*, affects the function of Peyer's patch cells. *Int J Immunopharmacol.* (1991) 13:437–42. doi: 10.1016/0192-0561(91)90014-X
83. Abid Obaid K, Ahmad S, Manzoor Khan H, Ali Mahdi A, Khanna R. Protective effect of L. donovani antigens using glucan as an adjuvant. *Int J Immunopharmacol.* (1989) 11:229–35. doi: 10.1016/0192-0561(89)90159-8
84. Nguyen ND, Van Dang P, Le AQ, Nguyen TKL, Pham DH, Van Nguyen N, et al. Effect of oligochitosan and oligo-β-glucan supplementation on growth, innate immunity, and disease resistance of striped catfish (*Pangasianodon hypophthalmus*). *Biotechnol Appl Biochem.* (2017) 64:564–71. doi: 10.1002/bab.1513
85. Tian X, Shao Y, Wang Z, Guo Y. Effects of dietary yeast β-glucans supplementation on growth performance, gut morphology, intestinal *Clostridium perfringens* population and immune response of broiler chickens challenged with necrotic enteritis. *Anim Feed Sci Technol.* (2016) 215:144–55. doi: 10.1016/j.anifeedsci.2016.03.009
86. Shao Y, Wang Z, Tian X, Guo Y, Zhang H. Yeast β-d-glucans induced antimicrobial peptide expressions against Salmonella infection in broiler chickens. *Int J Biol Macromol.* (2016) 85:573–84. doi: 10.1016/j.ijbiomac.2016.01.031
87. Liu AG, Ford NA, Hu FB, Zelman KM, Mozaffarian D, Kris-Etherton PM. A healthy approach to dietary fats: understanding the science and taking action to reduce consumer confusion. *Nutr J.* (2017) 16:1–15. doi: 10.1186/s12937-017-0271-4
88. Agans R, Gordon A, Kramer DL, Perez-Burillo S, Rufián-Henares JA, Paliy O. Dietary fatty acids sustain growth of human gut microbiota. *Appl Environ Microbiol.* (2018) 84:e01525-18. doi: 10.1128/AEM.01525-18
89. Ridlon JM, Wolf PG, Gaskins HR. Taurocholic acid metabolism by gut microbes and colon cancer. *Gut Microbes.* (2016) 7:201–15. doi: 10.1080/19490976.2016.1150414
90. Ghazalpour A, Cespedes I, Bennett BJ, Allayee H. Expanding role of gut microbiota in lipid metabolism. *Curr Opin Lipidol.* (2016) 27:141–7. doi: 10.1097/MOL.0000000000000278
91. Quin C, Gibson DL. Dietary lipids and enteric infection in rodent models. In: Patel VB, editor. *The Molecular Nutrition of Fats.* Elsevier (2019). p. 49–64. doi: 10.1016/B978-0-12-811297-7.00004-4
92. DeCoffe D, Quin C, Gill SK, Tasnim N, Brown K, Godovannyi A, et al. Dietary lipid type, rather than total number of calories, alters outcomes of enteric infection in mice. *J Infect Dis.* (2016) 213:1846–56. doi: 10.1093/infdis/jiw084
93. Devkota S, Wang Y, Musch M. 43 dietary fat-induced taurocholic acid production promotes pathobiont and colitis in IL-10$^{-/-}$ mice. *Gastroenterology.* (2012) 142:S—12. doi: 10.1016/S0016-5085(12)60043-2
94. Gunsalus KTW, Tornberg-Belanger SN, Matthan NR, Lichtenstein AH, Kumamoto CA. Manipulation of host diet to reduce gastrointestinal colonization by the opportunistic pathogen candida albicans. *mSphere.* (2016) 1:e00020–15. doi: 10.1128/mSphere.00020-15
95. Shilling M, Matt L, Rubin E, Visitacion MP, Haller NA, Grey SF, et al. Antimicrobial effects of virgin coconut oil and its medium-chain fatty acids on *Clostridium difficile*. *J Med Food.* (2013) 16:1079–85. doi: 10.1089/jmf.2012.0303
96. Kaliannan K, Wang B, Li XY, Kim KJ, Kang JX. A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endotoxemia. *Sci Rep.* (2015) 5:11276. doi: 10.1038/srep11276
97. Hekmatdoost A, Wu X, Morampudi V, Innis SM, Jacobson K. Dietary oils modify the host immune response and colonic tissue damage following *Citrobacter rodentium* infection in mice. *AJP Gastrointest Liver Physiol.* (2013) 304:G917–28. doi: 10.1152/ajpgi.00292.2012
98. Husson MO, Ley D, Portal C, Gottrand M, Hueso T, Desseyn JL, et al. Modulation of host defence against bacterial and viral infections by omega-3 polyunsaturated fatty acids. *J Infect.* (2016) 73:523–35. doi: 10.1016/j.jinf.2016.10.001
99. Jones GJB, Roper RL. The effects of diets enriched in omega-3 polyunsaturated fatty acids on systemic vaccinia virus infection. *Sci Rep.* (2017) 7:15999. doi: 10.1038/s41598-017-16098-7
100. Lavallee CM, Lim DW, Wizzard PR, Mazurak VC, Mi S, Curtis JM, et al. Impact of clinical use of parenteral lipid emulsions on bile acid metabolism and composition in neonatal piglets. *JPEN J Parenter Enter Nutr.* (2018) 43:668–76. doi: 10.1002/jpen.1437
101. Li P, Yin Y, Li D, Woo Kim S, Wu G. Amino acids and immune function. *Br J Nutr.* (2007) 98:237. doi: 10.1017/S000711450769936X
102. Ren W, Rajendran R, Zhao Y, Tan B, Wu G, Bazer FW, et al. Amino acids as mediators of metabolic cross talk between host and pathogen. *Front Immunol.* (2018) 9:319. doi: 10.3389/fimmu.2018.00319
103. Jonker R, Engelen MPKJ, Deutz NEP. Role of specific dietary amino acids in clinical conditions. *Br J Nutr.* (2012) 108(Suppl.):S139–48. doi: 10.1017/S0007114512002358
104. Bartelt LA, Bolick DT, Kolling GL, Roche JK, Zaenker EI, Lara AM, et al. Cryptosporidium priming is more effective than vaccine for protection against cryptosporidiosis in a murine protein malnutrition model. *PLoS Negl Trop Dis.* (2016) 10:e0004820. doi: 10.1371/journal.pntd.0004820
105. Ochi T, Feng Y, Kitamoto S, Nagao-Kitamoto H, Kuffa P, Atarashi K, et al. Diet-dependent, microbiota-independent regulation of IL-10-producing lamina propria macrophages in the small intestine. *Sci Rep.* (2016) 6:27634. doi: 10.1038/srep27634
106. Ma N, Tian Y, Wu Y, Ma X. Contributions of the interaction between dietary protein and gut microbiota to intestinal health. *Curr Protein Pept Sci.* (2017) 18:795–808. doi: 10.2174/1389203718666170216153505
107. Yao CK, Muir JG, Gibson PR. Review article: insights into colonic protein fermentation, its modulation and potential health implications. *Aliment Pharmacol Ther.* (2016) 43:181–96. doi: 10.1111/apt.13456
108. Opapeju FO, Krause DO, Payne RL, Rademacher M, Nyachoti CM. Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis. *J Anim Sci.* (2009) 87:2635–43. doi: 10.2527/jas.2008-1310

109. Windey K, de Preter V, Verbeke K. Relevance of protein fermentation to gut health. *Mol Nutr Food Res.* (2012) 56:184–96. doi: 10.1002/mnfr.201100542
110. Corpet DE, Yin Y, Zhang XM, Rémésy C, Stamp D, Medline A, et al. Colonic protein fermentation and promotion of colon carcinogenesis by thermolyzed casein. *Nutr Cancer.* (1995) 23:271–81. doi: 10.1080/01635589509514381
111. Llewellyn SR, Britton GJ, Contijoch EJ, Vennaro OH, Mortha A, Colombel JF, et al. Interactions between diet and the intestinal microbiota alter intestinal permeability and colitis severity in mice. *Gastroenterology.* (2018) 154:1037–46.e2. doi: 10.1053/j.gastro.2017.11.030
112. Lang JM, Pan C, Cantor RM, Tang WHW, Garcia-Garcia JC, Kurtz I, et al. Impact of individual traits, saturated fat, and protein source on the gut microbiome. *MBio.* (2018) 9:1–14. doi: 10.1128/mBio.01604-18
113. Bergeron N, Chiu S, Williams PT, King SM, Krauss RM. Effects of red meat, white meat, and nonmeat protein sources on atherogenic lipoprotein measures in the context of low compared with high saturated fat intake: a randomized controlled trial. *Am J Clin Nutr.* 110:24–33. (2019). doi: 10.1093/ajcn/nqz035
114. Ford JT, Wong CW, Colditz IG. Effects of dietary protein types on immune responses and levels of infection with *Eimeria vermiformis* in mice. *Immunol Cell Biol.* (2001) 79:23–8. doi: 10.1046/j.1440-1711.2001.00788.x
115. Zhu Y, Lin X, Zhao F, Shi X, Li H, Li Y, et al. Meat, dairy and plant proteins alter bacterial composition of rat gut bacteria. *Sci Rep.* (2015) 5:15220. doi: 10.1038/srep16546
116. Zhou Q, Verne ML, Fields JZ, Lefante JJ, Basra S, Salameh H, et al. Randomised placebo-controlled trial of dietary glutamine supplements for postinfectious irritable bowel syndrome. *Gut.* (2018) 68:996–1002. doi: 10.1136/gutjnl-2017-315136
117. Ren W, Duan J, Yin J, Liu G, Cao Z, Xiong X, et al. Dietary l-glutamine supplementation modulates microbial community and activates innate immunity in the mouse intestine. *Amino Acids.* (2014) 46:2403–13. doi: 10.1007/s00726-014-1793-0
118. Chen S, Xia Y, Zhu G, Yan J, Tan C, Deng B, et al. Glutamine supplementation improves intestinal cell proliferation and stem cell differentiation in weanling mice. *Food Nutr Res.* (2018) 62:2403–13. doi: 10.29219/fnr.v62.1439
119. Ren W, Chen S, Yin J, Duan J, Li T, Liu G, et al. Dietary arginine supplementation of mice alters the microbial population and activates intestinal innate immunity. *J Nutr.* (2014) 144:988–95. doi: 10.3945/jn.114.192120
120. Zambom de Souza AZ, Zambom AZ, Abboud KY, Reis SK, Tannihão F, Guadagnini D, et al. Oral supplementation with l-glutamine alters gut microbiota of obese and overweight adults: a pilot study. *Nutrition.* (2015) 31:884–9. doi: 10.1016/j.nut.2015.01.004
121. Koliada A, Syzenko G, Moseiko V, Budovska L, Puchkov K, Perederiy V, et al. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiol.* (2017) 17:120. doi: 10.1186/s12866-017-1027-1
122. Liu G, Ren W, Fang J, Hu CAA, Guan G, Al-Dhabi NA, et al. l-Glutamine and l-arginine protect against enterotoxigenic *Escherichia coli* infection via intestinal innate immunity in mice. *Amino Acids.* (2017) 49:1945–54. doi: 10.1007/s00726-017-2410-9
123. Conejero R, Bonet A, Grau T, Esteban A, Mesejo A, Montejo JC, et al. Effect of a glutamine-enriched enteral diet on intestinal permeability and infectious morbidity at 28 days in critically ill patients with systemic inflammatory response syndrome: a randomized, single-blind, prospective, multicenter study. *Nutrition.* (2002) 18:716–21. doi: 10.1016/S0899-9007(02)00847-X
124. Sader HS, Castanheira M, Mendes RE, Flamm RK. Frequency and antimicrobial susceptibility of Gram-negative bacteria isolated from patients with pneumonia hospitalized in ICUs of US medical centres (2015–17). *J Antimicrob Chemother.* (2018) 73:3053–9. doi: 10.1093/jac/dky279
125. Mottaghi A, Yeganeh MZ, Golzarand M, Jambarsang S, Mirmiran P. Efficacy of glutamine-enriched enteral feeding formulae in critically ill patients: a systematic review and meta-analysis of randomized controlled trials. *Asia Pac J Clin Nutr.* (2016) 25:504–12. doi: 10.6133/apjcn.092015.24
126. Biolo G. Protein metabolism and requirements. *World Rev Nutr Diet.* (2013) 105:12–20. doi: 10.1159/000341545
127. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol.* (2010) 185:3190–8. doi: 10.4049/jimmunol.0903670
128. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. *Nat Commun.* (2018) 9:1–10. doi: 10.1038/s41467-018-05470-4
129. El-Zaatari M, Chang Y-M, Zhang M, Franz M, Shreiner A, McDermott AJ, et al. Tryptophan catabolism restricts IFN-γ-expressing neutrophils and *Clostridium difficile* immunopathology. *J Immunol.* (2014) 193:807–16. doi: 10.4049/jimmunol.1302913
130. Dong YW, Feng L, Jiang WD, Liu Y, Wu P, Jiang J, et al. Dietary threonine deficiency depressed the disease resistance, immune and physical barriers in the gills of juvenile grass carp (*Ctenopharyngodon idella*) under infection of *Flavobacterium columnare*. *Fish Shellfish Immunol.* (2018) 72:161–73. doi: 10.1016/j.fsi.2017.10.048
131. Law GK, Bertolo RF, Adjiri-Awere A, Pencharz PB, Ball RO. Adequate oral threonine is critical for mucin production and gut function in neonatal piglets. *Am J Physiol Gastrointest Liver Physiol.* (2007) 292:G1293–301. doi: 10.1152/ajpgi.00221.2006
132. Diether N, Willing B. Microbial fermentation of dietary protein: an important factor in diet–microbe–host interaction. *Microorganisms.* (2019) 7:19. doi: 10.3390/microorganisms7010019
133. Ayseli MT, Ipek Ayseli Y. Flavors of the future: health benefits of flavor precursors and volatile compounds in plant foods. *Trends Food Sci Technol.* (2016) 48:69–77. doi: 10.1016/j.tifs.2015.11.005
134. Kemperman RA, Bolca S, Roger LC, Vaughan EE. Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities. *Microbiology.* (2010) 156:3224–31. doi: 10.1099/mic.0.042127-0
135. Gilani GS, Xiao CW, Cockell KA. Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. *Br J Nutr.* (2012) 108(Suppl. 2):S315–32. doi: 10.1017/S0007114512002371
136. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev.* (2009) 2:270–8. doi: 10.4161/oxim.2.5.9498
137. Dueñas M, Muñoz-González I, Cueva C, Jiménez-Girón A, Sánchez-Patán F, Santos-Buelga C, et al. A survey of modulation of gut microbiota by dietary polyphenols. *Biomed Res Int.* (2015) 2015:850902. doi: 10.1155/2015/850902
138. Willing BP, Pepin DM, Marcolla CS, Forgie AJ, Diether NE, Bourrie BCT. Bacterial resistance to antibiotic alternatives: a wolf in sheep's clothing? *Anim Front.* (2018) 8:39–47. doi: 10.1093/af/vfy003
139. Lee SH, Lillehoj HS, Jang SI, Lillehoj EP, Min W, Bravo DM. Dietary supplementation of young broiler chickens with Capsicum and turmeric oleoresins increases resistance to necrotic enteritis. *Br J Nutr.* (2013) 110:840–7. doi: 10.1017/S0007114512006083
140. Kaleeswaran B, Ilavenil S, Ravikumar S. Dietary supplementation with *Cynodon dactylon* (L.) enhances innate immunity and disease resistance of Indian major carp, *Catla catla* (Ham.). *Fish Shellfish Immunol.* (2011) 31:953–62. doi: 10.1016/j.fsi.2011.08.013
141. Wang HK, Yeh CH, Iwamoto T, Satsu H, Shimizu M, Totsuka M. Dietary flavonoid naringenin induces regulatory T cells via an aryl hydrocarbon receptor mediated pathway. *J Agric Food Chem.* (2012) 60:2171–8. doi: 10.1021/jf204625y
142. Kiss EA, Vonarbourg C, Kopfmann S, Hobeika E, Finke D, Esser C, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science.* (2011) 334:1561–5. doi: 10.1126/science.1214914
143. Qiu J, Heller JJ, Guo X, Chen ZE, Fish K, Fu Y-X, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity.* (2012) 36:92–104. doi: 10.1016/j.immuni.2011.11.011
144. Julliard W, De Wolfe TJ, Fechner JH, Safdar N, Agni R, Mezrich JD. Amelioration of *Clostridium difficile* infection in mice by dietary supplementation with indole-3-carbinol. *Ann Surg.* (2017) 265:1183–91. doi: 10.1097/SLA.0000000000001830
145. Nicolosi D, Tempera G, Genovese C, Furneri P. Anti-adhesion activity of A2-type proanthocyanidins (a cranberry major component) on uropathogenic *E. coli* and *P. mirabilis* strains. *Antibiotics.* (2014) 3:143–54. doi: 10.3390/antibiotics3020143

