IMMUNOBIOTICS: INTERACTIONS OF BENEFICIAL MICROBES WITH THE IMMUNE SYSTEM

EDITED BY: Julio Villena and Haruki Kitazawa

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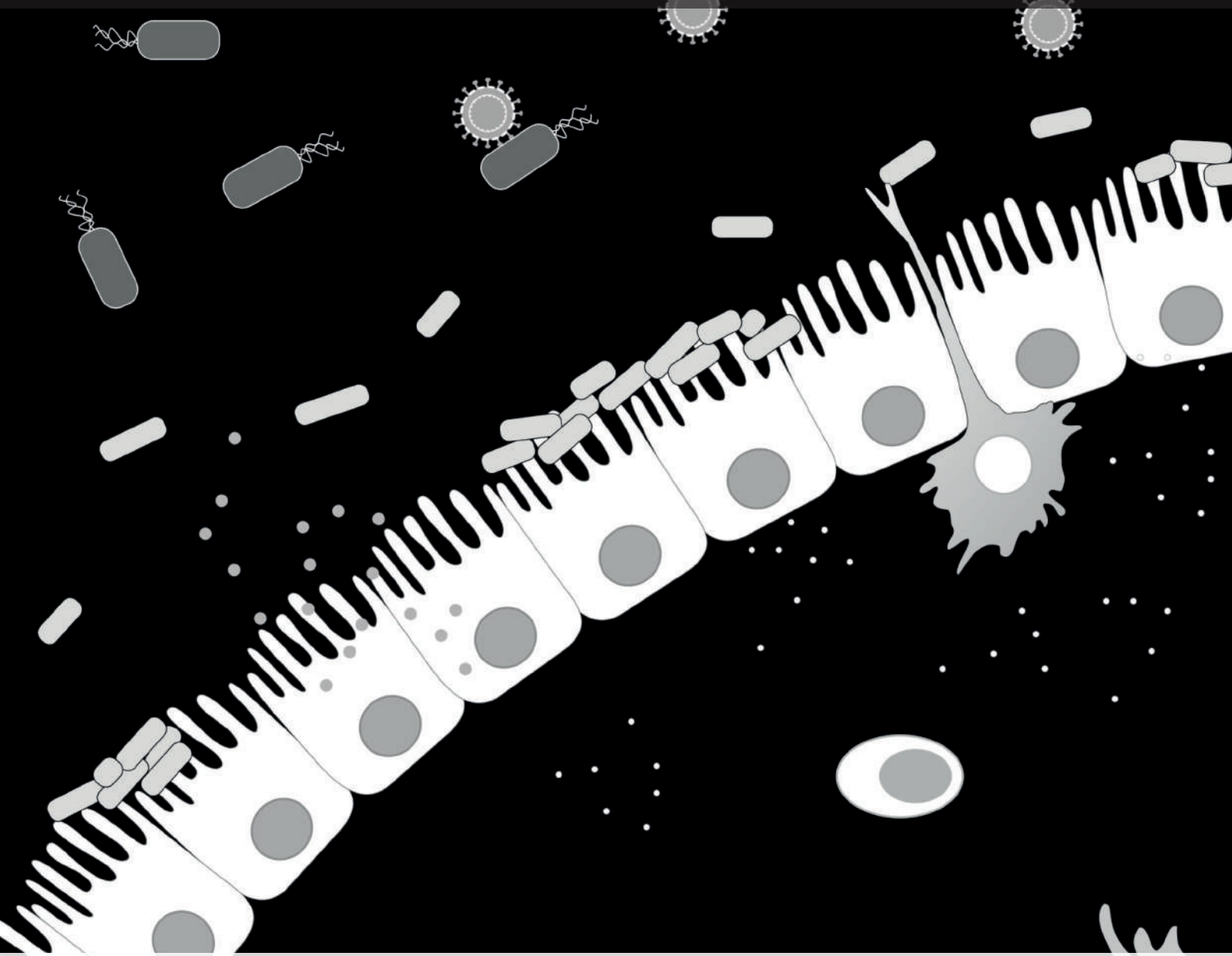