146. Luís Â, Domingues F, Pereira L, Luís Â. Can cranberries contribute to reduce the incidence of urinary tract infections? A systematic review with meta-analysis and trial sequential analysis of clinical trials. *J Urol.* (2017) 198:614–21. doi: 10.1016/j.juro.2017.03.078
147. Howell AB. Bioactive compounds in cranberries and their role in prevention of urinary tract infections. *Mol Nutr Food Res.* (2007) 51:732–7. doi: 10.1002/mnfr.200700038
148. Peron G, Sut S, Pellizzaro A, Brun P, Voinovich D, Castagliuolo I, Dall'Acqua S. The antiadhesive activity of cranberry phytocomplex studied by metabolomics: intestinal PAC-A metabolites but not intact PAC-A are identified as markers in active urines against uropathogenic *Escherichia coli*. *Fitoterapia.* (2017) 122:67–75. doi: 10.1016/j.fitote.2017.08.014
149. Tian L, Tan Y, Chen G, Wang G, Sun J, Ou S, et al. Metabolism of anthocyanins and consequent effects on the gut microbiota. *Crit Rev Food Sci Nutr.* (2019) 59:982–91. doi: 10.1080/10408398.2018.1533517
150. Yokota K, Kimura H, Ogawa S, Akihiro T. Analysis of A-type and B-type highly polymeric proanthocyanidins and their biological activities as nutraceuticals. *J Chem.* (2013) 2013:352042. doi: 10.1155/2013/352042
151. Anhê FF, Roy D, Pilon G, Dudonné S, Matamoros S, Varin TV, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased *Akkermansia* spp. population in the gut microbiota of mice. *Gut.* (2015) 64:872–83. doi: 10.1136/gutjnl-2014-307142
152. Forgie AJ, Gao Y, Ju T, Pepin DM, Yang K, Gänzle MG, et al. Pea polyphenolics and hydrolysis processing alter microbial community structure and early pathogen colonization in mice. *J Nutr Biochem.* (2019) 67:101–10. doi: 10.1016/j.jnutbio.2019.01.012
153. Wlodarska M, Willing BP, Bravo DM, Finlay BB. Phytonutrient diet supplementation promotes beneficial Clostridia species and intestinal mucus secretion resulting in protection against enteric infection. *Sci Rep.* (2015) 5:9253. doi: 10.1038/srep09253
154. Shenkin A. Micronutrients in health and disease. *Postgrad Med J.* (2006) 82:559–67. doi: 10.1136/pgmj.2006.047670
155. Skrypnik K, Suliburska J. Association between the gut microbiota and mineral metabolism. *J Sci Food Agric.* (2018) 98:2449–60. doi: 10.1002/jsfa.8724
156. Djoko KY, Ong Clynn Y, Walker MJ, McEwan AG. The role of copper and zinc toxicity in innate immune defense against bacterial pathogens. *J Biol Chem.* (2015) 290:18954–61. doi: 10.1074/jbc.R115.647099
157. Bolick DT, Kolling GL, Moore JH, de Oliveira LA, Tung K, Philipson C, et al. Zinc deficiency alters host response and pathogen virulence in a mouse model of enteroaggregative escherichia coli-induced diarrhea. *Gut Microbes.* (2015) 5:618–27. doi: 10.4161/19490976.2014.969642
158. Rondanelli M, Miccono A, Lamburghini S, Avanzato I, Riva A, Allegrini P, et al. Self-care for common colds: the pivotal role of vitamin D, vitamin C, zinc, and echinacea in three main immune interactive clusters (physical barriers, innate and adaptive immunity) involved during an episode of common colds - practical advice on dosages. *Evid Based Complement Altern Med.* (2018) 2018:5813095. doi: 10.1155/2018/5813095
159. Hemilä H. Zinc lozenges and the common cold: a meta-analysis comparing zinc acetate and zinc gluconate, and the role of zinc dosage. *JRSM Open.* (2017) 8:205427041769429. doi: 10.1177/2054270417694291
160. Douglas R, Hemilä H, Chalker E, Treacy B. Cochrane review: vitamin C for preventing and treating the common cold. *Evid Based Child Heal A Cochrane Rev J.* (2008) 3:672–720. doi: 10.1002/ebch.266
161. Bora SA, Kennett MJ, Smith PB, Patterson AD, Cantorna MT. The gut microbiota regulates endocrine vitamin D metabolism through fibroblast growth factor 23. *Front Immunol.* (2018) 9:408. doi: 10.3389/fimmu.2018.00408
162. Tolkien Z, Stecher L, Mander AP, Pereira DIA, Powell JJ. Ferrous sulfate supplementation causes significant gastrointestinal side-effects in adults: a systematic review and meta-analysis. *PLoS ONE.* (2015) 10:e0117383. doi: 10.1371/journal.pone.0117383
163. Kortman GAM, Boleij A, Swinkels DW, Tjalsma H. Iron availability increases the pathogenic potential of *Salmonella typhimurium* and other enteric pathogens at the intestinal epithelial interface. *PLoS ONE.* (2012) 7:e29968. doi: 10.1371/journal.pone.0029968
164. Natoli M, Felsani A, Ferruzza S, Sambuy Y, Canali R, Scarino ML. Mechanisms of defence from Fe(II) toxicity in human intestinal Caco-2 cells. *Toxicol Vitr.* (2009) 23:1510–5. doi: 10.1016/j.tiv.2009.06.016
165. Kortman GAM, Mulder MLM, Richters TJW, Shanmugam NKN, Trebicka E, Boekhorst J, et al. Low dietary iron intake restrains the intestinal inflammatory response and pathology of enteric infection by food-borne bacterial pathogens. *Eur J Immunol.* (2015) 45:2553–67. doi: 10.1002/eji.201545642
166. Hurrell R, Egli I. Iron bioavailability and dietary reference values. *Am J Clin Nutr.* (2010) 91:1461S—7S. doi: 10.3945/ajcn.2010.28674F
167. Liu Z, Petersen R, Devireddy L. Impaired neutrophil function in 24p3 null mice contributes to enhanced susceptibility to bacterial infections. *J Immunol.* (2013) 190:4692–706. doi: 10.4049/jimmunol.1202411
168. Cordonnier C, Le Bihan G, Emond-Rheault JG, Garrivier A, Harel J, Jubelin G. Vitamin B12 uptake by the gut commensal bacteria bacteroides thetaiotaomicron limits the production of shiga toxin by enterohemorrhagic *Escherichia coli*. *Toxins.* (2016) 8:E14. doi: 10.3390/toxins8 010014
169. Wexler AG, Schofield WB, Degnan PH, Folta-Stogniew E, Barry NA, Goodman AL. Human gut Bacteroides capture vitamin B12 via cell surface-exposed lipoproteins. *Elife.* (2018) 7:e37138. doi: 10.7554/eLife.37138
170. Degnan PH, Barry NA, Mok KC, Taga ME, Goodman AL. Human gut microbes use multiple transporters to distinguish vitamin B12 analogs and compete in the gut. *Cell Host Microbe.* (2014) 15:47–57. doi: 10.1016/j.chom.2013.12.007

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Infectious Threats, the Intestinal Barrier, and Its Trojan Horse: Dysbiosis

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The ecosystem of the gut microbiota consists of diverse intestinal species with multiple metabolic and immunologic activities and it is closely connected with the intestinal epithelia and mucosal immune response, with which it builds a complex barrier against intestinal pathogenic bacteria. The microbiota ensures the integrity of the gut barrier through multiple mechanisms, either by releasing antibacterial molecules (bacteriocins) and anti-inflammatory short-chain fatty acids or by activating essential cell receptors for the immune response. Experimental studies have confirmed the role of the intestinal microbiota in the epigenetic modulation of the gut barrier through posttranslational histone modifications and regulatory mechanisms induced by epithelial miRNA in the epithelial lumen. Any quantitative or functional changes of the intestinal microbiota, referred to as dysbiosis, alter the immune response, decrease epithelial permeability and destabilize intestinal homeostasis. Consequently, the overgrowth of pathobionts (*Staphylococcus, Pseudomonas,* and Escherichia *coli*) favors intestinal translocations with Gram negative bacteria or their endotoxins and could trigger sepsis, septic shock, secondary peritonitis, or various intestinal infections. Intestinal infections also induce epithelial lesions and perpetuate the risk of bacterial translocation and dysbiosis through epithelial ischemia and pro-inflammatory cytokines. Furthermore, the decline of protective anaerobic bacteria (*Bifidobacterium* and *Lactobacillus*) and inadequate release of immune modulators (such as butyrate) affects the release of antimicrobial peptides, de-represses microbial virulence factors and alters the innate immune response. As a result, intestinal germs modulate liver pathology and represent a common etiology of infections in HIV immunosuppressed patients. Antibiotic and antiretroviral treatments also promote intestinal dysbiosis, followed by the selection of resistant germs which could later become a source of infections. The current article addresses the strong correlations between the intestinal barrier and the microbiota and discusses the role of dysbiosis in destabilizing the intestinal barrier and promoting infectious diseases.

Keywords: dysbiosis, intestinal microbiota, epigenetic modulation, short chain fatty acids, immunity, sepsis, HIV infection, liver infections

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INTRODUCTION

The intestinal barrier defines the morpho-functional unit responsible for the defense of the intestinal mucosa and consists of the intestinal microbiota, intestinal epithelial cells (IECs) and mucosal immunity tightly linked through a complex network of cytokines, antimicrobial peptides (AMPs), metabolic products, and numerous regulatory molecules (Meng et al., 2017). Given that the intestinal mucosa is the largest body surface at risk of infectious threats, the anatomic and functional homeostasis of the intestinal barrier is a key step in the anti-infectious defense of the human organism.

The intestinal microbiota represents the first defense line of the intestinal barrier. The microbiota entails millions of microogranisms colonizing the gastrointestinal tract most of which are bacteria. This large number of microorganisms withstand the unfavorable intestinal habitat thanks to their symbiotic relationships with the human organism. These symbiotic host-commensal relationships develop after birth and enable the metabolic, immune and antiinfectious processes through which the microbiota contributes to gut homeostasis (O'Hara and Shanahan, 2006). The structural and functional stability of commensal populations is regulated through numerous signaling molecules (quorum sensing) and cellular regulators (miRNAs) as well as through other physiologic and pathologic factors. Qualitative or quantitative alterations of this microbial community broadly defined as dysbiosis impair the relationships between the host and commensal species, modify the balance between commensals and pathogens, decrease the intestinal barrier protection and favor infectious pathogens (McDonald et al., 2016). Consequently, the microbiota loses its anti-infectious role and becomes the weak link responsible for persistent infections.

The article discusses gut barrier defense mechanisms, the key anti-infectious role of microbiota inside of the gut barrier and the impact of dysbiosis in the life-threatening infections.

THE GUT BARRIER AND ANTIBACTERIAL DEFENSE MECHANISMS

Commensal Flora – The First Line of Defense

The intestinal microbiota encompasses all microbial species populating the gastrointestinal tract. Molecular techniques in healthy individuals have revealed a diverse ecosystem, containing nine bacterial phyla, out of which four are dominant: *Firmicutes, Bacteroidetes, Actinobacteria,* and *Proteobacteria* (Rajilić-Stojanović et al., 2007). The structure of the microbiota is gradually delineated after the age of three through symbiotic relationships with the organism and ensures the intestinal dominance of specific commensals ("symbionts") belonging to the *Firmicutes* and *Bacteroidetes* phylum or *Bifidobacteriales* order. Symbionts will compete with pathogens or potential pathogenic germs ("pathobionts") for pre-existent intestinal niches ("niche competition") as well as for intestinal nutrients ("nutritive competition"). This competition ensures the structural stability of the microbiota and is referred to as "colonization resistance." Colonization resistance employs a network of specific molecules with a critical anti-infectious roles (Sassone-Corsi and Raffatellu, 2015; Sorbara and Pamer, 2019). Among these are molecules with important metabolic and antimicrobial roles such as short chain fatty acids (SCFAs) and bacteriocins.

Short chain fatty acids are end products of the anaerobic fermentation of intestinal microbiota and the major energy source for colonocytes (Roediger, 1980). SCFAs effects are mediated by G protein-coupled receptors (GPRs) (GPR41, GPR43, and GPR109A) expressed on immune cells and a variety of tissues including gut epithelial cells. The predominant SCFAs are present at high mM levels in the colon (butyrate), entero-hepatic circulation (propionate), and systemic circulation (acetate) and are responsible for epithelial protection and regulation of the inflammatory intestinal response, reviewed in Donohoe et al. (2011) and Fukuda et al. (2011). SCFAs ensure a low antibacterial pH around colonic cells, favor mucus synthesis, and contribute to IECs integrity through the upregulation of tight junction proteins, stabilization of HIF transcription factor, IL-18 release, and NLR pyrin domain 3 (NLRP3) inflammasome modulation (Wang et al., 2012; Kelly et al., 2015; Macia et al., 2015; Feng Y. et al., 2018). At the same time SCFAs contribute to the antibacterial defense against pathogenic species through neutrophil recruitment, the release of cytokines and AMPs while also inducing the intestinal immunotolerant response against commensal species as further discussed.

Gut bacteria also, particularly *Firmicutes, Proteobacteria,* and *Bacteroidetes*, synthesize multiple microbicidal molecules (bacteriocins) with a broad spectrum of activity (Todorov et al., 2014; Drissi et al., 2015). Bacteriocin-producing strains such as lactic acid bacteria are commonly used as probiotics in the food industry (Parada et al., 2007). Certain bacteriocins (nissin, pediocin) have been approved for oral or topical use and others are studied as oral alternatives to antibiotics (reviewed in Cavera et al., 2015). Nevertheless, the oral use of bacteriocins is still uncertain due to a lack of data on their action mechanisms as well as on their efficiency, cytotoxicity, stability, and immunogenicity.

Dysbiosis modifies not only the balance between commensals and pathogens but also the release of antimicrobial molecules. In turn this disturbs the process of colonization resistance and allows the invasion of the intestinal epithelium by various pathogens.

The Intestinal Epithelium – The Second Anti-infectious Defense Line

The intestinal epithelium has its own defense mechanisms, both structural and functional. The intestinal epithelial monolayer includes several subsets of IECs united through tight apical junctions, externally covered with a mucus layer. IECs come into direct contact with the lamina propria and immune cells (Goto and Ivanov, 2013). All intestinal epithelial lineages arise from HOPX-quiescent stem cells with immunoregulatory

and tumor suppressing properties (Takeda et al., 2011). IECs include entero-absorptive enterocytes, mucus secreting goblet cells, antigen-sampling cells (M cells), and Paneth cells, each exhibiting specific surface receptors.

The IECs express specific receptors namely "pattern recognition receptors" (PRRs) such as Nod-like receptors (NLRs), toll-like receptors (TLRs) and other PRR families located on the cell membrane or in the cytoplasm which recognize specific microbial-associated molecular patterns (MAMP). Once the activation of these receptors induces cytoplasmic signal transduction cascades and further promotes the NF-kappaB (NF-kB) pathway along with other cellular transcription factors, inflammasome activation and pro-inflammatory cytokines (IL-17, IL-18, IL-22) (Hirota et al., 2011; Jones and Neish, 2011; Levy et al., 2015). The IECs and particularly Paneth cells residing at the bottom of the intestinal crypts release AMPs (human-cathelicidin, defensins) with microbicidal, immunomodulatory and wound-healing properties. Thus IECs further contribute along with microbial colonization resistance to the stabilization of the gut barrier. Therefore Paneth cell MyD88 expression is an essential mechanism for the restriction of intestinal translocation and penetration by enteric commensals or pathogens (Vaishnava et al., 2008).

Short chain fatty acids and GPR43/109A stimulation also protects epithelial integrity via inflammasome activation and epigenetic immunomodulation of FoxP3+ regulatory T cells (Treg) proliferation as shown below (Macia et al., 2015).

Dysbiosis induced by infectious or non-infectious causes (ischemia, inflammation, tumors, or various treatments) favors the distruption of the intestinal epitelium (Kitajima et al., 1999; Nagpal and Yadav, 2017; Stewart A. S. et al., 2017). The injured epithelium permits the translocation of various intestinal bacteria and of their toxins including endotoxins (lipopolysaccharide, LPS). In turn these translocations lead to bacteraemia, endotoxemia and life-threatening infections (sepsis, primitive peritonitis, portal encephalopathy in cirrhotic patients, or immune activation in human immunodeficiency virus (HIV) infection.

The Intestinal Immunity – The Third Anti-infectious Defense Line

The intestinal epithelium represents the largest epithelial surface in the human body. It connects with the intestinal ecosystem containing commensal and pathogenic species. Commensal germs need to be immunologically accepted, whereas intestinal pathogens must be eliminated. This differentiation requires the activation of an extremely well-coordinated and efficient immune system. Considering that intestinal commensals are present in the human body ever since birth they represent, in fact, the first contact between the immune system and the exterior environment. As such, this large and diverse microbiota will serve as one of the body's defense mechanisms against the invasion of pathogenic germs. At the same time, the microbiota develops mutually beneficial relationships with the organism and together with the intestinal epithelium and gut-associated lymphoid tissue (GALT) the microbiota forms a complex intestinal barrier against infectious threats.

These symbiotic relationships are facilitated by the presence of PRRs and by the immunomodulatory capacity of the intestinal microbiota against the antigenic structures exposed by the intestinal microbiota.

The Role of Cellular Receptors

The intestinal epithelium expresses numerous types of receptors able to recognize different MAMPs expressed by microbial species and to convert them into gut signals for inflammatory cascades (Kim et al., 2004). Thus TLR 1,2,4,5,6 (extracellular sensors) and NLR1,2 and TLR9 (cytoplasmic sensors) are expressed on epithelial cells and act complementary, promoting both innate and adaptive immunity (Kim et al., 2004; Abreu et al., 2005). MAMPs are expressed by commensal species since birth and examples of MAMPs include the LPS found in the outer membrane of Gram-negative bacteria, lipoteichoic acid present on the gram positive bacteria wall, peptidoglycan, a component of the bacterial wall, flagellin, a component of intestinal flagellated bacteria, or release outer membrane vesicles that function as PRR ligands (Cañas et al., 2018). PRRs activation in the presence of MAMPs stimulates the NF-kB signal transduction pathway, induces pro-inflammatory interleukins and enables an innate immune response lastly maintaining a state of controlled inflammation (Hayashi et al., 2001; Bambou et al., 2004; Vora et al., 2004; Rhayat et al., 2019). In the absence of MAMPs or of specific receptors (germ free animals or genetic mutations of receptors) the organism fails to detect microbial antigens and does not mount adequate defense mechanisms (Abreu et al., 2005). Hence, it is considered that the rich and diverse commensal flora plays an active role in the proper development of the immune system since birth.

Antigen-presenting cells (APC) belonging to GALT such as macrophages and dendritic cells (DCs) also exhibit cell receptors which regulate numerous genes and modulate the release of NF-κB transcription factor, immunomodulatory cytokines and AMP. Depending on the type of activated receptor DCs may generate a Th1/Th17 pro-inflammatory response. Thus, to exemplify, the NLR2 induced by commensal bacteria appears to play a central role in the downregulation of the GALT inflammatory activity and the ability of DCs to induce a polarized $CD4^{+}$Th1 response in mice and human experiments (Butler et al., 2007; Barreau et al., 2010).

By activating the IECs or APCs receptors, the commensal flora induces a tolerogenic response in DCs, characterized by the release of immunosuppressive cytokines (IL10, IL4, TGF-β), promotion of Treg cells and of $CD4^{+}$Th2 phenotype in the periphery (Iwasaki and Kelsall, 1999; Stagg et al., 2003; Atarashi et al., 2013; Maharshak et al., 2013; Martin-Gallausiaux et al., 2018). The induction of mucosal immune tolerance protects the epithelia from a detrimental inflammatory immune response and contributes to the immune system's maturation (Jung et al., 2019). Consequently any disturbance involving this process plays an essential role in intestinal inflammatory processes.

The activation of cellular receptors is intricately linked to the pro-inflammatory response of mesenteric DCs and is particularly

involved in the maintenance of the Th17/Th1 response directed against pathogens (reviewed in Sorini et al., 2018). What is interesting is that DCs may activate Treg cells to certain antigens such as LPS of the commensal flora but not LPS of the pathogenic flora (Shirai et al., 2004). Thus the stimulation of mice with *E. coli*-derived LPS activates inflammatory mechanisms (IL-12 production, Th1 response) whereas the stimulation with LPS derived from *Poprhyromonas gingivalis* induces IL-4 production and a Th2 response (Pulendran et al., 2001). Similarly TLR recognize flagellin antigens of pathogenic Salmonella species and trigger an inflammatory response against them, while commensal species lacking this antigen do not induce an inflammatory response (Gewirtz et al., 2001).

All these aspects evince that cell receptors represent a filter for intestinal signals and play a deciding role in the induction of specific defense mechanisms adapted to the commensal flora. This role of cell receptors is indeed significant considering the density and variety of the intestinal bacteria. Dysbsiosis eliminates commensal species that trigger Treg-cell polarization and therefore leads to an excessive Th1 or Th2 response, further promoting an inflammatory or autoimmune response.

The Immunomodulatory Role of GALT

Gut-associated lymphoid tissue is the most important lymphatic network in humans and involves isolated or aggregated lymphoid follicles (Peyer's Patches), intra-epithelial lymphocytes, macrophages, DCs, mesenteric ganglia, secretory IgA (sIgA) cells and lymphatics.

Peyer's patches localized in the mucosa and submucosa of the small intestine are covered by a "follicle-associated epithelium" containing specialized "M cells". These cells engulf and transport antigens from the intestinal lumen to intestinal DCs where T cell lymphocytes (LT) are primed (Alpan et al., 2001). LT subsequently return to the intestinal lymphatic compartment for their effector function. At the same time, M cells initiate mucosal sIgA production and humoral responses (Rios et al., 2016). Thus, Peyer's patches directly mediate the interaction between the intestinal flora and the humoral or cellular immune response. Enteric pathogens are adapted to invade and destroy follicle-associated epithelium, especially M cells, further interfering with T-cell differentiation and the immune response; these germs adhere to the intestinal epithelium through different mechanisms (Phillips et al., 2000), invade the enterocytes (Kühbacher et al., 2018), or invade and destroy M cells (Clark et al., 1994; Autenrieth and Firsching, 1996; Penheiter et al., 1997; Corr et al., 2006).

Gut-associated lymphoid tissue also harbors a tolerogenic DCs population namely $CD103^+$DCs involved in Foxp3 expression and intestinal conversion of naïve $CD4^+$T cells into Treg cells. Commensal flora stimulates $CD103^+$ DCs through the activation of specific receptors or through its own metabolic products such as SCFAs (Furusawa et al., 2013; Nastasi et al., 2015; Kaisar et al., 2017). Thus, butyrate stimulates intestinal DCs, ensures intestinal immune tolerance through IL-10 release (Liu et al., 2012) and T cell polarization toward Treg cells while maintaining the balance between the immunosuppressive IL-10-secreting $CD4^+$T cells, IL-17-secreting Th17 cells, and the $CD4^+$Th1 effector cells (Arpaia et al., 2013; Furusawa et al., 2013). Additionally, SCFAs attenuate the excessive inflammatory response induced by LPS-producing Gram negative bacteria (Cox et al., 2009).

The dysregulation of the Th1/Th17 immune response against commensal species is associated with intestinal inflammatory diseases. On the other hand the excessive polarization toward a Treg response attenuates the immune response to infections (Brenchley et al., 2004). Another example of GALT dysregulation occurs in HIV due to the destruction of the intestinal gut barrier and the ensuing chronic inflammatory response. GALT depends on the microbiota and it is worth noting that the development of GALT and the activation of T cell lymphocytes and B cell lymphocytes cannot occur in the absence of signals released by the intestinal flora.

Experiments on commensals belonging to *Clostridium* and *Bacteroides* species further highlighted the ability of the intestinal microbiota to maintain the intestinal homeostasis and to orchestrate an adequate T-cell response through specific MAMPs during bacterial invasion (Ivanov et al., 2008; Lécuyer et al., 2014). Thus, *Bacteroides fragilis* strains expressing polysaccharide A binding TLR2 on $CD4^+$T cells and gut-indigenous *Clostridium* belonging to XIVa and IV clusters favor Tregs, suppress Th-17 cells and facilitate mucosal tolerance toward the colon microbiota (Mazmanian et al., 2008; Round et al., 2011; Atarashi et al., 2013). On the other hand segmented filamentous bacteria, a genetic relative of the genus *Clostridium* promote both effector Th17 CD4+T cells (Ivanov et al., 2009; Farkas et al., 2015; Schnupf et al., 2017) and sIgA antibodies during intestinal invasion (Lécuyer et al., 2014). Other microbial species also modulate the Treg/Th17 axis, potentially controlling the intestinal inflammation and tolerance (Pandiyan et al., 2019). This aspect also explains the disequilibrium between these species during dysbiosis as well as the expansion of pathogens and of mucous lesions.

Epigenetic Regulatory Implications

Modulation of gene expression by epigenetic mechanisms in the intestinal environment was studied especially for neoplasms and inflammatory processes (Vdovikova et al., 2018). Epigenetic mechanisms modulate gene transcription by various processes: DNA methylation, histone modifications and modulation of long non-coding RNA and microRNA expression. However the mechanisms through which bacteria are either affected or induce gut epigenetic changes are not well understood.

Histone modifications are correlated with the activation or repression of genetic transcription through the modulation of two antagonistic enzymes, namely histone acetyl transferases (HATs) inducing histone acetylation, and histone deacetylases (HDACs) inducing histone deacetylation. During acetylation, the chromatin structure loosens and can be accessed by transcription factors. HDAC inhibitors increase histone acetylation and subsequently regulate gene expression in numerous immune cells such as epithelial cells, neutrophils, APCs and T cells. On the contrary, deacetylation of histones by HDACs prevents gene transcription. Thus studies performed *in vitro* showed that HDAC inhibitors promote the release of several transcription

factors such is NFκB, MyoD, p53, or HSP90 (Vinolo et al., 2011; Kumar S. A. et al., 2018; Banik et al., 2019). The NF-kB imbalance has been correlated with numerous inflammatory and antiapoptotic mechanisms that interfere with viral evasion (Le Negrate, 2012; Carrasco Pro et al., 2018), septic shock (Liu et al., 1999; Han et al., 2002), or inflammatory diseases (Makarov, 2001; Lawrence, 2009). Likewise, p53 regulates the cell cycle and apoptosis, functioning as a tumor suppressor (Aubrey et al., 2018). P53-mediated apoptosis was associated with the spread of viral infections (Lazo and Santos, 2011; Aloni-Grinstein et al., 2018), while HSP90 reverse transcriptase mediated activity was, most likely, associated with extensive hepatitis B virus (HBV) infection (Hu and Seeger, 1996). Several viruses among which HIV, HBV, hepatitis C virus, Epstein Barr virus, cytomegalovirus, herpes simplex virus and human T-lymphotropic virus have NF-kB activation strategies and cell apoptosis blockage (Santoro et al., 2003) while others, especially the oncogenic ones have p53 suppression mechanisms (Sato and Tsurumi, 2013). It has been demonstrated that HDAC inhibitors could block stellate cell activation thus hindering liver fibrosis in experimental animal models (Park et al., 2014; Ding et al., 2018).

By inhibiting HDAC, SCFAs could therefore control numerous infectious and immune processes (Vinolo et al., 2011; Zhou et al., 2017b; Sun et al., 2018). Although the role of HDAC inhibitors hasn't been completely elucidated yet, most experimental studies evinced an anti-inflammatory dose dependent effect (Yin et al., 2001; Le Poul et al., 2003; Weber and Kerr, 2006; Asarat et al., 2016; Kaisar et al., 2017; Li et al., 2018) and an immunosuppressive role of SCFAs in tumors (Villagra et al., 2010; Tang et al., 2011). Still, while other HDAC inhibitors are already used in oncologic therapies, the exact role of SCFAs in the immune and tumor processes remains purely theoretical (Meijer et al., 2010; Matthews et al., 2012; Ulven, 2012).

MicroRNAs (miRNAs) are small, evolutionary conserved non-coding RNAs of approximately 19-23 nucleotides involved in the post-transcriptional regulation of cellular mRNAs. The biogenesis of mature miRNA includes a two-step cleavage process from primary miRNAs (pri-miRNA). The mature miRNA is then loaded into the effector complex RNA-induced complex (RISC). RISC interacts with target mRNA and induce mRNA cleavage or translational repression hence controlling diverse metabolic or cellular pathways including cell cycle progression, differentiation, apoptosis, immune regulation or oncogenesis (Dalmasso et al., 2011; Singh et al., 2012; Nakata et al., 2017). miRNAs are released extracellularly by most eukaryotic cells and various types of small non-coding RNAs (sRNAs) around 50–200 nt in length have been observed within extracellular vesicles released by gram negative bacteria ("outer membrane vesicles") (Gong et al., 2011). The general conception is that sRNA, as well as microRNA-size small RNAs (msRNAs) of 15–25 nucleotides in length such as msRNA observed in the model bacterium *E. coli* (Kang et al., 2013) act as post-transcriptional regulators and function as signaling molecules for bacterial growth and virulence under experimental conditions (Ortega et al., 2012; Zhao et al., 2017) and virulence mechanisms, at least under experimental conditions (Padalon-Brauch et al., 2008; Choi et al., 2017). The role of these structures in intestinal homeostasis, has been, for now, scarcely covered, most information having been acquired from studies on conventional or germ-free mice.

Intestinal miRNAs are released by IECs (Liu et al., 2016) and are regulated by intestinal microbiota through TLR/MyD88-dependent pathway (Dalmasso et al., 2011; Singh et al., 2012; reviewed in Eulalio et al., 2012; Williams et al., 2017). Liu and Weiner (2016) conducted an extensive study identifying numerous types of extracellular miRNAs circulating in exosomes in the gut lumen and feces of mice and humans. The study proved that IECs and Hopx-expressing cells are the main sources of exosomal intestinal miRNAs and also highlighted their uptake by intestinal bacteria and potential role in post-transcriptional regulation of bacterial genes.

Studies on mice indicate a certain pattern of miRNA gut compartimentalization after bacterial colonization (Dalmasso et al., 2011) and reported the dominance of miR-143,-145 in the jejunum and cecum and of miR-200b in the large intestine and caecum (Singh et al., 2012). On the other hand the human cells or animal experimental models with pathogenic species (*Helicobacter pylori, Citrobacter rodentium, Listeria monocytogenes, Francisella tularensis,* and *Salmonella enterica*) induce a different miRNA panel mainly represented by miR-155 and miR-146 (Eulalio et al., 2012; Archambaud et al., 2013; Staedel and Darfeuille, 2013).

On a molecular level, there is a growing interest for the intestinal role of miRNAs and their regulatory implications in the gut-barrier functionality but data on this topic is divergent, scarce and fragmented. Cellular and extracellular miRNAs from the intestinal lumen modulate the epithelial integrity, inflammatory response, and probably bacterial gene mRNAs through insufficiently known mechanisms (reviewed in Belcheva, 2017).

Studies on human intestinal cells have documented the miRNA importance in the protection of epithelial tight junctions (e.g., miRNA-122) (Ye et al., 2011), epithelial regeneration (e.g., miR-143, miR-145) (Chivukula et al., 2014), and proliferation (miR-30 family members) (Peck et al., 2016), the modulation of epithelial integrity (e.g., miR-122) (Ye et al., 2011), mucin gene expression (Mo et al., 2016), and epithelial permeability (e.g., miR-21-5p) (Nakata et al., 2017).

Intestinal miRNAs are also key regulators of the immune response against infections. Host miRNAs orchestrate the immune response through PRR families and TLR signaling pathways (miR-146a) (Xue et al., 2011, 2014), while the microbiota downregulates the expression of miR-10a and miR-107 in host DCs, decreases the release of proinflammatory cytokines in mice and controls the excessive inflammatory response in human and mice (e.g., miRNA-146a and miR-193a-3p) (Taganov et al., 2006; Nahid et al., 2011; Singh et al., 2012; Dai et al., 2015). MiRNAs could also potentially influence the microbiota and alleviate colonic inflammation as was suggested by a negative correlation on ulcerative colitis between the release of miR-193a-3p and colonic inflammation (Dai et al., 2015). This concept was further explored in a study in which the oral ingestion of endotoxin in mice led to the upregulation of epithelial miR-146 and promoted innate immune tolerance and epithelial protection in the postnatal

period (Chassin et al., 2010). On a similar note mi-RNA-155 defective mice failed to develop a protective immunity toward *H. pylori* or *Salmonella* Typhimurium (Rodriguez et al., 2007).

Pathogenic bacteria also release membrane or outer-membrane vesicles containing sRNAs that modulate the host miRNA profile and gene expression (Gu et al., 2017). Some studies on intestinal inflammation due to enteropathogens revealed the upregulation of miR-16,-21,-223,-594 and miRNA-31 or downregulation of miR-124 within the human intestinal lumen (Wu et al., 2010; Koukos et al., 2013; Lin et al., 2014). Their biological relevance is under study.

Various miRNAs have been correlated with intestinal oncogenesis according to human studies. In this respect miR-182,-503 and miR-17∼92 clusters modulate glycan production and correlate with the growth of certain bacteria which could potentially initiate the microenvironmental changes in colorectal cancer (Yuan et al., 2018). Gut miRNAs could therefore induce and perpetuate dysbiosis favoring various infections or intestinal cancers (Liu et al., 2016; Williams et al., 2017). Furthermore, single nucleotide polymorphisms of miRNA-146a,-27a genes promote various infections associated with inflammatory and neoplasic intestinal changes (Song et al., 2013; Shao et al., 2014). Butyrate was also shown to affect miRNA-106b expression in IECs and to modulate carcinogenesis (Hu et al., 2011). Therefore, it is probable that epigenetic regulators such as miRNAs could play a key role in the interaction between host and microbiota and miRNA dysregulations.

DYSBIOSIS, THE TROJAN HORSE INSIDE THE GUT BARRIER

Dysbiosis and Enteral Infections

Enteric pathogens alter the intestinal barrier, antagonize the intestinal microbiota, and trigger enteral infections through various mechanisms including increased intestinal inflammation, the release of bacteriocins and upregulation of AMPs and toxin delivery secretory systems (T6SS, T3SS) as well as the exploitation of nutrients or intestinal niches (reviewed in Rolhion and Chassaing, 2016; Sorbara and Pamer, 2018).

Dysbiosis occurs as a consequence of different enteroinvasive or entorotoxinogenic species which distrupt colonization resistance, reduce the protective species belonging to the *Bacteroidetes* and *Firmicutes* phylum (especially the *Clostridiales incertae sedis* XI or the IV/XIVa cluster) and allow the overgrowth of species belonging to the *Proteobacteria* phylum (especially *Enterococcaceae* and *Enterobacteriaceae* families) (Vincent et al., 2013; Livanos et al., 2018). Antibiotic treatment of intestinal infections additionally modifies the structure of the microbiota and drastically disturbs the process of "colonization resistance" (Panda et al., 2014).

As a result, the first alterations due to dysbiosis include the loss of commensals synthesizing bacteriocins and SCFAs such as *Bacteroides thuringiensis* and the *Lachnospiraceae* and *Ruminococcaceae* families (Rea et al., 2010) along with the loss of *Clostridia* commensals competing with pathobionts for the same intestinal niche (Sailhamer et al., 2009; Buffie et al., 2015; Geerlings et al., 2018) and of *Bacteroides thetaiotaomicron* and *Akkermansia muciniphila* which protect the gut barrier (Hooper et al., 2001; Donato et al., 2010; Martín et al., 2019). In these cases, the normally scarce pathobionts eventually become the dominant species and display various virulence mechanisms. Consequently, pathobionts or their endotoxins trigger intestinal infections, gut sepsis or postantibiotic colitis (Brown et al., 2013; Pérez-Cobas et al., 2013). Moreover, antibiotic treatment favors the development of multidrug-resistance species (MDR) and in turn these become invasive and pathogenic after extraluminal translocation (Hirakata et al., 2002; Ayres et al., 2012). Hence, *Pseudomonas* MDR species with efflux pump systems like MexAB-OprM develop quorum sensing machinery to sense host stress and express multiple virulence determinants (Hirakata et al., 2002). The wide distribution of efflux pump systems in MDR intestinal species after antibiotic treatment increases the virulence of these species and their associated risk of extraluminal translocation (Nishino et al., 2006; also reviewed in Nikaido, 1996). Therefore dysbiosis may be both the cause and the consequence of enteral infection.

The restoration of the microbiota is particularly difficult and is best ensured through fecal microbiota transplantation (FMT) from a healthy donor. The effectiveness of FMT in the management of *C. difficile* enterocolitis or sepsis dysbiosis further underlines the critical role of the microbiota for gut health (Gough et al., 2011; Rao and Young, 2015; Wei et al., 2016).

Dysbiosis and Sepsis

Sepsis is a life-threatening organ dysfunction caused by a dysregulated inflammatory response to infectious agents or their proinflamatory products. Sepsis has a progressive and fatal course to generalized microvascular injury, cellular hypoxia, and shock (Singer et al., 2016).Under certain conditions enteric bacteria could elicit a dysregulated systemic inflammatory response causing sepsis (Deitch et al., 1994; Mainous et al., 1995). The germs most commonly encountered in gut-derived sepsis are Gram negative bacilli producing either endotoxins or pore-forming exotoxins (Wallace et al., 2000) and probably one of the most common species involved in these cases is *E. coli*, a common gut resident (MacFie et al., 1999). The presence of pore-forming exotoxins is followed by the efflux of cellular potassium and activation of NLRP3 inflammasome. In turn these contribute to cellular apoptosis and loss of epithelial integrity and permit the access of toxins and pathogens to the portal and systemic circulation. This further stimulates the releases of various pro-inflammatory and blood coagulation mediators and a cytokine storm followed by vasoconstriction, ischemia and peripheral necrosis and finally organ dysfunction and shock (reviewed in Los et al., 2013; Sonnen and Henneke, 2013). The excessive activation of the CD14/TLR4/MD2 complex by bacterial endotoxins also induces the inflammasome signaling pathway, along with caspase activation (Hotchkiss et al., 1999), cells lysis and disproportionate release of pro-inflammatory cytokines, further eliciting and exacerbating sepsis, reviewed in Lamkanfi (2011), Gao et al. (2018), and Skirecki and Cavaillon (2019).