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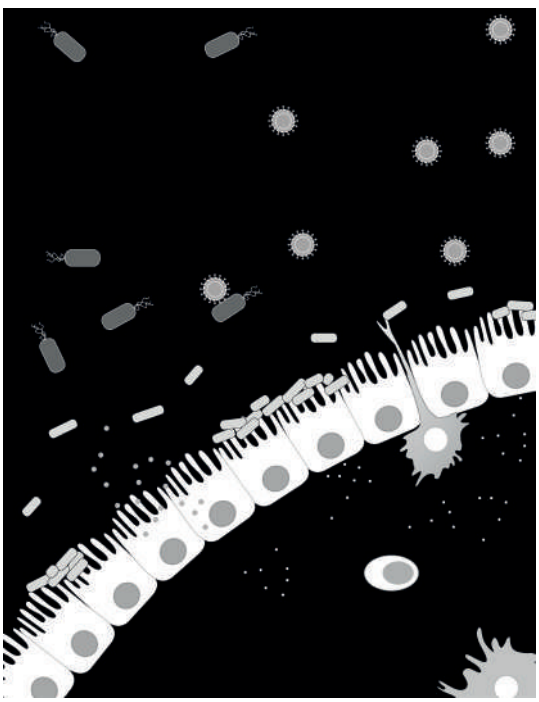
IMMUNOBIOTICS: INTERACTIONS OF BENEFICIAL MICROBES WITH THE IMMUNE SYSTEM

Topic Editors:
Julio Villena, Immunobiotics Research Group, Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Argentina
Haruki Kitazawa, International Education and Research Center for Food and Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Japan



Schematic representation of the complex interactions of immunomodulatory beneficial microbes with the host cells. Image by Leonardo Albarracin and Julio Villena.

The term "immunobiotics" has been proposed to define microbial strains able to beneficially regulate the mucosal immune system. Research in immunobiotics has significantly evolved as researchers employed cutting-edge technologies to investigate the complex interactions of these beneficial microorganisms with the immune system. During the last decade, our understanding of immunobiotics-host interaction was profoundly transformed by the discovery of microbial molecules and host receptors involved in the modulation of gut associated immune system, as well as the systemic and distant mucosal immune systems.

In recent years, there has been a substantial increase in the number of reports describing the beneficial effects of immunobiotics in diseases such as intestinal and respiratory infections, allergy, inflammatory bowel disease, obesity, immunosuppression, and several other immune-mediated conditions. Evidence is also emerging of immunobiotics related molecules with immunomodulatory functions leading to the production of pharmabiotics, which may positively influence human or animal health.

Therefore, research in immunobiotics continue to contribute not only to food but also medical and pharmaceutical fields. The compilation of research articles included in this ebook should help reader to have an overview of the recent advances in immunobiotics.

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EDITORIAL
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Editorial: Immunobiotics—Interactions of Beneficial Microbes with the Immune System

Julio Villena[1,2,3]* *and Haruki Kitazawa*[3,4]*

[1] Immunobiotics Research Group, Tucuman, Argentina, [2] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [3] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [4] Livestock Immunology Unit, International Education and Research Center for Food and Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

Keywords: immunobiotics, lactic acid bacteria, mucosal immunology, innate immunity, beneficial microbes

Editorial on the Research Topic

Immunobiotics—Interactions of Beneficial Microbes with the Immune System

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Edited by:
Ulrich Emil Schaible, Forschungszentrum Borstel (LG), Germany

Reviewed by:
Ashok Munjal, Barkatullah University, India
Matthias Hauptmann, Research Center Borstel, Germany

***Correspondence:**
Julio Villena
jcvillena@cerela.org.ar;
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp

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The term "immunobiotic" has been proposed to define microbial strains that are able to beneficially regulate the immune system of the host. Over the past few years, we have witnessed the emergence of robust development in the application of immunobiotics to combat infections, and researchers have found that the use of beneficial microbes is an interesting alternative to prevent and reduce the severity of infections in humans and animals. In a study by Villena et al., the advances in the application of immunobiotics for preventing intestinal viral infections are analyzed. The capacity of immunobiotics to beneficially modulate the intestinal activation of toll-like receptor 3 (TLR3) and to reduce the local inflammatory-tissue damage is highlighted. Complementing this article, Albarracín et al. reported that immunobiotics substantially modify the immunotranscriptomic response of intestinal epithelial cells after activation of TLR3, inducing an improvement of type-I interferons and antiviral factors and a differential modulation of cytokines, chemokines, and adhesion molecules. Moreover, Kandasamy et al. reviewed the specific effects of Gram-positive and Gram-negative immunobiotics in modulating intestinal immunity against rotavirus and emphasized that immunomodulatory functions of beneficial microbes are species and strain specific.