Sepsis alters the intestinal barrier through multiple factors which ultimately promote dysbiosis including intestinal ischemia

and inflammatory lesions, aggressive care, antibiotic treatments, intestinal comorbidities, parenteral nutrition, etc. (reviewed in Hassoun et al., 2001; Alverdy and Luo, 2017; Fay et al., 2017). Gut vascular dysfunctions in sepsis and particularly intestinal ischemia stimulates pro-inflammatory cytokines, activates HIF-1 alpha factor, disrupts the epithelial tight junction and finally induces colonic cell apoptosis (Hassoun et al., 2001; Li et al., 2009; Yoseph et al., 2016). All of these events increase the permeability of intestinal epithelia and promote intestinal translocations (Diebel et al., 2003). The intestinal translocation of bacteria or microbial products such as endotoxin-LPS or MAMPs perpetuates and aggravates the systemic pro-inflammatory response further speeding the progression to organ failure and death (Shimizu et al., 2006, 2011; also reviewed in Deitch and Berg, 1987; Doig et al., 1998; Meng et al., 2017).

Dysbiosis entails the decrease of anaerobes *Bifidobacterium* and *Lactobacillus* (the main producers of SCFAs) accompanied by a detrimental increase of intestinal pathobionts such as *E. coli* or *Staphylococcus* and *Pseudomonas* species (Shimizu et al., 2006, 2011; Hayakawa et al., 2011; Ayres et al., 2012) replacing the protective microbiome. Additionally, the reduction of SCFAs favor the cascade release of LPS-induced pro-inflammatory mediators, LPS-triggered macrophage migration and massive release of nitric oxide by neutrophils (PMN), thus promoting intestinal inflammation and destabilizing the gut-barrier (Maa et al., 2010; Wang et al., 2017). Certain strains or specific phylogenetic groups of intestinal Gram negative bacilli (*E. coli* and *Pseudomonas aeruginosa*) acquire additional virulence genes during colonization or even change their morphotype after translocation to express virulence genes and to avoid host defense mechanisms giving rise to gut-derived sepsis (Johnson et al., 2001; Zaborina et al., 2007; Hickey et al., 2018). Thus, dysbiosis induces and supports sepsis associated mechanisms. In favor of this hypothesis, Souza et al. (2004) administered LPS to germ free mice and reported the absence of a severe inflammatory response and low mortality. Literature data on sepsis dysbiosis in newborns has confirmed significant changes of the microbiome, a decreased bacterial diversity, pre-sepsis gut colonization with invasive species (Carl et al., 2014), intestinal translocations (Madan et al., 2012; Mai et al., 2013; Taft et al., 2015; Stewart C. J. et al., 2017) or the activation of virulence and antibiotic resistance factors in intestinal germs (Mittal and Coopersmith, 2014). Of note, these alterations continue to occur throughout the progression of sepsis.

The administration of probiotics in sepsis (*Lactobacillus rhamnosus* and *Bifidobacterium longum*) decreases epithelial apoptosis as well as the release of cytokines and bacterial translocations in experimental mice models (Khailova et al., 2013; Panpetch et al., 2017). Probiotics can also restore the lactic acid-producing flora and ensure colonization resistance toward pathogenic flora. According to Haak and Wiersinga (2017), probiotics further influence the prognosis in sepsis by down-regulating colonic TLR-2/TLR-4 via MyD88 and by mitigating the systemic pro-inflammatory response. However, their benefit to reduce the probability of sepsis in critically ill patients is questionable (Jain et al., 2004; Kotzampassi et al., 2006; Jacobi et al., 2011; Panigrahi et al., 2017). On the other hand, data on prebiotic supplementation in infections is discordant and clear recommendations are missing (Srinivasjois et al., 2013; Chi et al., 2019).

Dysbiosis and Liver Infections

The liver is connected to the intestine through the portal vein, biliary tract, and numerous signaling molecules, together building the "gut-liver axis." As a result, the liver is continuously exposed to microbial and metabolic molecules produced by intestinal bacteria (SCFAs, MAMPs) and further releases its own antibacterial products (bile salts) involved in the intestinal homeostasis (reviewed in Macpherson et al., 2016). Thus intestinal MAMPs reach the portal system and hepatic sinusoids and are further processed by Kuppfer cells and liver-resident T cells. SCFAs released by the microbiota are absorbed in the colon and filtered intrahepatically where most modulate the immune response, oncogenesis and are involved in the epigenetic control of liver pathogens through HDAC inhibition and transcriptional changes (Candido et al., 1978). As HDAC inhibitors, SCFAs decrease HBV replication (Pollicino et al., 2006), mediate hepatitis C virus replication (Taguwa et al., 2008, 2009), impede liver necrosis and prevent hepatocellular carcinoma, reviewed in Koumbi and Karayiannis (2015). Recently, butyrate was shown to regulate the expression of tumor-suppresive miRNAs (miRNA-26a,-26a-1,-192, etc.) through the bile acid nuclear receptor, farnesoid X receptor (FXR) while also promoting hepatocyte apoptosis through miR-22 up-regulation in hepatic cells (Pant et al., 2017; reviewed in Feng Q. et al., 2018).

Host liver miRNAs are able to regulate the replication of viral hepatitis yet the precise roles of intestinal miRNAs in the regulation of hepatotropic viruses remain unclear (Bandiera et al., 2016; Feng Q. et al., 2018; reviewed in Fan and Tang, 2014; Li et al., 2016). Hepatocytes also release immunoregulatory molecules such as primary bile acids (BA). A small proportion of primary BA are dehydroxylated in the intestine through bile salt hydrolases and converted to secondary BA entering the entero-hepatic circulation. The latter exert a bacteriostatic effect on the intestinal barrier and an anti-fibrotic and anti-inflammatory intrahepatic activity (Wu et al., 2012).

Dysbiosis is closely related to liver pathology. The liver provides an immunotolerogenic environment with an attenuated immune responsiveness and an increased risk of persistent infections (Crispe, 2014). Chronic liver diseases and their neurologic complications (hepatic encephalopathy and coma) are induced and aggravated by changes of the gut microbiota such as the overgrowth of pathobionts (*Enterobacteriaceae, Bacteroidaceae,* and *Enterococcus* species) and the dramatic decline of commensals such as *Roseburia*, *Bifidobacterium*, and *Lactobacillales* (Wu et al., 2012; Bajaj et al., 2014; Schnabl and Brenner, 2014). On the other hand cirrhosis modifies the composition of the microbiota, namely it decreases commensal *Clostridia* species (the clusters XI and XIVab), the *Bacteroidales-Prevotella* group and butyrate-producing *Roseburia* genus (Lu et al., 2011; Bajaj et al., 2012; also reviewed in Schnabl, 2013; Kang and Cai, 2017). HBV associated cirrhosis significantly reduces the *Bifidobacterium/Enterobacteriaceae*

ratio and increases the bacterial virulence through adhesins and toxins (Lu et al., 2011). The ensuing dysbiosis compromises the integrity of the intestinal barrier and favors bacterial translocations, bacteraemia, or endotoxemia (Mainous et al., 1991; Lin et al., 1995; Bajaj et al., 2014). Given the reduced phagocytosis and canalization of porto-systemic shunts in this setting, bacterial translocations increase the risk of primitive peritonitis and septic shock.

Translocated bacteria and their derived products enter the portal circulation and liver sinusoids, promote liver inflammation (Seki et al., 2007) and aggravate the fibrogenic and oncogenic potential of liver diseases, reviewed in Yang and Seki (2012).

The mechanisms through which intestinal dysbiosis promotes liver injury employ the activation of TLR signaling pathways, reviewed in Seki and Schnabl (2012). Hence, LPS binding to TLR4 receptors of Kuppfer cells triggers a cascade of inflammatory cytokines (Su et al., 2000) and plays a key role in hepatic injury. Bacterial DNA activates TLR9 signaling pathways in Kuppfer and stellate cells which further induces liver inflammation and fibrogenesis in animal models (Miura et al., 2010). NLRP3 and NLRP6 inflammasome deficiency due to intestinal dysbiosis activate TLR4 and TLR9 pathways and exacerbate hepatic inflammation (Henao-Mejia et al., 2012). Therefore dysbiosis ensures a pro-inflammatory intrahepatic environment (Richer et al., 2013; Miura and Ohnishi, 2014; Carrasco Pro et al., 2018) and supports the progression to liver cirrhosis. The imbalance of BA synthesis in dysbiosis contributes to liver inflammation, fibrosis and carcinogenesis as well as to small intestine bacterial overgrowth, which in turn prompts an intestinal inflammatory response (Gunnarsdottir et al., 2003; Jun et al., 2010; Ridlon et al., 2014).

Considering the pathogenic role of microbiota in liver diseases various authors investigated therapeutic and prophylactic interventions. Studies on the latter explored the therapeutic use of probiotics (mostly *Lactobacillus* and *Bifidobacterium* species), antibiotics (Rifaximine), gut-derived hormones and even FMT for the manipulation of the gut-liver axis (Ponziani et al., 2015; Zhou et al., 2017a; Román et al., 2019; also reviewed in Wiest et al., 2017). Concurrently, the administration of conjugated BA in rats could normalize bile secretion and reduce intestinal bacterial overgrowth and translocations (Lorenzo-Zúñiga et al., 2003).

Dysbiosis and HIV Infection

Human immunodeficiency virus is a retrovirus with $CD4^+$T cell tropism leading to a severe immunodeficiency and AIDS, followed by secondary infections, reactivations of opportunistic germs and HIV-associated cancers. HIV induces systemic immune activation, alters the intestinal barrier and promotes irreversible metabolic, cardiovascular and neurologic changes (Steele et al., 2014). As most experiments on non-human primate model of acquired immunodeficiency have shown, HIV/simian immunodeficiency virus (SIV) induces an ongoing activation of the mucosal immune response and a rapid and massive destruction of CD4+T lymphocytes belonging to the GALT system. The cells most affected are the Th17/22 $CD4^+$T intestinal subsets involved in the maintenance of the gut barrier (Veazey et al., 1998; Guadalupe et al., 2003; Brenchley et al., 2004, 2006; McGowan et al., 2004; Kim et al., 2013). Thus, HIV reconfigures the intestinal barrier early during the infection through immune mechanisms which promote gut alterations, chronic inflammation, and immunodeficiency.

Human immunodeficiency virus-associated intestinal lesions ("HIV enteropathy") initially arise due to the imbalance of the epithelial barrier turnover (Sankaran et al., 2008). In time, the increasing inflammation and intestinal permeability contribute to HIV enteropathy and persist despite antiretroviral therapy (ART) (Olsson et al., 2000; Guadalupe et al., 2003; Neff et al., 2018). The histopathological lesions described in this setting involve intestinal inflammation and mucosal atrophy that nevertheless differ from changes recorded in other intestinal immune-mediated diseases (Magro et al., 2013). On a molecular level, animal studies showed that during HIV enteropathy, IECs display a downregulation of host genes and of miRNAs associated with the homeostasis of the epithelial barrier (Sankaran et al., 2005), epithelial permeability (miRNA-21,-130a,-212) (Gaulke et al., 2014; Zhang et al., 2015; Kumar V. et al., 2018), inflammatory signaling (miR-150) (Kumar et al., 2016), and immune activation syndrome (miR-34a) (Mohan et al., 2015). Some of these miRNAs also regulate the gut microbiota (Johnston et al., 2018) or inflammatory pathways associated with irritable bowel syndrome (Zhou et al., 2010, 2015; Fourie et al., 2014). A study performed on the intestinal lamina propria and leukocytes of SIV-infected macaques indicated that the host miRNA gut profile displays a bitemporal variation initially dictated by the rapid reduction of SIV replication and T-cell activation and afterwards correlated with the consequent boost of the inflammatory response (Kumar et al., 2016). Under these conditions gut epithelia gradually lose their protective role and permit the translocation of bacterial immunogenic molecules (Chege et al., 2011; Marchetti et al., 2013) or enteropathogens (Zeitz et al., 1998; Nazli et al., 2010). In the presence of an impaired immune response, these translocations recur and re-enforce the systemic inflammatory response activating $CD4^+$T cells and HIV replication in a vicious circle (Kotler et al., 1993; Brenchley et al., 2006; Cassol et al., 2010; Dillon et al., 2014). Thus dysbiosis perpetuates enteric and opportunistic infections and aggravates the course of HIV.

Studies on HIV associated dysbiosis are discordant, hindering comparisons, yet available data shows a major depletion of *Bacteroides* in favor of *Proteobacteria* (Dillon et al., 2014), the enrichment of pathobionts (*Enterococcus, Streptococcus, Staphylococcus, Salmonella*, and *Escherichia* species) and the significant reduction of symbionts throughout the entire intestinal tract (Nishitsuji et al., 2017).Whereas the higher population of *Enterobacteriaceae* increases the risk of translocations, epithelial cells exposed to *E. coli* could increase the susceptibility of $CD4^+$Th17 and Th-1 lymphocytes to HIV and viral replication (Dillon et al., 2012).

Intestinal dysbiosis occurs early during HIV and is aggravated by ART itself (Ling et al., 2016; Pinto-Cardoso et al., 2017; also reviewed in Pinto-Cardoso et al., 2018). Hence, patients

with immunologic failure on ART, predominantly host species of *Enterobacteriaceae* instead of *Lactobacillus* known for its immunomodulatory and anti-inflammatory activity (Merlini et al., 2011). Otherwise, butyrate synthesized by dominant species in HIV patients (*Fusobacterium nucleatum, Clostridium cochlearium,* and *Eubacterium multiforme*) could reactivate both unintegrated HIV-1 genomes and latent HIV proviruses through HDAC inhibition along with other transcription factors and concurrently explains HIV immunosuppression during ART (Kantor et al., 2009; Imai et al., 2012; Lee et al., 2018).The role of SCFAs in the regulation of mucosal immunity during HIV is controversial due to their implication in the reactivation of various infections as well as in the attenuation of mucosal inflammation and microbial translocation (Das et al., 2015; Ye and Karn, 2015; Dillon et al., 2017). Consequently, certain authors proposed the use of chemical HDAC inhibitors but not of SCFAs along with ART for a successful antiretroviral treatment of HIV reservoirs as well as for the attenuation of the LPS-induced inflammatory response (Archin et al., 2009, 2012; also reviewed in Shirakawa et al., 2013; McManamy et al., 2014).

In conclusion, HIV pathogenesis involves the loss of the immunomodulatory gastrointestinal activity, bacterial translocations and intestinal dysbiosis, independent of ART. Current therapeutic strategies are modest and based on few studies in humans or macaques: the administration of probiotics (Hummelen et al., 2011; Ortiz et al., 2016; also reviewed in Hummelen et al., 2010; D'Angelo et al., 2017; Kazemi et al., 2018), anti-inflammatory drugs (reviewed in Deeks et al., 2013), FMT (Hensley-McBain et al., 2016; Vujkovic-Cvijin et al., 2017) stimulating the immune response with a T lymphocyte-adjuvated-DNA vaccine (Fuller et al., 2012), or using an oral recombinant vaccine with *Cl. perfringens* expressing HIV-1 Gag protein (Pegu et al., 2011).

CONCLUSION

The microbiota, the intestinal epithelia and mucosal immunity form an anti-infectious barrier rigorously regulated through complex mechanisms. The host-microbiota-pathogen interactions employ numerous cell receptors and molecules with antibacterial and anti-inflammatory roles, which also modulate the epigenetic and immune response. Together with epithelial and immune cells, these signaling molecules form a network that is essential for intestinal homeostasis and anti-infectious defense. Dysbiosis renders these defense mechanisms non-functional. It consequently aggravates gastrointestinal infections, favors bacterial and LPS translocations in sepsis, unbalances the immune defense in hepatitis viruses and HIV replications and controls the progression of infectious diseases to an unknown extent. A better knowledge on the interactions driving the antimicrobial response of the intestinal barrier is therefore crucial to improve the current anti-infectious armamentarium.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the acquisition, analysis, and critical revision of the manuscript, gave their consent for the publication, and agreed to be responsible for the accuracy and integrity of the manuscript.

REFERENCES

Abreu, M. T., Fukata, M., and Arditi, M. (2005). TLR signaling in the gut in health and disease. *J. Immunol.* 174, 4453–4460. doi: 10.4049/jimmunol.174.8.4453

Aloni-Grinstein, R., Charni-Natan, M., Solomon, H., and Rotter, V. (2018). P53 and the viral connection: back into the future ‡. *Cancers* 10:E178. doi: 10.3390/cancers10060178

Alpan, O., Rudomen, G., Matzinger, P., and Kapsenberg, M. L. (2001). The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. *J. Immunol.* 166, 4843–4852. doi: 10.4049/jimmunol.166.8.4843

Alverdy, J. C., and Luo, J. N. (2017). The Influence of host stress on the mechanism of infection: lost microbiomes, emergent pathobiomes, and the role of interkingdom signaling. *Front. Microbiol.* 8:322. doi: 10.3389/fmicb.2017.00322

Archambaud, C., Sismeiro, O., Toedling, J., Soubigou, G., Bécavin, C., Lechat, P., et al. (2013). The intestinal microbiota interferes with the microRNA response upon oral Listeria infection. *MBio.* 4:e00707-13. doi: 10.1128/mBio.00707-13

Archin, N. M., Keedy, K. S., Espeseth, A., Dang, H., Hazuda, D. J., and Margolis, D. M. (2009). Expression of latent human immunodeficiency type 1 is induced by novel and selective histone deacetylase inhibitors. *AIDS* 23, 1799–1806. doi: 10.1097/QAD.0b013e32832ec1dc

Archin, N. M., Liberty, A. L., Kashuba, A. D., Choudhary, S. K., Kuruc, J. D., Crooks, A. M., et al. (2012). Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature* 487, 482–485. doi: 10.1038/nature11286

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., et al. (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504, 451–455. doi: 10.1038/nature12726

Asarat, M., Apostolopoulos, V., Vasiljevic, T., and Donkor, O. (2016). Short-chain fatty acids regulate cytokines and Th17/Treg cells in human peripheral blood mononuclear cells in vitro. *Immunol. Invest.* 45, 205–222. doi: 10.3109/08820139.2015.1122613

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500, 232–236. doi: 10.1038/nature12331

Aubrey, B. J., Kelly, G. L., Janic, A., Herold, M. J., and Strasser, A. (2018). How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death Differ.* 25, 104–113. doi: 10.1038/cdd.2017.169

Autenrieth, I. B., and Firsching, R. (1996). Penetration of M cells and destruction of Peyer's patches by Yersinia enterocolitica: an ultrastructural and histological study. *J. Med. Microbiol.* 44, 285–294. doi: 10.1099/00222615-44-4-285

Ayres, J. S., Trinidad, N. J., and Vance, R. E. (2012). Lethal inflammasome activation by a multidrug-resistant pathobiont upon antibiotic disruption of the microbiota. *Nat. Med.* 18, 799–806. doi: 10.1038/nm.2729

Bajaj, J. S., Heuman, D. M., Hylemon, P. B., Sanyal, A. J., White, M. B., Monteith, P., et al. (2014). Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J. Hepatol.* 60, 940–947. doi: 10.1016/j.jhep.2013.12.019

Bajaj, J. S., Hylemon, P. B., Ridlon, J. M., Heuman, D. M., Daita, K., White, M. B., et al. (2012). Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 303, G675–G685. doi: 10.1152/ajpgi.00152.2012

Bambou, J.-C., Giraud, A., Menard, S., Begue, B., Rakotobe, S., Heyman, M., et al. (2004). In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial Ccells by a commensal *Escherichia coli* strain. *J. Biol. Chem.* 279, 42984–42992.