The effect of immunobiotics on the gut innate and adaptive immune responses to enteric pathogens has been recognized conclusively. However, the influence of immunobiotics on the immune responses in distal mucosal sites and its impact in the outcome of respiratory infections has recently been exposed. In this regard, some studies have demonstrated the potential of beneficial microbes in enhancing respiratory antiviral immunity. Zelaya et al. provide an update on the modulation of respiratory immunity by immunobiotics, and their impact on influenza virus infection. Interestingly, the article highlights the recent findings demonstrating the capacity of some immunobiotic strains to reduce the severity of viral disease through the regulation of the immune-coagulative responses in the respiratory tract. Research indicates that beneficial microbes would be able to influence not only the outcome of viral infections but also secondary bacterial pneumonia. In this regard, Clua et al. demonstrate that the nasal priming with inactivated immunobiotics or purified peptidoglycan improved the resistance to primary respiratory syncytial virus infection, and secondary pneumococcal pneumonia in infant mice. Researchers show that a differential modulation of lung immune cell populations and cytokine production are involved in the protective effects induced by inactivated immunobiotics. Interestingly, the approach of using immunobiotics for modulating respiratory immune responses can be extended for the protection of immunocompromised hosts, as elegantly reviewed by Salva and Alvarez.

Several research works have also reported that immunobiotic intervention had beneficial effects on chronic inflammatory conditions of the gastrointestinal tract. As reviewed by Carvalho et al. and Shigemori and Shimosato, studies in several animal models have provided evidence of the health benefits of certain bacterial species in the alleviation of intestinal inflammation. It was reported that the beneficial effects induced by immunobiotics could be achieved by several mechanisms including the modulation mucosal cytokine profiles, IgA levels, expression patterns of cell surface molecules of antigen presenting cells, or gut microbiota composition, as shown by Carasi et al. and Bene et al. Strikingly, lactate that has long been considered as a metabolic by-product of cells is now seen as a potential beneficial microbiota metabolite with immunomodulatory functions. In this regard, Iraporda et al. revealed that the local treatment with lactate prevents intestinal inflammation in the TNBS-induced colitis model.

In the past few years, researchers have been trying to genetically improve the beneficial microbes designed to express anti-inflammatory factors such as cytokines and enzymes, and they have used this genetically modified immunobiotics as a promising strategy in the treatment of inflammatory bowel diseases and mucositis (Carvalho et al.; Shigemori and Shimosato). Of note, the use of microbes to alleviate intestinal inflammation has not been limited to classical immunobiotics strains such as lactic acid bacteria. Researchers have started to search new beneficial strains in other sources as shown by two articles in this research topic. Indrelid et al. reported that *Methylococcus capsulatus* prevents experimentally induced colitis in a murine model of inflammatory bowel disease by influencing dendritic cell maturation, cytokine production, and subsequent T-cell activation, proliferation, and differentiation. In addition, Diling et al. demonstrated that a protein isolated from the fungus *Hericium erinaceus* exhibited immunomodulatory activity in LPS-activated macrophages *in vitro* by decreasing the overproduction of inflammatory cytokines. Moreover, *in vivo* studies showed that the immunomodulatory fungal protein reduced intestinal inflammation in TNBS-treated animals.

Intestinal dysbiosis, metabolic endotoxemia, and systemic inflammation have been associated with metabolic disorders, such as obesity, insulin resistance, and type-2 diabetes. In this regard, Leite et al. by performing a clinical trial in type-2 diabetes patients and healthy controls observed that the prevalence of Gram-negative species in the gut and the increased plasma IL-6 could be linked to insulin resistance. On the other hand, alterations of microbiota in other mucosal tissues in type-2 diabetes patients have been less explored. Interestingly, Ling et al. reported for the first time that dysbiosis of the urinary microbiota was associated with increased levels of urinary IL-8 in female type-2 diabetes patients. These and other studies suggest that modulation of microbiota could have the potential to reduce inflammation and diminish the severity of alterations in metabolic disorders. In agreement, Fabersani et al. demonstrated that some immunobiotic Gram-positive strains are able to differentially modulate the production of adipokines and leptin by macrophages and adipocytes. Of note, although most studies of the microbiota influence on metabolic alterations have focused on obesity and diabetes, recent findings show that intestinal dysbiosis could be also implicated in inflammatory and metabolic alterations of other diseases. In the case of systemic lupus erythematosus, Rodríguez-Carrio et al. show that intestinal dysbiosis is associated with altered serum levels of free fatty acids and endothelial activation in these patients, opening the door to a new potential application of immunobiotics that must be explored in-depth.

Research in immunobiotics continues to evolve as many laboratories are employing cutting-edge technology to investigate the complex interactions of these beneficial microorganisms with the immune system (Albarracín et al.; Kang et al.; Adachi et al.). During the past decade, our understanding of immunobiotics–host interaction was profoundly transformed by the discovery of microbial molecules and host receptors involved in the modulation of gut-associated immune system, as well as the systemic and distant mucosal immune systems. The compilation of research articles included in this research topic gives an overview of the recent advances in immunobiotics and help reader to locate them (Ko et al.; Wan et al.; Dong et al.; van Beek et al.; Górska et al.; Yu et al.).

AUTHOR CONTRIBUTIONS

All authors have made a substantial, direct, and intellectual contribution to the work and approved the final version of the manuscript for publication.

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REVIEW
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Intestinal Innate Antiviral Immunity and Immunobiotics: Beneficial Effects against Rotavirus Infection

Julio Villena[1,2,3], Maria Guadalupe Vizoso-Pinto[1,4] and Haruki Kitazawa[2,3,5]**

[1] Immunobiotics Research Group, Tucuman, Argentina, [2] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [3] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [4] Faculty of Medicine, INSIBIO (UNT-CONICET), National University of Tucuman, Tucuman, Argentina, [5] Livestock Immunology Unit, International Education and Research Center for Food and Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

The mucosal tissues of the gastrointestinal tract are the main portal entry of pathogens such as rotavirus (RV), which is a leading cause of death due to diarrhea among young children across the globe and a major cause of severe acute intestinal infection in livestock animals. The interactions between intestinal epithelial cells (IECs) and immune cells with RVs have been studied for several years, and now, it is known that the innate immune responses triggered by this virus can have both beneficial and detrimental effects for the host. It was demonstrated that natural RV infection in infants and experimental challenges in mice result in the intestinal activation of pattern recognition receptors (PRRs) such as toll-like receptor 3 (TLR3) and striking secretion of proinflammatory mediators that can lead to increased local tissue damage and immunopathology. Therefore, modulating desregulated intestinal immune responses triggered by PRRs activation are a significant promise for reducing the burden of RV diseases. The ability of immunoregulatory probiotic microorganisms (immunobiotics) to protect against intestinal infections, such as those caused by RVs, is among the oldest effects studied for these important group of beneficial microbes. In this review, we provide an update of the current status on the modulation of intestinal antiviral innate immunity by immunobiotics and their beneficial impact on RV infection. In addition, we describe the research of our group that demonstrated the capacity of immunobiotic strains to beneficially modulated TLR3-triggered immune response in IECs, reduce the disruption of intestinal homeostasis caused by intraepithelial lymphocytes, and improve the resistance to RV infections.

Keywords: immunobiotics, rotavirus, inflammation, TLR3, intestinal epithelial cells, intraepithelial lymphocytes

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Edited by:
Amy Rasley, Lawrence Livermore National Laboratory, USA

Reviewed by:
Benoit Chassaing, Georgia State University, USA
Karl William Boehme, University of Arkansas for Medical Sciences, USA

****Correspondence:***
Julio Villena
jcvillena@cerela.org.ar;
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp

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INTRODUCTION

One of the leading causes of children mortality is preventable infectious diseases (1, 2). Rotavirus (RVs), calicivirus, astrovirus, and adenovirus account to the viral etiologic agents of gastroenteritis in humans (3, 4). RV, a naked double-strand RNA (dsRNA) virus, is the most common cause of severe dehydrating diarrhea in children (5, 6). The main symptoms of RVs gastroenteritis are nausea, low-grade fever, vomit, and acute watery diarrhea. Even though two oral vaccines containing attenuated live viruses are being used globally, Rotarix (GlaxoSmithKline) and RotaTeq (Merck), the epidemic in the developing world is far from being controlled (6, 7). Vaccine effectiveness

is reduced in developing areas, and some possible reasons are children infected at an early age, high viral challenge loads, and the lack of transferred maternal antibodies (8, 9).

Some lactic acid bacteria (LAB) strains are able to impact on human and animal health by modulating the mucosal and systemic immune systems. Those immunoregulatory probiotic LAB, known as immunobiotics, provide protection against viral infections by modulating innate and adaptive antiviral immunity. Thus, several reports have shown that immunobiotic LAB shorten the duration of diarrhea, reduce the number of episodes, diminish RVs shedding, normalize gut permeability, and increase the production of RVs-specific antibodies (10–12).

The purpose of this review is to provide an update of the current status on the modulation of intestinal antiviral innate immunity by immunobiotics, and their beneficial impact on RVs infection. We also highlight some results of our group, which demonstrate the capacity of immunobiotic strains to beneficially modulate toll-like receptor (TLR)-3-triggered immune response in intestinal epithelial cells (IECs), reduce the disruption of intestinal homeostasis caused by intraepithelial lymphocytes (IELs), and improve the resistance to RVs infection.