Bandiera, S., Pernot, S., El Saghire, H., Durand, S. C., Thumann, C., Crouchet, E., et al. (2016). Hepatitis C virus-induced upregulation of microRNA miR-146a-5p in hepatocytes promotes viral infection and deregulates metabolic pathways associated with liver disease pathogenesis. *J. Virol.* 90, 6387–6400. doi: 10.1128/JVI.00619-16

Banik, D., Moufarrij, S., and Villagra, A. (2019). Immunoepigenetics combination therapies: an overview of the role of HDACs in cancer immunotherapy. *Int. J. Mol. Sci.* 20:E2241. doi: 10.3390/ijms20092241

Barreau, F., Madre, C., Meinzer, U., Berrebi, D., Dussaillant, M., Merlin, F., et al. (2010). *Nod2* regulates the host response towards microflora by modulating T cell function and epithelial permeability in mouse Peyer's patches. *Gut* 59, 207–217. doi: 10.1136/gut.2008.171546

Belcheva, A. (2017). MicroRNAs at the epicenter of intestinal homeostasis. *BioEssays* 39:1600200. doi: 10.1002/bies.201600200

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., et al. (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* 12, 1365–1371.

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., et al. (2004). CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* 200, 749–759. doi: 10.1084/jem.20040874

Brown, K. A., Khanafer, N., Daneman, N., and Fisman, D. N. (2013). Meta-analysis of antibiotics and the risk of community-associated Clostridium difficile infection. *Antimicrob. Agents Chemother.* 57, 2326–2332. doi: 10.1128/AAC.02176-12

Buffie, C. G., Bucci, V., Stein, R. R., McKenney, P. T., Ling, L., Gobourne, A., et al. (2015). Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature* 517, 205–208. doi: 10.1038/nature13828

Butler, M., Chaudhary, R., van Heel, D. A., Playford, R. J., and Ghosh, S. (2007). NOD2 activity modulates the phenotype of LPS-stimulated dendritic cells to promote the development of T-helper type 2-like lymphocytes — Possible implications for NOD2-associated Crohn's disease. *J. Crohn's Colitis* 1, 106–115. doi: 10.1016/j.crohns.2007.08.006

Cañas, M.-A., Fábrega, M.-J., Giménez, R., Badia, J., and Baldomà, L. (2018). Outer membrane vesicles from probiotic and commensal *Escherichia coli* activate NOD1-mediated immune responses in intestinal epithelial cells. *Front. Microbiol.* 9:498. doi: 10.3389/fmicb.2018.00498

Candido, E. P., Reeves, R., and Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell* 14, 105–113. doi: 10.1016/0092-8674(78)90305-7

Carl, M. A., Ndao, I. M., Springman, A. C., Manning, S. D., Johnson, J. R., Johnston, B. D., et al. (2014). Sepsis from the gut: the enteric habitat of bacteria that cause late-onset neonatal bloodstream infections. *Clin. Infect. Dis.* 58, 1211–1218. doi: 10.1093/cid/ciu084

Carrasco Pro, S., Lindestam Arlehamn, C. S., Dhanda, S. K., Carpenter, C., Lindvall, M., Faruqi, A. A., et al. (2018). Microbiota epitope similarity either dampens or enhances the immunogenicity of disease-associated antigenic epitopes. *PLoS One* 13:e0196551. doi: 10.1371/journal.pone.0196551

Cassol, E., Malfeld, S., Mahasha, P., van der Merwe, S., Cassol, S., Seebregts, C., et al. (2010). Persistent microbial translocation and immune activation in HIV-1–infected south africans receiving combination cntiretroviral therapy. *J. Infect. Dis.* 202, 723–733. doi: 10.1086/655229

Cavera, V. L., Arthur, T. D., Kashtanov, D., and Chikindas, M. L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *Int. J. Antimicrob. Agents* 46, 494–501. doi: 10.1016/j.ijantimicag.2015.07.011

Chassin, C., Kocur, M., Pott, J., Duerr, C. U., Gütle, D., Lotz, M., et al. (2010). miR-146a mediates protective innate immune tolerance in the neonate Intestine. *Cell Host Microbe.* 8, 358–368. doi: 10.1016/j.chom.2010.09.005

Chege, D., Sheth, P. M., Kain, T., Kim, C. J., Kovacs, C., Loutfy, M., et al. (2011). Sigmoid Th17 populations, the HIV latent reservoir, and microbial translocation in men on long-term antiretroviral therapy. *AIDS* 25, 741–749. doi: 10.1097/QAD.0b013e328344cefb

Chi, C., Buys, N., Li, C., Sun, J., and Yin, C. (2019). Effects of prebiotics on sepsis, necrotizing enterocolitis, mortality, feeding intolerance, time to full enteral feeding, length of hospital stay, and stool frequency in preterm infants: a meta-analysis. *Eur. J. Clin. Nutr.* 73, 657–670. doi: 10.1038/s41430-018-0377-6

Chivukula, R. R., Shi, G., Acharya, A., Mills, E. W., Zeitels, L. R., Anandam, J. L., et al. (2014). An essential mesenchymal function for miR-143/145 in intestinal epithelial regeneration. *Cell* 157, 1104–1116. doi: 10.1016/j.cell.2014.03.055

Choi, J.-W., Um, J.-H., Cho, J.-H., and Lee, H.-J. (2017). Tiny RNAs and their voyage via extracellular vesicles: secretion of bacterial small RNA and eukaryotic microRNA. *Exp. Biol. Med.* 242, 1475–1481. doi: 10.1177/1535370217723166

Clark, M. A., Jepson, M. A., Simmons, N. L., and Hirst, B. H. (1994). Preferential interaction of *Salmonella* typhimurium with mouse Peyer's patch M cells. *Res. Microbiol.* 145, 543–552. doi: 10.1016/0923-2508(94)90031-0

Corr, S., Hill, C., and Gahan, C. G. M. (2006). An in vitro cell-culture model demonstrates internalin- and hemolysin-independent translocation of Listeria monocytogenes across M cells. *Microb. Pathog.* 41, 241–250. doi: 10.1016/j.micpath.2006.08.003

Cox, M. A., Jackson, J., Stanton, M., Rojas-Triana, A., Bober, L., Laverty, M., et al. (2009). Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E(2) and cytokines. *World J. Gastroenterol.* 15, 5549–5557.

Crispe, I. N. (2014). Immune tolerance in liver disease. *Hepatology* 60, 2109–2117. doi: 10.1002/hep.27254

Dai, X., Chen, X., Chen, Q., Shi, L., Liang, H., Zhou, Z., et al. (2015). MicroRNA-193a-3p reduces intestinal inflammation in response to microbiota via down-regulation of colonic PepT1. *J. Biol. Chem.* 290, 16099–16115. doi: 10.1074/jbc.M115.659318

Dalmasso, G., Nguyen, H. T. T., Yan, Y., Laroui, H., Charania, M. A., Ayyadurai, S., et al. (2011). Microbiota modulate host gene expression via microRNAs. *PLoS One* 6:e19293. doi: 10.1371/journal.pone.0019293

D'Angelo, C., Reale, M., and Costantini, E. (2017). Microbiota and probiotics in health and HIV Infection. *Nutrients* 9:E615. doi: 10.3390/nu9060615

Das, B., Dobrowolski, C., Shahir, A.-M., Feng, Z., Yu, X., Sha, J., et al. (2015). Short chain fatty acids potently induce latent HIV-1 in T-cells by activating P-TEFb and multiple histone modifications. *Virology* 474, 65–81. doi: 10.1016/j.virol.2014.10.033

Deeks, S. G., Tracy, R., and Douek, D. C. (2013). Systemic effects of inflammation on health during chronic HIV infection. *Immunity* 39, 633–645. doi: 10.1016/j.immuni.2013.10.001

Deitch, E. A., and Berg, R. (1987). Bacterial translocation from the gut: a mechanism of infection. *J. Burn Care Rehabil.* 8, 475–482. doi: 10.1097/00004630-198711000-00005

Deitch, E. A., Xu, D., Franko, L., Ayala, A., and Chaudry, I. H. (1994). Evidence favoring the role of the gut as a cytokine-generating organ in rats subjected to hemorrhagic shock. *Shock* 1, 141–145. doi: 10.1097/00024382-199402000-00010

Diebel, L. N., Liberati, D. M., Dulchavsky, S. A., Diglio, C. A., and Brown, W. J. (2003). Enterocyte apoptosis and barrier function are modulated by SIgA after exposure to bacteria and hypoxia/reoxygenation. *Surgery* 134, 574–580. discussion 580-1, doi: 10.1016/s0039-6060(03)00302-7

Dillon, S. M., Kibbie, J., Lee, E. J., Guo, K., Santiago, M. L., Austin, G. L., et al. (2017). Low abundance of colonic butyrate-producing bacteria in HIV infection is associated with microbial translocation and immune activation. *AIDS* 31, 511–521. doi: 10.1097/QAD.0000000000001366

Dillon, S. M., Lee, E. J., Kotter, C. V., Austin, G. L., Dong, Z., Hecht, D. K., et al. (2014). An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal Immunol.* 7, 983–994. doi: 10.1038/mi.2013.116

Dillon, S. M., Manuzak, J. A., Leone, A. K., Lee, E. J., Rogers, L. M., McCarter, M. D., et al. (2012). HIV-1 infection of human intestinal lamina propria CD4+ T cells in vitro is enhanced by exposure to commensal *Escherichia coli*. *J. Immunol.* 189, 885–896. doi: 10.4049/jimmunol.1200681

Ding, D., Chen, L.-L., Zhai, Y.-Z., Hou, C.-J., Tao, L.-L., Lu, S.-H., et al. (2018). Trichostatin A inhibits the activation of Hepatic stellate cells by increasing C/EBP-α acetylation in vivo and in vitro. *Sci. Rep.* 8:4395. doi: 10.1038/s41598-018-22662-6

Doig, C. J., Sutherland, L. R., Dean Sandham, J., Fick, G. H., Verhoef, M., and Meddings, J. B. (1998). Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically Ill ICU

patients. *Am. J. Respir. Crit. Care Med.* 158, 444–451. doi: 10.1164/ajrccm.158.2.9710092

Donato, K. A., Gareau, M. G., Wang, Y. J. J., and Sherman, P. M. (2010). Lactobacillus rhamnosus GG attenuates interferon- and tumour necrosis factor- -induced barrier dysfunction and pro-inflammatory signalling. *Microbiology* 156, 3288–3297. doi: 10.1099/mic.0.040139-0

Donohoe, D. R., Garge, N., Zhang, X., Sun, W., O'Connell, T. M., Bunger, M. K., et al. (2011). The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab.* 13, 517–526. doi: 10.1016/j.cmet.2011.02.018

Drissi, F., Buffet, S., Raoult, D., and Merhej, V. (2015). Common occurrence of antibacterial agents in human intestinal microbiota. *Front. Microbiol.* 6:441. doi: 10.3389/fmicb.2015.00441

Eulalio, A., Schulte, L., and Vogel, J. (2012). The mammalian microRNA response to bacterial infections. *RNA Biol.* 9, 742–750. doi: 10.4161/rna.20018

Fan, H.-X., and Tang, H. (2014). Complex interactions between microRNAs and hepatitis B/C viruses. *World J. Gastroenterol.* 20, 13477–13492. doi: 10.3748/wjg.v20.i37.13477

Farkas, A. M., Panea, C., Goto, Y., Nakato, G., Galan-Diez, M., Narushima, S., et al. (2015). Induction of Th17 cells by segmented filamentous bacteria in the murine intestine. *J. Immunol. Methods* 421, 104–111. doi: 10.1016/j.jim.2015.03.020

Fay, K. T., Ford, M. L., and Coopersmith, C. M. (2017). The intestinal microenvironment in sepsis. *Biochim. Biophys. Acta. - Mol. Basis Dis.* 1863, 2574–2583. doi: 10.1016/j.bbadis.2017.03.005

Feng, Q., Chen, W.-D., and Wang, Y.-D. (2018). Gut microbiota: an integral moderator in health and disease. *Front. Microbiol.* 9:151. doi: 10.3389/fmicb.2018.00151

Feng, Y., Wang, Y., Wang, P., Huang, Y., and Wang, F. (2018). Short-chain fatty acids manifest stimulative and protective effects on intestinal barrier function through the inhibition of NLRP3 inflammasome and autophagy. *Cell Physiol. Biochem.* 49, 190–205. doi: 10.1159/000492853

Fourie, N. H., Peace, R. M., Abey, S. K., Sherwin, L. B., Rahim-Williams, B., Smyser, P. A., et al. (2014). Elevated circulating miR-150 and miR-342-3p in patients with irritable bowel syndrome. *Exp. Mol. Pathol.* 96, 422–425. doi: 10.1016/j.yexmp.2014.04.009

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469, 543–547. doi: 10.1038/nature09646

Fuller, D. H., Rajakumar, P., Che, J. W., Narendran, A., Nyaundi, J., Michael, H., et al. (2012). Therapeutic DNA vaccine induces broad T cell responses in the gut and sustained protection from viral rebound and AIDS in SIV-infected rhesus macaques. *PLoS One* 7:e33715. doi: 10.1371/journal.pone.0033715

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504, 446–450. doi: 10.1038/nature12721

Gao, Y.-L., Zhai, J.-H., and Chai, Y.-F. (2018). Recent advances in the molecular mechanisms underlying pyroptosis in sepsis. *Mediators Inflamm.* 2018:5823823. doi: 10.1155/2018/5823823

Gaulke, C. A., Porter, M., Han, Y.-H., Sankaran-Walters, S., Grishina, I., George, M. D., et al. (2014). Intestinal epithelial barrier disruption through altered mucosal microRNA expression in human immunodeficiency virus and simian immunodeficiency virus infections. *J. Virol.* 88, 6268–6280. doi: 10.1128/JVI.00097-14

Geerlings, S. Y., Kostopoulos, I., de Vos, W. M., and Belzer, C. (2018). Akkermansia muciniphila in the human gastrointestinal tract: when, where, and how? *Microorganisms* 6:E75. doi: 10.3390/microorganisms6030075

Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J., and Madara, J. L. (2001). Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J. Immunol.* 167, 1882–1885. doi: 10.4049/jimmunol.167.4.1882

Gong, H., Vu, G.-P., Bai, Y., Chan, E., Wu, R., Yang, E., et al. (2011). A *Salmonella* small non-coding RNA facilitates bacterial invasion and intracellular replication by modulating the expression of virulence factors. *PLoS Pathog.* 7:e1002120. doi: 10.1371/journal.ppat.1002120

Goto, Y., and Ivanov, I. I. (2013). Intestinal epithelial cells as mediators of the commensal-host immune crosstalk. *Immunol. Cell Biol.* 91, 204–214. doi: 10.1038/icb.2012.80

Gough, E., Shaikh, H., and Manges, A. R. (2011). Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent Clostridium difficile infection. *Clin. Infect. Dis.* 53, 994–1002. doi: 10.1093/cid/cir632

Gu, H., Zhao, C., Zhang, T., Liang, H., Wang, X.-M., Pan, Y., et al. (2017). *Salmonella* produce microRNA-like RNA fragment Sal-1 in the infected cells to facilitate intracellular survival. *Sci. Rep.* 7:2392. doi: 10.1038/s41598-017-02669-1

Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil, A., et al. (2003). Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J. Virol.* 77, 11708–11717. doi: 10.1128/jvi.77.21.11708-11717.2003

Gunnarsdottir, S. A., Sadik, R., Shev, S., Simren, M., Sjovall, H., Stotzer, P.-O., et al. (2003). Small intestinal motility disturbances and bacterial overgrowth in patients with liver cirrhosis and portal hypertension. *Am. J. Gastroenterol.* 98, 1362–1370. doi: 10.1016/s0002-9270(03)00250-8

Haak, B. W., and Wiersinga, W. J. (2017). The role of the gut microbiota in sepsis. *Lancet Gastroenterol. Hepatol.* 2, 135–143. doi: 10.1016/S2468-1253(16)30119-4

Han, S.-J., Ko, H.-M., Choi, J.-H., Seo, K. H., Lee, H.-S., Choi, E.-K., et al. (2002). Molecular mechanisms for lipopolysaccharide-induced biphasic Aactivation of nuclear factor-κB (NF-κB). *J. Biol. Chem.* 277, 44715–44721. doi: 10.1074/jbc.m202524200

Hassoun, H. T., Kone, B. C., Mercer, D. W., Moody, F. G., Weisbrodt, N. W., and Moore, F. A. (2001). Post-injury multiple organ multiple organ failure:the role of the gut: shock. *Shock* 15, 1–10. doi: 10.1097/00024382-200115010-00001

Hayakawa, M., Asahara, T., Henzan, N., Murakami, H., Yamamoto, H., Mukai, N., et al. (2011). Dramatic changes of the gut flora immediately after severe and sudden Insults. *Dig. Dis. Sci.* 56, 2361–2365. doi: 10.1007/s10620-011-1649-3

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., et al. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099–1103. doi: 10.1038/35074106

Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W. Z., Strowig, T., et al. (2012). Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 482, 179–185. doi: 10.1038/nature10809

Hensley-McBain, T., Zevin, A. S., Manuzak, J., Smith, E., Gile, J., Miller, C., et al. (2016). Effects of fecal microbial transplantation on microbiome and immunity in simian immunodeficiency virus-infected macaques. *J. Virol.* 90, 4981–4989. doi: 10.1128/JVI.00099-16

Hickey, C., Schaible, B., Nguyen, S., Hurley, D., Srikumar, S., Fanning, S., et al. (2018). Increased virulence of bloodstream over peripheral isolates of P. aeruginosa identified through post-transcriptional regulation of virulence factors. *Front. Cell Infect. Microbiol.* 8:357. doi: 10.3389/fcimb.2018.00357

Hirakata, Y., Srikumar, R., Poole, K., Gotoh, N., Suematsu, T., Kohno, S., et al. (2002). Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. *J. Exp. Med.* 196, 109–118. doi: 10.1084/jem.20020005

Hirota, S. A., Ng, J., Lueng, A., Khajah, M., Parhar, K., Li, Y., et al. (2011). NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflamm. Bowel Dis.* 17, 1359–1372. doi: 10.1002/ibd.21478

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291, 881–884. doi: 10.1126/science.291.5505.881

Hotchkiss, R. S., Swanson, P. E., Freeman, B. D., Tinsley, K. W., Cobb, J. P., Matuschak, G. M., et al. (1999). Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. *Crit. Care Med.* 27, 1230–1251. doi: 10.1097/00003246-199907000-00002

Hu, J., and Seeger, C. (1996). Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. *Proc. Natl. Acad. Sci. U.S.A.* 93, 1060–1064. doi: 10.1073/pnas.93.3.1060

Hu, S., Dong, T. S., Dalal, S. R., Wu, F., Bissonnette, M., Kwon, J. H., et al. (2011). The microbe-derived short chain fatty acid butyrate targets miRNA-dependent p21 gene expression in human colon cancer. *PLoS One* 6:e16221. doi: 10.1371/journal.pone.0016221

Hummelen, R., Changalucha, J., Butamanya, N. L., Koyama, T. E., Cook, A., Habbema, J. D. F., et al. (2011). Effect of 25 weeks probiotic supplementation on immune function of HIV patients. *Gut. Microbes.* 2, 80–85. doi: 10.4161/gmic.2.2.15787

Hummelen, R., Vos, A. P., Land, B., Norren, K., and Reid, G. (2010). Altered host-microbe interaction in HIV: a target for intervention with pro- and prebiotics. *Int. Rev. Immunol.* 29, 485–513. doi: 10.3109/08830185.2010.505310

Imai, K., Yamada, K., Tamura, M., Ochiai, K., and Okamoto, T. (2012). Reactivation of latent HIV-1 by a wide variety of butyric acid-producing bacteria. *Cell Mol. Life Sci.* 69, 2583–2592. doi: 10.1007/s00018-012-0936-2

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139, 485–498. doi: 10.1016/j.cell.2009.09.033

Ivanov, I. I., Frutos, R., de, L., Manel, N., Yoshinaga, K., Rifkin, D. B., et al. (2008). Specific microbiota direct the differentiation of Th17 cells in the mucosa of the small intestine. *Cell Host Microbe* 4, 337–349. doi: 10.1016/j.chom.2008.09.009

Iwasaki, A., and Kelsall, B. L. (1999). Freshly isolated Peyer's Patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J. Exp. Med.* 190, 229–240. doi: 10.1084/jem.190.2.229

Jacobi, C. A., Schulz, C., and Malfertheiner, P. (2011). Treating critically ill patients with probiotics: beneficial or dangerous? *Gut. Pathog.* 3:2. doi: 10.1186/1757-4749-3-2

Jain, P. K., McNaught, C. E., Anderson, A. D., MacFie, J., and Mitchell, C. J. (2004). Influence of synbiotic containing *Lactobacillus acidophilus* La5, *Bifidobacterium* lactis Bb 12, *Streptococcus thermophilus*, *Lactobacillus* bulgaricus and oligofructose on gut barrier function and sepsis in critically ill patients: a randomised controlled trial. *Clin. Nutr.* 23, 467–475. doi: 10.1016/j.clnu.2003.12.002

Johnson, J. R., O'Bryan, T. T., Kuskowski, M., and Maslow, J. N. (2001). Ongoing horizontal and vertical transmission of virulence genes and papA alleles among *Escherichia coli* blood isolates from patients with diverse-source bacteremia. *Infect. Immun.* 69, 5363–5374. doi: 10.1128/iai.69.9.5363-5374.2001

Johnston, D. G. W., Williams, M. A., Thaiss, C. A., Cabrera-Rubio, R., Raverdeau, M., McEntee, C., et al. (2018). Loss of microRNA-21 influences the gut microbiota, causing reduced susceptibility in a murine model of colitis. *J. Crohn's Colitis* 12, 835–848. doi: 10.1093/ecco-jcc/jjy038

Jones, R. M., and Neish, A. S. (2011). Recognition of bacterial pathogens and mucosal immunity. *Cell Microbiol.* 13, 670–676. doi: 10.1111/j.1462-5822.2011.01579.x

Jun, D. W., Kim, K. T., Lee, O. Y., Chae, J. D., Son, B. K., Kim, S. H., et al. (2010). Association between small intestinal bacterial overgrowth and peripheral bacterial DNA in cirrhotic patients. *Dig. Dis. Sci.* 55, 1465–1471. doi: 10.1007/s10620-009-0870-9

Jung, J., Surh, C. D., and Lee, Y. J. (2019). Microbial colonization at early life promotes the development of diet-induced CD8αβ intraepithelial T cells. *Mol. Cells.* 42, 313–320. doi: 10.14348/molcells.2019.2431

Kaisar, M. M. M., Pelgrom, L. R., van der Ham, A. J., Yazdanbakhsh, M., and Everts, B. (2017). Butyrate Conditions Human Dendritic Cells to Prime Type 1 Regulatory T Cells via both Histone Deacetylase Inhibition and G Protein-Coupled Receptor 109A Signaling. *Front. Immunol.* 8:1429. doi: 10.3389/fimmu.2017.01429

Kang, S.-M., Choi, J.-W., Lee, Y., Hong, S.-H., and Lee, H.-J. (2013). Identification of microRNA-Size, Small RNAs in *Escherichia coli*. *Curr. Microbiol.* 67, 609–613. doi: 10.1007/s00284-013-0411-9

Kang, Y., and Cai, Y. (2017). Gut microbiota and hepatitis-B-virus-induced chronic liver disease: implications for faecal microbiota transplantation therapy. *J. Hosp. Infect.* 96, 342–348. doi: 10.1016/j.jhin.2017.04.007

Kantor, B., Ma, H., Webster-Cyriaque, J., Monahan, P. E., and Kafri, T. (2009). Epigenetic activation of unintegrated HIV-1 genomes by gut-associated short chain fatty acids and its implications for HIV infection. *Proc. Natl. Acad. Sci. U.S.A.* 106, 18786–18791. doi: 10.1073/pnas.0905859106

Kazemi, A., Djafarian, K., Speakman, J. R., Sabour, P., Soltani, S., and Shab-Bidar, S. (2018). Effect of probiotic supplementation on CD4 cell count in HIV-infected patients: a systematic review and meta-analysis. *J. Diet Suppl.* 15, 776–788. doi: 10.1080/19390211.2017.1380103

Kelly, C. J., Zheng, L., Campbell, E. L., Saeedi, B., Scholz, C. C., Bayless, A. J., et al. (2015). Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe* 17, 662–671. doi: 10.1016/j.chom.2015.03.005

Khailova, L., Frank, D. N., Dominguez, J. A., and Wischmeyer, P. E. (2013). Probiotic administration reduces mortality and improves intestinal epithelial homeostasis in experimental sepsis. *Anesthesiology* 119, 166–177. doi: 10.1097/ALN.0b013e318291c2fc

Kim, C. J., McKinnon, L. R., Kovacs, C., Kandel, G., Huibner, S., Chege, D., et al. (2013). Mucosal Th17 cell function is altered during HIV infection and is an independent predictor of systemic immune activation. *J. Immunol.* 191, 2164–2173. doi: 10.4049/jimmunol.1300829

Kim, J. G., Lee, S. J., and Kagnoff, M. F. (2004). Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by toll-like receptors. *Infect. Immun.* 72, 1487–1495. doi: 10.1128/iai.72.3.1487-1495.2004

Kitajima, S., Takuma, S., and Morimoto, M. (1999). Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. *Exp. Anim.* 48, 137–143. doi: 10.1538/expanim.48.137

Kotler, D. P., Reka, S., and Clayton, F. (1993). Intestinal mucosal inflammation associated with human immunodeficiency virus infection. *Dig. Dis. Sci.* 38, 1119–1127. doi: 10.1007/bf01295730

Kotzampassi, K., Giamarellos-Bourboulis, E. J., Voudouris, A., Kazamias, P., and Eleftheriadis, E. (2006). Benefits of a synbiotic formula (Synbiotic 2000Forte®) in critically Ill trauma patients: early results of a randomized controlled trial. *World J. Surg.* 30, 1848–1855. doi: 10.1007/s00268-005-0653-1

Koukos, G., Polytarchou, C., Kaplan, J. L., Morley–Fletcher, A., Gras–Miralles, B., Kokkotou, E., et al. (2013). MicroRNA-124 regulates STAT3 expression and is down-regulated in colon tissues of pediatric patients with ulcerative colitis. *Gastroenterology* 145, 842.e2–852.e2. doi: 10.1053/j.gastro.2013.07.001

Koumbi, L., and Karayiannis, P. (2015). The epigenetic control of hepatitis B virus modulates the outcome of infection. *Front. Microbiol.* 6:1491. doi: 10.3389/fmicb.2015.01491

Kühbacher, A., Novy, K., Quereda, J. J., Sachse, M., Moya-Nilges, M., Wollscheid, B., et al. (2018). Listeriolysin O-dependent host surfaceome remodeling modulates Listeria monocytogenes invasion. *Pathog. Dis.* 76:fty082. doi: 10.1093/femspd/fty082

Kumar, S. A., Bishayee, A., and Pandey, A. K. (2018). Targeting histone deacetylases with natural and synthetic agents: an emerging anticancer strategy. *Nutrients* 10:E731. doi: 10.3390/nu10060731

Kumar, V., Mansfield, J., Fan, R., MacLean, A., Li, J., and Mohan, M. (2018). miR-130a and miR-212 disrupt the intestinal epithelial barrier through modulation of PPARγ and occludin expression in chronic simian immunodeficiency virus-infected rhesus macaques. *J. Immunol.* 200, 2677–2689. doi: 10.4049/jimmunol.1701148

Kumar, V., Torben, W., Kenway, C. S., Schiro, F. R., and Mohan, M. (2016). Longitudinal examination of the intestinal lamina propria cellular compartment of simian immunodeficiency virus-infected Rhesus macaques provides broader and deeper insights into the link between aberrant microRNA expression and persistent immune activati. *J. Virol.* 90, 5003–5019. doi: 10.1128/JVI.00189-16

Lamkanfi, M. (2011). Emerging inflammasome effector mechanisms. *Nat. Rev. Immunol.* 11, 213–220. doi: 10.1038/nri2936

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect. Biol.* 1:a001651. doi: 10.1101/cshperspect.a001651

Lazo, P. A., and Santos, C. R. (2011). Interference with p53 functions in human viral infections, a target for novel antiviral strategies? *Rev. Med. Virol.* 21, 285–300. doi: 10.1002/rmv.696

Le Negrate, G. (2012). Viral interference with innate immunity bypreventing NF-kB activity. *Cell Microbiol.* 14, 168–181. doi: 10.1111/j.1462-5822.2011.01720.x

Le Poul, E., Loison, C., Struyf, S., Springael, J.-Y., Lannoy, V., Decobecq, M.-E., et al. (2003). Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J. Biol. Chem.* 278, 25481–25489. doi: 10.1074/jbc.m301403200

Lécuyer, E., Rakotobe, S., Lengliné-Garnier, H., Lebreton, C., Picard, M., Juste, C., et al. (2014). Segmented filamentous bacterium uses secondary and tertiary lymphoid tissues to induce gut IgA and specific T helper 17 cell responses. *Immunity* 40, 608–620. doi: 10.1016/j.immuni.2014.03.009

Lee, S. C., Chua, L. L., Yap, S. H., Khang, T. F., Leng, C. Y., Raja Azwa, R. I., et al. (2018). Enrichment of gut-derived Fusobacterium is associated with suboptimal immune recovery in HIV-infected individuals. *Sci. Rep.* 8:14277. doi: 10.1038/s41598-018-32585-x

Levy, M., Thaiss, C. A., Katz, M. N., Suez, J., and Elinav, E. (2015). Inflammasomes and the microbiota—partners in the preservation of mucosal homeostasis. *Semin. Immunopathol.* 37, 39–46. doi: 10.1007/s00281-014-0451-7

Li, H., Jiang, J.-D., and Peng, Z.-G. (2016). MicroRNA-mediated interactions between host and hepatitis C virus. *World J. Gastroenterol.* 22, 1487–1496. doi: 10.3748/wjg.v22.i4.1487

Li, M., van Esch, B. C. A. M., Henricks, P. A. J., Garssen, J., and Folkerts, G. (2018). Time and concentration dependent effects of short chain fatty acids on lipopolysaccharide- or tumor necrosis factor α-induced endothelial activation. *Front. Pharmacol.* 9:233. doi: 10.3389/fphar.2018.00233

Li, Q., Zhang, Q., Wang, C., Liu, X., Li, N., and Li, J. (2009). Disruption of tight junctions during polymicrobial sepsis *in vivo*. *J. Pathol.* 218, 210–221. doi: 10.1002/path.2525

Lin, J., Welker, N. C., Zhao, Z., Li, Y., Zhang, J., Reuss, S. A., et al. (2014). Novel specific microRNA biomarkers in idiopathic inflammatory bowel disease unrelated to disease activity. *Mod. Pathol.* 27, 602–608. doi: 10.1038/modpathol.2013.152

Lin, R. S., Lee, F. Y., Lee, S. D., Tsai, Y. T., Lin, H. C., Lu, R. H., et al. (1995). Endotoxemia in patients with chronic liver diseases: relationship to severity of liver diseases, presence of esophageal varices, and hyperdynamic circulation. *J. Hepatol.* 22, 165–172. doi: 10.1016/0168-8278(95)80424-2

Ling, Z., Jin, C., Xie, T., Cheng, Y., Li, L., and Wu, N. (2016). Alterations in the fecal microbiota of patients with HIV-1 infection: an observational study in a chinese population. *Sci. Rep.* 6:30673. doi: 10.1038/srep30673

Liu, L., Li, L., Min, J., Wang, J., Wu, H., Zeng, Y., et al. (2012). Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells. *Cell Immunol.* 277, 66–73. doi: 10.1016/j.cellimm.2012.05.011

Liu, S., da Cunha, A. P., Rezende, R. M., Cialic, R., Wei, Z., Bry, L., et al. (2016). The host shapes the gut microbiota via fecal microRNA. *Cell Host Microbe* 19, 32–43. doi: 10.1016/j.chom.2015.12.005

Liu, S., and Weiner, H. L. (2016). Control of the gut microbiome by fecal microRNA. *Microb. Cell.* 3, 176–177. doi: 10.15698/mic2016.04.492

Liu, S. F., Ye, X., and Malik, A. B. (1999). Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents In vivo expression of proinflammatory genes. *Circulation* 100, 1330–1337. doi: 10.1161/01.cir.100.12.1330

Livanos, A. E., Snider, E. J., Whittier, S., Chong, D. H., Wang, T. C., Abrams, J. A., et al. (2018). Rapid gastrointestinal loss of Clostridial Clusters IV and XIVa in the ICU associates with an expansion of gut pathogens. *PLoS One.* 13:e0200322. doi: 10.1371/journal.pone.0200322

Lorenzo-Zúñiga, V., Bartolí, R., Planas, R., Hofmann, A. F., Viñado, B., Hagey, L. R., et al. (2003). Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. *Hepatology* 37, 551–557. doi: 10.1053/jhep.2003.50116

Los, F. C. O., Randis, T. M., Aroian, R. V., and Ratner, A. J. (2013). Role of pore-forming toxins in bacterial infectious diseases. *Microbiol. Mol. Biol. Rev.* 77, 173–207. doi: 10.1128/MMBR.00052-12

Lu, H., Wu, Z., Xu, W., Yang, J., Chen, Y., and Li, L. (2011). Intestinal microbiota was assessed in cirrhotic patients with hepatitis B virus infection. *Microb. Ecol.* 61, 693–703. doi: 10.1007/s00248-010-9801-8

Maa, M.-C., Chang, M. Y., Hsieh, M.-Y., Chen, Y.-J., Yang, C.-J., Chen, Z.-C., et al. (2010). Butyrate reduced lipopolysaccharide-mediated macrophage migration by suppression of Src enhancement and focal adhesion kinase activity. *J. Nutr. Biochem.* 21, 1186–1192. doi: 10.1016/j.jnutbio.2009.10.004

MacFie, J., O'Boyle, C., Mitchell, C. J., Buckley, P. M., Johnstone, D., and Sudworth, P. (1999). Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. *Gut* 45, 223–228. doi: 10.1136/gut.45.2.223

Macia, L., Tan, J., Vieira, A. T., Leach, K., Stanley, D., Luong, S., et al. (2015). Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat. Commun.* 6:6734. doi: 10.1038/ncomms7734

Macpherson, A. J., Heikenwalder, M., and Ganal-Vonarburg, S. C. (2016). The liver at the nexus of host-microbial interactions. *Cell Host Microbe* 20, 561–571. doi: 10.1016/j.chom.2016.10.016

Madan, J. C., Salari, R. C., Saxena, D., Davidson, L., O'Toole, G. A., Moore, J. H., et al. (2012). Gut microbial colonisation in premature neonates predicts neonatal sepsis. *Arch. Dis. Child Fetal. Neonatal. Ed.* 97, F456–F462. doi: 10.1136/fetalneonatal-2011-301373

Magro, F., Langner, C., Driessen, A., Ensari, A., Geboes, K., Mantzaris, G. J., et al. (2013). European consensus on the histopathology of inflammatory bowel disease. *J. Crohn's Colitis* 7, 827–851. doi: 10.1016/j.crohns.2013.06.001

Maharshak, N., Packey, C. D., Ellermann, M., Manick, S., Siddle, J. P., Huh, E. Y., et al. (2013). Altered enteric microbiota ecology in interleukin 10-deficient mice during development and progression of intestinal inflammation. *Gut Microbes* 4, 316–324. doi: 10.4161/gmic.25486

Mai, V., Torrazza, R. M., Ukhanova, M., Wang, X., Sun, Y., Li, N., et al. (2013). Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants. *PLoS One* 8:e52876. doi: 10.1371/journal.pone.0052876

Mainous, M. R., Ertel, W., Chaudry, I. H., and Deitch, E. A. (1995). The gut: a cytokine-generating organ in systemic inflammation? *Shock* 4, 193–199. doi: 10.1097/00024382-199509000-00007

Mainous, M. R., Tso, P., Berg, R. D., and Deitch, E. A. (1991). Studies of the route, magnitude, and time course of bacterial translocation in a model of systemic inflammation. *Arch. Surg.* 126, 33–37.