INTESTINAL ANTIVIRAL INNATE IMMUNE RESPONSE AND ROTAVIRUS

Upon RVs internalization, the capsid uncoats loosing VP4 and VP7, the outer surface proteins, and yielding a transcriptionally active double-layered particle. The eleven segments of dsRNA viral genome are transcribed directing the synthesis of structural and non-structural proteins and serving as templates for the complementary strand of genomic RNA (13). The IEC senses viral dsRNA through pattern recognition receptors (PRRs), such as TLR3, retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), and cellular signaling cascades are activated to react to viral infection (14–16) (**Figure 1**). One of the major innate responses against dsRNA viruses relies

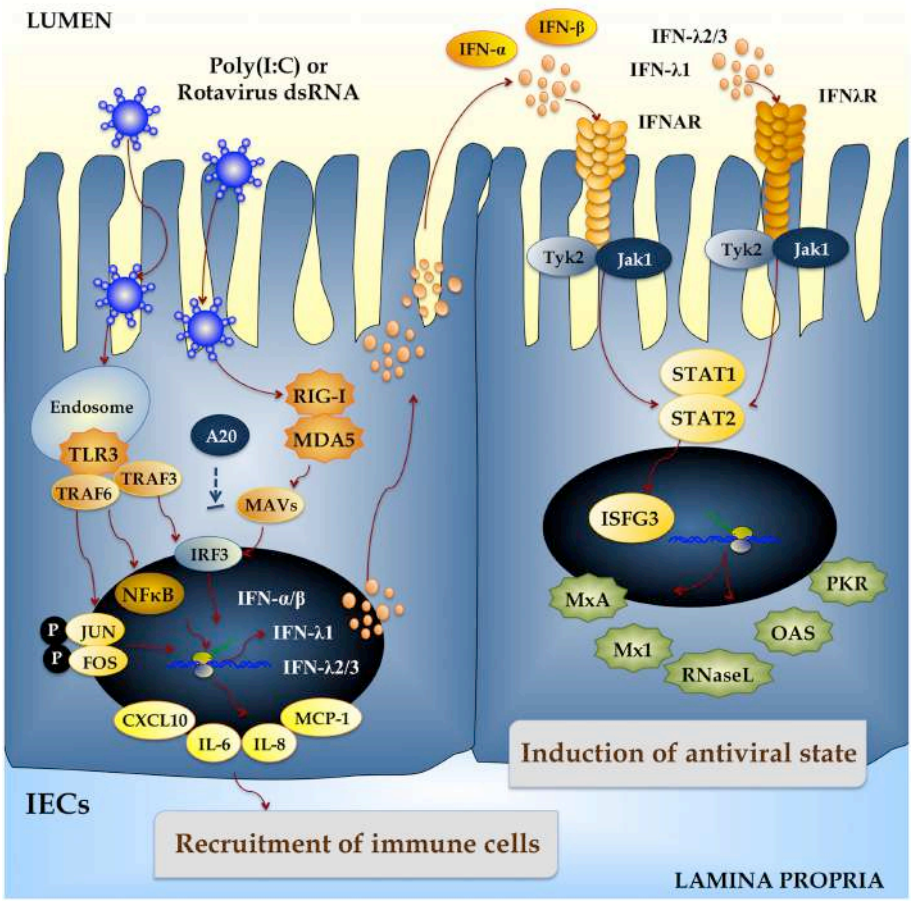

FIGURE 1 | Innate immune response against rotavirus in intestinal epithelial cells (IECs). Rotavirus double-strand genomic RNA activates toll-like receptor 3 (TLR3), retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), which are pattern recognition receptors (PRRs) expressed in IECs. Cellular signaling cascades are activated and converge at the level of interferon (IFN) regulatory factor-3 (IRF3) that upregulate the expression of type I (IFN-α, IFN-β) and type III (IFNλ1, IFNλ2/3) IFN, which in turn induces the synthesis of IFN-stimulated genes with antiviral activities (MxA, Mx1, RNase L, OAS, PKR). Antiviral PRRs also activate nuclear factor κB (NF-κB) pathway and induce the secretion of proinflammatory cytokines and chemokines (IL-6, IL-8, MCP-1, CXCL10). Those effects could be imitated *in vitro* and *in vivo* by administration of the dsRNA synthetic analog poly(I:C).

on the activation of those PRRs, which leads to the production of cytokines and chemokines by IECs and immune cells. Thus, RVs dsRNA triggers the production of IL-8, IP-10, IL-6, TNF-α, and IL-15 in IECs *via* the TLR3-, RIG-I-, and MDA5-activated pathways inducing recruitment and activation of macrophages and NK cells and stimulating adaptive B- and T-cell immune responses. As a result of PRRs activation, interferons (IFNs) and IFN-regulated gene products are also produced and they play a key role in establishing an antiviral state for virus clearance and restriction of spread (**Figure 1**). Type I and III IFNs limit RV infection *in vitro*, and their levels are augmented in RVs-infected children and animals (17–19). Both families of IFN are immediately produced upon RV infection, elicit responses on different types of receptors, and temporally and spatially regulated in the gastrointestinal tract (20). Another evidence suggesting that IFNs are crucial to limit RV infection relies on the fact that this virus has evolved mechanisms to manipulate IFNs signaling such as the type I IFNs damping NSP1 protein (21). While TLR3 mainly recognizes viral components such as viral nucleic acid in endosomal compartments, RIG-I and MDA-5 recognize cytoplasmatic dsRNA. These pathways converge at the level of IFN regulatory factor-3 (IRF3) (18, 22, 23). Upon IRF3 phosphorylation, antiviral responses initiate the activation of type I IFN, which in turn induces the synthesis of interferon-stimulated genes (ISGs), secretion of proinflammatory cytokines, and activation and maturation of antigen-presenting cells (APCs) (**Figure 2**).

Poly(I:C), a synthetic analog of dsRNA, when administered intraperitoneally to mice mimics the local intestinal immune response elicited by an enteric viral infection (24, 25). Both purified RVs dsRNA and poly(I:C) are able to induce severe mucosal damage in the gut *via* TLR3 activation including villous atrophy, mucosal erosion, and gut wall attenuation (24). IELs, which are mostly T cells distributed as single cells within the epithelial cell layer, play a critical role in disrupting epithelial homeostasis caused by abnormal TLR3 signaling (**Figure 3**) (24). Due to their key location at the interface between the inner intestinal tissue and the lumen, these specialized immune cells are important as a first line of defense against microbes and in maintaining the epithelial barrier homeostasis. The majority of IELs are $CD8^+$ being simply classified as $CD8\alpha\alpha^+$ or $CD8\alpha\beta^+$. The $CD8\alpha\beta^+$ IELs bear the hallmarks of adaptive immune cells, whereas the $CD8\alpha\alpha^+$ IELs are considered innate immune cells (26). When TLR3 is abnormally activated by poly(I:C) and RVs, genomic dsRNA, IL-15, and $CD3^+NK1.1^+CD8\alpha\alpha^+$ IELs are involved in the disruption of epithelial homeostasis. In addition, it was demonstrated that TLR3 activation in IECs induces the expression of retinoic acid early inducible-1 (RAE1), which mediates epithelial destruction and mucosal injury by interacting with the NKG2D receptor expressed on IELs (27) (**Figure 3**).

Thus, increasing our understanding of how PRRs such as TLR3 are activated and regulated in immune cells and IECs

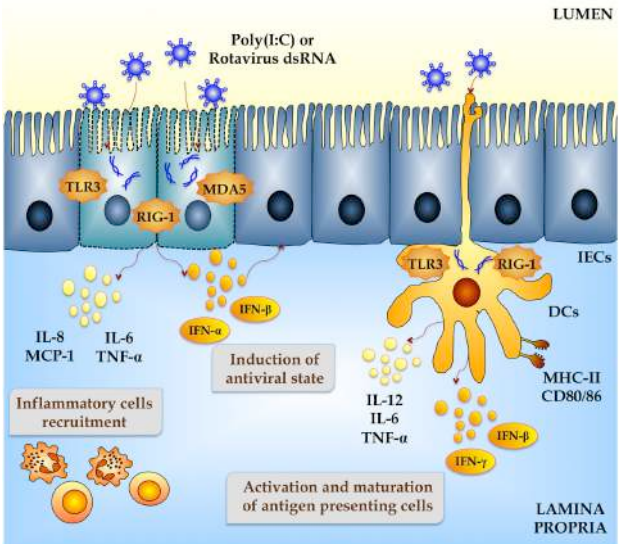

FIGURE 2 | Innate immune response against rotavirus in intestinal mucosa. Rotavirus double-strand genomic RNA activate toll-like receptor 3 (TLR3), retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), which are pattern recognition receptors (PRRs) expressed in intestinal epithelial cells (IECs) and dendritic cells (DCs). Activation of antiviral PRRs in the intestinal mucosa increases the production of type I IFN (IFN-α, IFN-β), IFN-γ, and proinflammatory cytokines and chemokines (TNF-α, IL-6, IL-8, IL-12, MCP-1), which improves the antiviral state in IECs, induces the recruitment and activation of immune cells and the maturation of DCs. Those effects could be imitated *in vitro* and *in vivo* by administration of the dsRNA synthetic analog poly(I:C).