Makarov, S. S. (2001). NF-kappa B in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction. *Arthritis Res.* 3, 200–206.

Marchetti, G., Tincati, C., and Silvestri, G. (2013). Microbial translocation in the pathogenesis of HIV infection and AIDS. *Clin. Microbiol. Rev.* 26, 2–18. doi: 10.1128/CMR.00050-12

Martín, R., Chamignon, C., Mhedbi-Hajri, N., Chain, F., Derrien, M., Escribano-Vázquez, U., et al. (2019). The potential probiotic Lactobacillus rhamnosus CNCM I-3690 strain protects the intestinal barrier by stimulating both mucus production and cytoprotective response. *Sci. Rep.* 9:5398. doi: 10.1038/s41598-019-41738-5

Martin-Gallausiaux, C., Béguet-Crespel, F., Marinelli, L., Jamet, A., Ledue, F., Blottière, H. M., et al. (2018). Butyrate produced by gut commensal bacteria activates TGF-beta1 expression through the transcription factor SP1 in human intestinal epithelial cells. *Sci. Rep.* 8:9742. doi: 10.1038/s41598-018-28048-y

Matthews, G. M., Howarth, G. S., and Butler, R. N. (2012). Short-chain fatty acids induce apoptosis in colon cancer cells associated with changes to intracellular redox state and glucose metabolism. *Chemotherapy* 58, 102–109. doi: 10.1159/000335672

Mazmanian, S. K., Round, J. L., and Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453, 620–625. doi: 10.1038/nature07008

McDonald, D., Ackermann, G., Khailova, L., Baird, C., Heyland, D., Kozar, R., et al. (2016). Extreme dysbiosis of the microbiome in critical illness. *mSphere* 1:e00199-16. doi: 10.1128/mSphere.00199-16

McGowan, I., Elliott, J., Fuerst, M., Taing, P., Boscardin, J., Poles, M., et al. (2004). Increased HIV-1 mucosal replication is associated with generalized mucosal cytokine activation. *J. Acquir. Immune. Defic. Syndr.* 37, 1228–1236. doi: 10.1097/01.qai.0000131846.12453.29

McManamy, M. E. M., Hakre, S., Verdin, E. M., and Margolis, D. M. (2014). Therapy for latent HIV-1 infection: the role of histone deacetylase inhibitors. *Antivir. Chem. Chemother.* 23, 145–149. doi: 10.3851/IMP2551

Meijer, K., De Vos, P., and Priebe, M. G. (2010). Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? *Curr. Opin. Clin. Nutr. Metab. Care.* 13, 715–721. doi: 10.1097/MCO.0b013e32833eebe5

Meng, M., Klingensmith, N. J., and Coopersmith, C. M. (2017). New insights into the gut as the driver of critical illness and organ failure. *Curr. Opin. Crit. Care.* 23, 143–148. doi: 10.1097/MCC.0000000000000386

Merlini, E., Bai, F., Bellistrì, G. M., Tincati, C., d'Arminio Monforte, A., and Marchetti, G. (2011). Evidence for polymicrobic flora translocating in peripheral blood of HIV-infected patients with poor immune response

to antiretroviral therapy. *PLoS One* 6:e18580. doi: 10.1371/journal.pone.0018580

Mittal, R., and Coopersmith, C. M. (2014). Redefining the gut as the motor of critical illness. *Trends Mol. Med.* 20, 214–223. doi: 10.1016/j.molmed.2013.08.004

Miura, K., Kodama, Y., Inokuchi, S., Schnabl, B., Aoyama, T., Ohnishi, H., et al. (2010). Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. *Gastroenterology* 139, 323.e7–334.e7. doi: 10.1053/j.gastro.2010.03.052

Miura, K., and Ohnishi, H. (2014). Role of gut microbiota and Toll-like receptors in nonalcoholic fatty liver disease. *World J. Gastroenterol.* 20:7381. doi: 10.3748/wjg.v20.i23.7381

Mo, J.-S., Alam, K. J., Kim, H.-S., Lee, Y.-M., Yun, K.-J., and Chae, S.-C. (2016). MicroRNA 429 regulates mucin gene expression and secretion in murine model of colitis. *J. Crohn's Colitis* 10, 837–849. doi: 10.1093/ecco-jcc/jjw033

Mohan, M., Kumar, V., Lackner, A. A., and Alvarez, X. (2015). Dysregulated miR-34a–SIRT1–acetyl p65 axis is a potential mediator of immune activation in the colon during chronic simian immunodeficiency virus infection of Rhesus macaques. *J. Immunol.* 194, 291–306. doi: 10.4049/jimmunol.1401447

Nagpal, R., and Yadav, H. (2017). Bacterial translocation from the gut to the distant organs: an overview. *Ann. Nutr. Metab.* 71, 11–16. doi: 10.1159/000479918

Nahid, M. A., Satoh, M., and Chan, E. K. L. (2011). Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. *J. Immunol.* 186, 1723–1734. doi: 10.4049/jimmunol.1002311

Nakata, K., Sugi, Y., Narabayashi, H., Kobayakawa, T., Nakanishi, Y., Tsuda, M., et al. (2017). Commensal microbiota-induced microRNA modulates intestinal epithelial permeability through the small GTPase ARF4. *J. Biol. Chem.* 292, 15426–15433. doi: 10.1074/jbc.M117.788596

Nastasi, C., Candela, M., Bonefeld, C. M., Geisler, C., Hansen, M., Krejsgaard, T., et al. (2015). The effect of short-chain fatty acids on human monocyte-derived dendritic cells. *Sci. Rep.* 5:16148. doi: 10.1038/srep16148

Nazli, A., Chan, O., Dobson-Belaire, W. N., Ouellet, M., Tremblay, M. J., Gray-Owen, S. D., et al. (2010). Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS Pathog.* 6:e1000852. doi: 10.1371/journal.ppat.1000852

Neff, C. P., Krueger, O., Xiong, K., Arif, S., Nusbacher, N., Schneider, J. M., et al. (2018). Fecal microbiota composition drives immune activation in HIV-infected individuals. *EBioMedicine* 30, 192–202. doi: 10.1016/j.ebiom.2018.03.024

Nikaido, H. (1996). Multidrug Efflux Pumps of Gram-Negative Bacteria [Internet]. *J. Bacteriol.* 178, 5853–5859. doi: 10.1128/jb.178.20.5853-5859.1996

Nishino, K., Latifi, T., and Groisman, E. A. (2006). Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* 59, 126–141. doi: 10.1111/j.1365-2958.2005.04940.x

Nishitsuji, K., Xiao, J., Nagatomo, R., Umemoto, H., Morimoto, Y., Akatsu, H., et al. (2017). Analysis of the gut microbiome and plasma short-chain fatty acid profiles in a spontaneous mouse model of metabolic syndrome. *Sci. Rep.* 7:15876. doi: 10.1038/s41598-017-16189-5

O'Hara, A. M., and Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Rep.* 7, 688–693. doi: 10.1038/sj.embor.7400731

Olsson, J., Poles, M., Spetz, A., Elliott, J., Hultin, L., Giorgi, J., et al. (2000). Human immunodeficiency virus type 1 infection is associated with significant mucosal inflammation characterized by increased expression of CCR5, CXCR4, and β-chemokines. *J. Infect Dis.* 182, 1625–1635. doi: 10.1086/317625

Ortega, ÁD., Gonzalo-Asensio, J., and García-del Portillo, F. (2012). Dynamics of *Salmonella* small RNA expression in non-growing bacteria located inside eukaryotic cells. *RNA Biol.* 9, 469–488. doi: 10.4161/rna.19317

Ortiz, A. M., Klase, Z. A., DiNapoli, S. R., Vujkovic-Cvijin, I., Carmack, K., Perkins, M. R., et al. (2016). IL-21 and probiotic therapy improve Th17 frequencies, microbial translocation and microbiome in ARV-treated, SIV-infected macaques. *Mucosal Immunol.* 9, 458–467. doi: 10.1038/mi.2015.75

Padalon-Brauch, G., Hershberg, R., Elgrably-Weiss, M., Baruch, K., Rosenshine, I., Margalit, H., et al. (2008). Small RNAs encoded within genetic islands of *Salmonella* typhimurium show host-induced expression and role in virulence. *Nucleic Acids Res.* 36, 1913–1927. doi: 10.1093/nar/gkn050

Panda, S., khader, I., Casellas, F., López Vivancos, J., Cors, M., Santiago, A., et al. (2014). Short-term effect of antibiotics on human gut microbiota. *PLoS One* 9:e95476. doi: 10.1371/journal.pone.0095476

Pandiyan, P., Bhaskaran, N., Zou, M., Schneider, E., Jayaraman, S., and Huehn, J. (2019). Microbiome dependent regulation of Tregs and Th17 cells in mucosa. *Front. Immunol.* 10:426. doi: 10.3389/fimmu.2019.00426

Panigrahi, P., Parida, S., Nanda, N. C., Satpathy, R., Pradhan, L., Chandel, D. S., et al. (2017). A randomized synbiotic trial to prevent sepsis among infants in rural India. *Nature* 548, 407–412. doi: 10.1038/nature23480

Panpetch, W., Chancharoenthana, W., Bootdee, K., Nilgate, S., Finkelman, M., Tumwasorn, S., et al. (2017). Lactobacillus rhamnosus L34 attenuates gut translocation-induced bacterial sepsis in murine models of leaky gut. *Infect Immun.* 86:e00700-17. doi: 10.1128/IAI.00700-17

Pant, K., Yadav, A. K., Gupta, P., Islam, R., Saraya, A., and Venugopal, S. K. (2017). Butyrate induces ROS-mediated apoptosis by modulating miR-22/SIRT-1 pathway in hepatic cancer cells. *Redox Biol.* 12, 340–349. doi: 10.1016/j.redox.2017.03.006

Parada, J. L., Caron, C. R., Medeiros, A. B. P., and Soccol, C. R. (2007). Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. *Brazilian Arch. Biol. Technol.* 50, 512–542. doi: 10.1590/s1516-89132007000300018

Park, K. C., Park, J. H., Jeon, J. Y., Kim, S. Y., Kim, J. M., Lim, C. Y., et al. (2014). A new histone deacetylase inhibitor improves liver fibrosis in BDL rats through suppression of hepatic stellate cells. *Br. J. Pharmacol.* 171, 4820–4830. doi: 10.1111/bph.12590

Peck, B. C. E., Sincavage, J., Feinstein, S., Mah, A. T., Simmons, J. G., Lund, P. K., et al. (2016). miR-30 Family Controls Proliferation and Differentiation of Intestinal Epithelial Cell Models by Directing a Broad Gene Expression Program That Includes SOX9 and the Ubiquitin Ligase Pathway. *J. Biol. Chem.* 291, 15975–15984. doi: 10.1074/jbc.M116.733733

Pegu, P., Helmus, R., Gupta, P., Tarwater, P., Caruso, L., Shen, C., et al. (2011). Induction of strong anti-HIV cellular immunity by a combination of Clostridium perfringens expressing HIV gag and virus like particles. *Curr. HIV Res.* 9, 613–622. doi: 10.2174/157016211798998808

Penheiter, K. L., Mathur, N., Giles, D., Fahlen, T., and Jones, B. D. (1997). Non-invasive *Salmonella* typhimurium mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches. *Mol. Microbiol.* 24, 697–709. doi: 10.1046/j.1365-2958.1997.3741745.x

Pérez-Cobas, A. E., Gosalbes, M. J., Friedrichs, A., Knecht, H., Artacho, A., Eismann, K., et al. (2013). Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. *Gut* 62, 1591–1601. doi: 10.1136/gutjnl-2012-303184

Phillips, A. D., Navabpour, S., Hicks, S., Dougan, G., Wallis, T., and Frankel, G. (2000). Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut* 47, 377–381. doi: 10.1136/gut.47.3.377

Pinto-Cardoso, S., Klatt, N. R., and Reyes-Terán, G. (2018). Impact of antiretroviral drugs on the microbiome: unknown answers to important questions. *Curr. Opin. HIV AIDS* 13, 53–60. doi: 10.1097/COH.0000000000000428

Pinto-Cardoso, S., Lozupone, C., Briceño, O., Alva-Hernández, S., Téllez, N., Adriana, A., et al. (2017). Fecal bacterial communities in treated HIV infected individuals on two antiretroviral regimens. *Sci. Rep.* 7:43741. doi: 10.1038/srep43741

Pollicino, T., Belloni, L., Raffa, G., Pediconi, N., Squadrito, G., Raimondo, G., et al. (2006). Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology* 130, 823–837. doi: 10.1053/j.gastro.2006.01.001

Ponziani, F. R., Gerardi, V., Pecere, S., D'Aversa, F., Lopetuso, L., Zocco, M. A., et al. (2015). Effect of rifaximin on gut microbiota composition in advanced liver disease and its complications. *World J. Gastroenterol.* 21:12322. doi: 10.3748/wjg.v21.i43.12322

Pulendran, B., Kumar, P., Cutler, C. W., Mohamadzadeh, M., Van Dyke, T., Banchereau, J., et al. (2001). Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *J. Immunol.* 167, 5067–5076. doi: 10.4049/jimmunol.167.9.5067

Rajilić-Stojanović, M., Smidt, H., and de Vos, W. M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. *Environ. Microbiol.* 9, 2125–2136. doi: 10.1111/j.1462-2920.2007.01369.x

Rao, K., and Young, V. B. (2015). Fecal microbiota transplantation for the management of Clostridium difficile infection. *Infect Dis. Clin. North Am.* 29, 109–122. doi: 10.1016/j.idc.2014.11.009

Rea, M. C., Sit, C. S., Clayton, E., O'Connor, P. M., Whittal, R. M., Zheng, J., et al. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proc. Natl. Acad. Sci.* 107, 9352–9357. doi: 10.1073/pnas.0913554107

Rhayat, L., Maresca, M., Nicoletti, C., Perrier, J., Brinch, K. S., Christian, S., et al. (2019). Effect of Bacillus subtilis strains on intestinal barrier function and inflammatory response. *Front. Immunol.* 10:564. doi: 10.3389/fimmu.2019.00564

Richer, M. J., Nolz, J. C., and Harty, J. T. (2013). Pathogen-specific inflammatory milieux tune the antigen sensitivity of CD8+ T cells by enhancing T cell receptor signaling. *Immunity* 38, 140–152. doi: 10.1016/j.immuni.2012.09.017

Ridlon, J. M., Kang, D. J., Hylemon, P. B., and Bajaj, J. S. (2014). Bile acids and the gut microbiome. *Curr. Opin. Gastroenterol.* 30, 332–338. doi: 10.1097/mog.0000000000000057

Rios, D., Wood, M. B., Li, J., Chassaing, B., Gewirtz, A. T., and Williams, I. R. (2016). Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria. *Mucosal Immunol.* 9, 907–916. doi: 10.1038/mi.2015.121

Rodriguez, A., Vigorito, E., Clare, S., Warren, M. V., Couttet, P., Soond, D. R., et al. (2007). Requirement of bic/microRNA-155 for normal immune function. *Science* 316, 608–611. doi: 10.1126/science.1139253

Roediger, W. E. (1980). Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut* 21, 793–798. doi: 10.1136/gut.21.9.793

Rolhion, N., and Chassaing, B. (2016). When pathogenic bacteria meet the intestinal microbiota. *Philos. Trans. R. Soc. B Biol. Sci.* 371:20150504. doi: 10.1098/rstb.2015.0504

Román, E., Nieto, J. C., Gely, C., Vidal, S., Pozuelo, M., Poca, M., et al. (2019). Effect of a multistrain probiotic on cognitive function and risk of falls in patients with cirrhosis: a randomized trial. *Hepatol. Commun.* 3, 632–645. doi: 10.1002/hep4.1325

Round, J. L., Lee, S. M., Li, J., Tran, G., Jabri, B., Chatila, T. A., et al. (2011). The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 332, 974–977. doi: 10.1126/science.1206095

Sailhamer, E. A., Carson, K., Chang, Y., Zacharias, N., Spaniolas, K., Tabbara, M., et al. (2009). Fulminant Clostridium difficile colitis. *Arch. Surg.* 144, 433–439. doi: 10.1001/archsurg.2009.51

Sankaran, S., George, M. D., Reay, E., Guadalupe, M., Flamm, J., Prindiville, T., et al. (2008). Rapid onset of intestinal epithelial barrier dysfunction in primary human immunodeficiency virus infection is driven by an imbalance between immune response and mucosal repair and regeneration. *J. Virol.* 82, 538–545. doi: 10.1128/jvi.01449-07

Sankaran, S., Guadalupe, M., Reay, E., George, M. D., Flamm, J., Prindiville, T., et al. (2005). Gut mucosal T cell responses and gene expression correlate with protection against disease in long-term HIV-1-infected nonprogressors. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9860–9865. doi: 10.1073/pnas.0503463102

Santoro, M. G., Rossi, A., Amici, C., and Schneider, R. J. (2003). NF-kappaB and virus infection: who controls whom. *EMBO J.* 22, 2552–2560. doi: 10.1093/emboj/cdg267