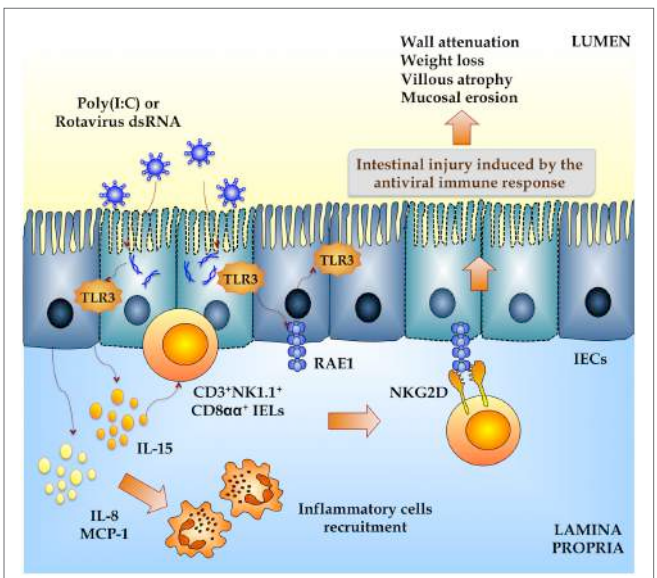

FIGURE 3 | Inflammatory damage of the intestinal mucosa induced by rotavirus in a toll-like receptor 3-deppendent manner. Both purified rotavirus double-strand genomic RNA and poly(I:C) induce severe mucosal intestinal damage *via* TLR3 activation and intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IELs) interaction. Activation of TLR3 in IECs increases the expression of proinflammatory cytokines and chemokines (MCP-1, IL-8, IL-15) and retinoic acid early inducible-1 (RAE1). IL-15 produced by IECs induce the recruitment of $CD3^+NK1.1^+CD8\alpha\alpha^+$ IELs, which mediates epithelial destruction and mucosal injury by the NKG2D receptor expressed on these cells that is able to recognize RAE1. This intestinal TLR3-IECs-IELs interaction induces villous atrophy, mucosal erosion, and gut wall attenuation.

may help designing effective therapies for the prevention and/or treatment of viral diseases.

BENEFICIAL EFFECTS OF IMMUNOBIOTICS IN ROTAVIRUS INFECTION

Several studies have demonstrated that immunobiotics are able to improve the outcome of RV infection in human and livestock animals.

Effects of Immunobiotics in Humans

Lactobacillus rhamnosus GG is probably the most studied probiotic bacteria in the context of intestinal viral infections. Isolauri et al. (28) first described for more than 20 years, a protective effect of *L. rhamnosus* GG strain in RVs gastroenteritis in infants and children. In this study, the patients who received either a *L. rhamnosus* GG-fermented milk product or a *L. rhamnosus* GG freeze-dried powder after oral rehydration presented a significantly shorter duration of diarrhea when compared to the placebo group. Later, Majamaa et al. (29) conducted a study, in which 6- to 35-month-old children with RVs gastroenteritis received either *L. rhamnosus* GG, *Lactobacillus acidophilus* or a combination of *Streptococcus thermophilus* with *L. delbrückii* subsp. *bulgaricus* twice daily for 5 days. Only children who received *L. rhamnosus* GG had shorter diarrhea duration. The protective effect was related to augmented intestinal and serum IgA concentration, and a higher number of specific antibody-secreting cells to RVs. Additional studies showed that the consumption of *L. rhamnosus* GG is able to shorten the diarrheal phase in children suffering from RVs infection, an effect that was associated with increased concentrations of IgA antibodies as well (28–33). Furthermore, meta-analysis showed that the administration of *L. rhamnosus* GG to hospitalized children reduced the overall incidence of health care-associated diarrhea, including symptomatic RVs gastroenteritis (34). In spite of this evidence, it is important to notice that the shortening of diarrhea already at day 3 after probiotic treatment strongly suggests that the main therapeutic effect involves innate immune responses rather than the modulation of adaptive immunity (35).

Another example of probiotic treatment for alleviating RVs gastroenteritis was the use of *Lactobacillus reuteri*, which has been daily administered to hospitalized children with acute diarrhea for the length of hospitalization (up to 5 days). *L. reuteri* shortened both the duration of RVs diarrhea and the disease severity, when compared to the placebo group (36). *L. sporogenes* daily administered to newborns during 1 year, prevented the incidence and also diminished the duration of acute RVs diarrhea (37). Fang et al. (38) demonstrated that a minimal effective dose of *L. rhamnosus* significantly reduced fecal shedding RVs concentration in pediatric patients. Although the administration of lyophilized *Lactobacillus paracasei* strain ST11 daily for 5 days had a clinically significant benefit in the management of non-RVs-induced diarrhea, ST11 treatment against severe RVs diarrhea was ineffective (39). Children with RV infection who received milk-based formula supplemented with either *B. animalis* Bb12 alone or combined with *S. thermophilus* had fewer RVs infections (40).