Sassone-Corsi, M., and Raffatellu, M. (2015). No vacancy: How beneficial microbes cooperate with immunity to provide colonization resistance to pathogens. *J. Immunol.* 194, 4081–4087. doi: 10.4049/jimmunol.1403169

Sato, Y., and Tsurumi, T. (2013). Genome guardian p53 and viral infections. *Rev. Med. Virol.* 23, 213–220. doi: 10.1002/rmv.1738

Schnabl, B. (2013). Linking intestinal homeostasis and liver disease. *Curr. Opin. Gastroenterol.* 29, 264–270. doi: 10.1097/mog.0b013e32835ff948

Schnabl, B., and Brenner, D. A. (2014). Interactions between the intestinal microbiome and liver diseases. *Gastroenterology* 146, 1513–1524. doi: 10.1053/j.gastro.2014.01.020

Schnupf, P., Gaboriau-Routhiau, V., Sansonetti, P. J., and Cerf-Bensussan, N. (2017). Segmented filamentous bacteria, Th17 inducers and helpers in a hostile world. *Curr. Opin. Microbiol.* 35, 100–109. doi: 10.1016/j.mib.2017.03.004

Seki, E., De Minicis, S., Österreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A., et al. (2007). TLR4 enhances TGF-β signaling and hepatic fibrosis. *Nat. Med.* 13, 1324–1332. doi: 10.1038/nm1663

Seki, E., and Schnabl, B. (2012). Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut. *J. Physiol.* 590, 447–458. doi: 10.1113/jphysiol.2011.219691

Shao, Y., Li, J., Cai, Y., Xie, Y., Ma, G., Li, Y., et al. (2014). The functional polymorphisms of miR-146a are associated with susceptibility to severe sepsis in the Chinese population. *Mediators Inflamm.* 2014:916202. doi: 10.1155/2014/916202

Shimizu, K., Ogura, H., Goto, M., Asahara, T., Nomoto, K., Morotomi, M., et al. (2006). Altered gut flora and environment in patients with severe SIRS. *J. Trauma* 60, 126–133. doi: 10.1097/01.ta.0000197374.99755.fe

Shimizu, K., Ogura, H., Hamasaki, T., Goto, M., Tasaki, O., Asahara, T., et al. (2011). Altered gut flora are associated with septic complications and death in critically ill patients with systemic inflammatory response syndrome. *Dig. Dis. Sci.* 56, 1171–1177. doi: 10.1007/s10620-010-1418-8

Shirai, Y., Hashimoto, M., Kato, R., Kawamura, Y. I., Kirikae, T., Yano, H., et al. (2004). Lipopolysaccharide induces CD25-Positive, IL-10-producing lymphocytes without secretion of proinflammatory cytokines in the human colon: low MD-2 mRNA expression in colonic macrophages. *J. Clin. Immunol.* 24, 42–52. doi: 10.1023/b:joci.0000018062.01980.ba

Shirakawa, K., Chavez, L., Hakre, S., Calvanese, V., and Verdin, E. (2013). Reactivation of latent HIV by histone deacetylase inhibitors. *Trends Microbiol.* 21, 277–285. doi: 10.1016/j.tim.2013.02.005

Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., et al. (2016). The third international consensus definitions for sepsis and septic shock (sepsis-3). *JAMA* 315, 801–810.

Singh, N., Shirdel, E. A., Waldron, L., Zhang, R.-H., Jurisica, I., and Comelli, E. M. (2012). The murine caecal microRNA signature depends on the presence of the endogenous microbiota. *Int. J. Biol. Sci.* 8, 171–186. doi: 10.7150/ijbs.8.171

Skirecki, T., and Cavaillon, J.-M. (2019). Inner sensors of endotoxin – implications for sepsis research and therapy. *FEMS Microbiol. Rev.* 43, 239–256. doi: 10.1093/femsre/fuz004

Song, M., Su, H., Zhang, L., Ma, J., Li, J., Pan, K., et al. (2013). Genetic polymorphisms of miR-146a and miR-27a, H. pylori infection, and risk of gastric lesions in a chinese population. *PLoS One* 8:e61250. doi: 10.1371/journal.pone.0061250

Sonnen, A. F.-P., and Henneke, P. (2013). Role of pore-forming toxins in neonatal sepsis. *Clin. Dev. Immunol.* 2013:608456. doi: 10.1155/2013/608456

Sorbara, M. T., and Pamer, E. G. (2018). Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. *Mucosal Immunol.* 12, 1–9. doi: 10.1038/s41385-018-0053-0

Sorbara, M. T., and Pamer, E. G. (2019). Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. *Mucosal Immunol.* 12, 1–9. doi: 10.1038/s41385-018-0053-0

Sorini, C., Cardoso, R. F., Gagliani, N., and Villablanca, E. J. (2018). Commensal bacteria-specific CD4+ T cell responses in health and disease. *Front. Immunol.* 9:2667. doi: 10.3389/fimmu.2018.02667

Souza, D. G., Vieira, A. T., Soares, A. C., Pinho, V., Nicoli, J. R., Vieira, L. Q., et al. (2004). The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *J. Immunol.* 173, 4137–4146. doi: 10.4049/jimmunol.173.6.4137

Srinivasjois, R., Rao, S., and Patole, S. (2013). Prebiotic supplementation in preterm neonates: updated systematic review and meta-analysis of randomised controlled trials. *Clin. Nutr.* 32, 958–965. doi: 10.1016/j.clnu.2013.05.009

Staedel, C., and Darfeuille, F. (2013). MicroRNAs and bacterial infection. *Cell Microbiol.* 15, 1496–1507. doi: 10.1111/cmi.12159

Stagg, A. J., Hart, A. L., Knight, S. C., and Kamm, M. A. (2003). The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria. *Gut* 52, 1522–1529. doi: 10.1136/gut.52.10.1522

Steele, A. K., Lee, E. J., Vestal, B., Hecht, D., Dong, Z., Rapaport, E., et al. (2014). Contribution of intestinal barrier damage, microbial translocation and HIV-1 infection status to an inflammaging signature. *PLoS One* 9:e97171. doi: 10.1371/journal.pone.0097171

Stewart, A. S., Pratt-Phillips, S., and Gonzalez, L. M. (2017). Alterations in intestinal permeability: the role of the "Leaky Gut" in health and disease. *J. Equine Vet. Sci.* 52, 10–22. doi: 10.1016/j.jevs.2017.02.009

Stewart, C. J., Embleton, N. D., Marrs, E. C. L., Smith, D. P., Fofanova, T., Nelson, A., et al. (2017). Longitudinal development of the gut microbiome and metabolome in preterm neonates with late onset sepsis and healthy controls. *Microbiome* 5:75. doi: 10.1186/s40168-017-0295-1

Su, G. L., Klein, R. D., Aminlari, A., Zhang, H. Y., Steinstraesser, L., Alarcon, W. H., et al. (2000). Kupffer cell activation by lipopolysaccharide in rats: role

for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* 31, 932–936. doi: 10.1053/he.2000.5634

Sun, B., Jia, Y., Hong, J., Sun, Q., Gao, S., Hu, Y., et al. (2018). Sodium butyrate ameliorates high-fat-diet-induced non-alcoholic fatty liver disease through peroxisome proliferator-activated receptor α-mediated activation of β oxidation and suppression of inflammation. *J. Agric. Food Chem.* 66, 7633–7642. doi: 10.1021/acs.jafc.8b01189

Taft, D. H., Ambalavanan, N., Schibler, K. R., Yu, Z., Newburg, D. S., Deshmukh, H., et al. (2015). Center variation in intestinal microbiota prior to late-onset sepsis in preterm infants. *PLoS One* 10:e0130604. doi: 10.1371/journal.pone.0130604

Taganov, K. D., Boldin, M. P., Chang, K.-J., and Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U.S.A* 103, 12481–12486. doi: 10.1073/pnas.0605298103

Taguwa, S., Kambara, H., Omori, H., Tani, H., Abe, T., Mori, Y., et al. (2009). Cochaperone activity of human butyrate-induced transcript 1 facilitates hepatitis C virus replication through an Hsp90-dependent pathway. *J. Virol.* 83, 10427–10436. doi: 10.1128/JVI.01035-09

Taguwa, S., Okamoto, T., Abe, T., Mori, Y., Suzuki, T., Moriishi, K., et al. (2008). Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication. *J. Virol.* 82, 2631–2641. doi: 10.1128/jvi.02153-07

Takeda, N., Jain, R., LeBoeuf, M. R., Wang, Q., Lu, M. M., and Epstein, J. A. (2011). Interconversion between intestinal stem cell populations in distinct niches. *Science* 334, 1420–1424. doi: 10.1126/science.1213214

Tang, Y., Chen, Y., Jiang, H., Robbins, G. T., and Nie, D. (2011). G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer. *Int. J. Cancer* 128, 847–856. doi: 10.1002/ijc.25638

Todorov, S. D., Franco, B. D. G. M., and Wiid, I. J. (2014). *In vitro* study of beneficial properties and safety of lactic acid bacteria isolated from Portuguese fermented meat products. *Benef. Microbes* 5, 351–366. doi: 10.3920/BM2013.0030

Ulven, T. (2012). Short-chain free fatty acid receptors FFA2/GPR43 and FFA3/GPR41 as new potential therapeutic targets. *Front. Endocrinol.* 3:111. doi: 10.3389/fendo.2012.00111

Vaishnava, S., Behrendt, C. L., Ismail, A. S., Eckmann, L., and Hooper, L. V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20858–20863. doi: 10.1073/pnas.0808723105

Vdovikova, S., Gilfillan, S., Wang, S., Dongre, M., Wai, S. N., and Hurtado, A. (2018). Modulation of gene transcription and epigenetics of colon carcinoma cells by bacterial membrane vesicles. *Sci. Rep.* 8, 7434. doi: 10.1038/s41598-018-25308-9

Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., et al. (1998). Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 280, 427–431. doi: 10.1126/science.280.5362.427

Villagra, A., Sotomayor, E. M., and Seto, E. (2010). Histone deacetylases and the immunological network: implications in cancer and inflammation. *Oncogene* 29, 157–173. doi: 10.1038/onc.2009.334

Vincent, C., Stephens, D. A., Loo, V. G., Edens, T. J., Behr, M. A., Dewar, K., et al. (2013). Reductions in intestinal Clostridiales precede the development of nosocomial Clostridium difficile infection. *Microbiome* 1:18. doi: 10.1186/2049-2618-1-18

Vinolo, M. A. R., Rodrigues, H. G., Hatanaka, E., Sato, F. T., Sampaio, S. C., and Curi, R. (2011). Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J. Nutr. Biochem.* 22, 849–855. doi: 10.1016/j.jnutbio.2010.07.009

Vora, P., Youdim, A., Thomas, L. S., Fukata, M., Tesfay, S. Y., Lukasek, K., et al. (2004). Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *J. Immunol.* 173, 5398–5405. doi: 10.4049/jimmunol.173.9.5398

Vujkovic-Cvijin, I., Rutishauser, R. L., Pao, M., Hunt, P. W., Lynch, S. V., McCune, J. M., et al. (2017). Limited engraftment of donor microbiome via one-time fecal microbial transplantation in treated HIV-infected individuals. *Gut Microbes* 8, 440–450. doi: 10.1080/19490976.2017.1334034

Wallace, A. J., Stillman, T. J., Atkins, A., Jamieson, S. J., Bullough, P. A., Green, J., et al. (2000). E. coli hemolysin E (HlyE, ClyA, SheA): X-ray crystal structure of the toxin and observation of membrane pores by electron microscopy. *Cell* 100, 265–276. doi: 10.1016/s0092-8674(00)81564-0

Wang, F., Liu, J., Weng, T., Shen, K., Chen, Z., Yu, Y., et al. (2017). The inflammation induced by lipopolysaccharide can be mitigated by short-chain fatty acid, butyrate, through upregulation of IL-10 in septic shock. *Scand. J. Immunol.* 85, 258–263. doi: 10.1111/sji.12515

Wang, H.-B., Wang, P.-Y., Wang, X., Wan, Y.-L., and Liu, Y.-C. (2012). Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein claudin-1 transcription. *Dig. Dis. Sci.* 57, 3126–3135. doi: 10.1007/s10620-012-2259-4

Weber, T. E., and Kerr, B. J. (2006). Butyrate differentially regulates cytokines and proliferation in porcine peripheral blood mononuclear cells. *Vet. Immunol. Immunopathol.* 113, 139–147. doi: 10.1016/j.vetimm.2006.04.006

Wei, Y., Yang, J., Wang, J., Yang, Y., Huang, J., Gong, H., et al. (2016). Successful treatment with fecal microbiota transplantation in patients with multiple organ dysfunction syndrome and diarrhea following severe sepsis. *Crit. Care* 20:332.

Wiest, R., Albillos, A., Trauner, M., Bajaj, J. S., and Jalan, R. (2017). Targeting the gut-liver axis in liver disease. *J. Hepatol.* 67, 1084–1103. doi: 10.1016/j.jhep.2017.05.007

Williams, M. R., Stedtfeld, R. D., Tiedje, J. M., and Hashsham, S. A. (2017). MicroRNAs-Based Inter-Domain Communication between the Host and Members of the Gut Microbiome. *Front. Microbiol.* 8:1896. doi: 10.3389/fmicb.2017.01896

Wu, F., Zhang, S., Dassopoulos, T., Harris, M. L., Bayless, T. M., Meltzer, S. J., et al. (2010). Identification of microRNAs associated with ileal and colonic Crohn's disease. *Inflamm. Bowel Dis.* 16, 1729–1738. doi: 10.1002/ibd.21267

Wu, Z.-W., Lu, H.-F., Wu, J., Zuo, J., Chen, P., Sheng, J.-F., et al. (2012). *Assessment of the fecal lactobacilli population in patients with hepatitis B virus-related decompensated cirrhosis and hepatitis B cirrhosis treated with liver transplant, Microbial Ecology*. New York, NY: Springer, 929–937.

Xue, X., Cao, A. T., Cao, X., Yao, S., Carlsen, E. D., Soong, L., et al. (2014). Downregulation of microRNA-107 in intestinal CD11c + myeloid cells in response to microbiota and proinflammatory cytokines increases IL-23p19 expression. *Eur. J. Immunol.* 44, 673–682. doi: 10.1002/eji.201343717

Xue, X., Feng, T., Yao, S., Wolf, K. J., Liu, C.-G., Liu, X., et al. (2011). Microbiota downregulates dendritic cell expression of miR-10a, which targets IL-12/IL-23p40. *J. Immunol.* 187, 5879–5886. doi: 10.4049/jimmunol.1100535

Yang, L., and Seki, E. (2012). Toll-like receptors in liver fibrosis: cellular crosstalk and mechanisms. *Front. Physiol.* 3:138. doi: 10.3389/fphys.2012.00138

Ye, D., Guo, S., Al-Sadi, R., and Ma, T. Y. (2011). MicroRNA regulation of intestinal epithelial tight junction permeability. *Gastroenterology* 141, 1323–1333. doi: 10.1053/j.gastro.2011.07.005

Ye, F., and Karn, J. (2015). Bacterial short chain fatty acids push all the buttons needed to reactivate latent viruses. *Stem Cell Epigenet.* 2:e532.

Yin, L., Laevsky, G., and Giardina, C. (2001). Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. *J. Biol. Chem.* 276, 44641–44646. doi: 10.1074/jbc.m105170200

Yoseph, B. P., Klingensmith, N. J., Liang, Z., Breed, E. R., Burd, E. M., Mittal, R., et al. (2016). Mechanisms of intestinal barrier dysfunction in sepsis. *Shock* 46, 52–59. doi: 10.1097/SHK.0000000000000565

Yuan, C., Burns, M. B., Subramanian, S., and Blekhman, R. (2018). Interaction between host microRNAs and the gut microbiota in colorectal cancer. *mSystems* 3:e00205-17. doi: 10.1128/mSystems.00205-17

Zaborina, O., Lepine, F., Xiao, G., Valuckaite, V., Chen, Y., Li, T., et al. (2007). Dynorphin activates quorum sensing quinolone signaling in *Pseudomonas aeruginosa*. *PLoS Pathog.* 3:e35. doi: 10.1371/journal.ppat.0030035

Zeitz, M., Ullrich, R., Schneider, T., Kewenig, S., Hohloch, K., and Riecken, E. O. (1998). HIV/SIV enteropathy. *Ann. N.Y. Acad. Sci.* 859, 139–148. doi: 10.1111/j.1749-6632.1998.tb11118.x

Zhang, L., Shen, J., Cheng, J., and Fan, X. (2015). MicroRNA-21 regulates intestinal epithelial tight junction permeability. *Cell Biochem. Funct.* 33, 235–240. doi: 10.1002/cbf.3109

Zhao, C., Zhou, Z., Zhang, T., Liu, F., Zhang, C.-Y., Zen, K., et al. (2017). *Salmonella* small RNA fragment Sal-1 facilitates bacterial survival in infected cells via suppressing iNOS induction in a microRNA manner. *Sci. Rep.* 7:16979. doi: 10.1038/s41598-017-17205-4

Zhou, D., Pan, Q., Shen, F., Cao, H., Ding, W., Chen, Y., et al. (2017a). Total fecal microbiota transplantation alleviates high-fat diet-induced steatohepatitis in mice via beneficial regulation of gut microbiota. *Sci. Rep.* 7:1529. doi: 10.1038/s41598-017-01751-y

Zhou, D., Pan, Q., Xin, F.-Z., Zhang, R.-N., He, C.-X., Chen, G.-Y., et al. (2017b). Sodium butyrate attenuates high-fat diet-induced steatohepatitis in mice by improving gut microbiota and gastrointestinal barrier. *World J. Gastroenterol.* 23:60. doi: 10.3748/wjg.v23.i1.60

Zhou, Q., Costinean, S., Croce, C. M., Brasier, A. R., Merwat, S., Larson, S. A., et al. (2015). MicroRNA 29 targets nuclear Factor-κB–repressing factor and claudin 1 to increase intestinal permeability. *Gastroenterology* 148, 158.e–169.e.

Zhou, Q., Souba, W. W., Croce, C. M., and Verne, G. N. (2010). MicroRNA-29a regulates intestinal membrane permeability in patients with irritable bowel syndrome. *Gut* 59, 775–784. doi: 10.1136/gut.2009.181834

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