In Argentina, mucosal infections such as bronchitis and diarrhea are the most common infectious diseases in children (41–43). In a randomized controlled trial conducted by Villena et al. (44), *L. rhamnosus* CRL1505 (administered in a yogurt formulation) improved mucosal immunity and reduced the incidence and severity of intestinal and respiratory infection in children. Hence, the incidence of infectious events was reduced from 66% in the placebo group to 34% in the group that received the probiotic yogurt. Furthermore, there was also a significant reduction in the occurrence of indicators of disease severity such as fever and the need for antibiotic treatment in children receiving the probiotic yogurt (44). Therefore, the results of this trial suggested that consumption of yogurt containing *L. rhamnosus* CRL1505 was helpful to reduce the burden of common childhood morbidities, especially those associated to viral infections including RVs (44).

Effects of Immunobiotics in Livestock Animals

Apart from the beneficial effects of immunobiotics on humans, some studies have evaluated their antiviral and anti-inflammatory activities in animals. Zhang et al. (45) reported that probiotic administration to gnotobiotic pigs challenged with RVs did not yielded differences in virus titers with respect to the placebo group. Nonetheless, LAB administration downregulated the recruitment of viral-activated monocytes/macrophages into the intestinal tract thereby limiting the inflammation induced by the virus (45).

In another study, it was shown that systemic monocyte/macrophage and APCs responses were modulated by immunobiotics in the context of a RV infection (45). Probiotic LAB induced strong TLR2-expressing APCs responses in blood and spleen, RVs induced a TLR3 response in spleen, and TLR9 responses were induced by RVs (as measured in immune cells isolated from spleen) and LAB (as determined in blood circulating immune cells). Immunobiotics and RVs had an additive effect on TLR2- and TLR9-expressing APCs responses, consistent with the adjuvant effect of LAB. Immunobiotics augmented IFN-γ and IL-4 levels in serum, but suppressed TLR3- and TLR9-expressing APCs responses in spleen and the serum IFN-α response induced by virulent RVs (46).

During RVs infections in weaned pigs, there is evidence of disruption of the barrier function as evidenced by the decreased villus height and crypt depth, lower levels of IgA, IL-4, and mucin 1 as well a reduced transcription of ZO-1, occludin, and Bcl-2 in jejunal mucosa (47). Some of these effects have been partially associated with alterations of transforming growth factor (TGF)-β production (48). Azevedo et al. (48) demonstrated that immunobiotic LAB further enhanced the Th1 and Th2 cytokine responses to RV infection as indicated by significantly higher concentrations of IL-12, IFN-γ, IL-4, and IL-10 in RVs-infected gnotobiotic pigs. LAB also helped to maintain immunological homeostasis during RV infection by regulating TGF-β production. It was also shown that treatment of pigs with *L. rhamnosus* GG modulated TGF-β and promoted the enhancement of

intestinal epithelial tight junctions, which may contribute to the preservation and restoration of the gut homeostasis after RV infection (11). Further evidence was reported by Maragkoudakis et al. (12) demonstrating that *Lactobacillus casei* Shirota and *L. rhamnosus* GG protected porcine and goat epithelial cells from RVs and other transmissible gastroenteritis viruses.

CELLULAR AND MOLECULAR MECHANISMS OF IMMUNOBIOTICS ACTIONS

The interactions of IECs with luminal antigens and with immune cells play a central role in determining the type of immune response triggered by intestinal microorganisms (5, 6). A critical and virtually universal early innate response of host cells to viral infection is the secretion of factors belonging to the IFN family. The secretion of IFN results in the expression of several ISGs products with antiviral activities.

We showed in different studies that the originally established porcine intestinal epithelial cell line (PIE cells) is a useful tool for studying IFN response triggered by TLR3, RIG-I, and/or MDA-5 activation. These cells are permissive to porcine RVs and also respond to dsRNA and its synthetic analog poly(I:C) (49, 50). Furthermore, co-cultures of PIE cells with immune cells isolated from porcine Peyer's patches (PPs) provide an *in vitro* system to study the transduction of the signal from its detection by IECs to the effect on the under laying immune cells.

The response of PIE cells to poly(I:C) challenge was evaluated, and it was found that MCP-1, IL-8, TNF-α, IL-6, and both IFN-α and IFN-β were upregulated in PIE cells after stimulation (49). We also showed that after stimulation of co-cultures with poly(I:C), there was an upregulation of IFN-α, IFN-β, IFN-γ, IL-2, and IL-12p40 in immune cells (49). TLR3 was the receptor involved in the recognition of the luminal stimulus and the responsible to trigger the expression and release of cytokines, which in turn activated the underlying APCs and effector lymphocytes.

Rotavirus infection stimulates IFN-β and early antiviral gene expression by a signaling pathway that requires MAVS, an adaptor protein that is recruited to signaling complexes following activation of RIG-I or MDA-5 (51, 52). In addition, both RIG-I and MDA-5 are involved in recognizing RVs infection, as proven by the reduction of IFN-β induction when these factors are lost (51, 52). Taking into account those facts, we evaluated the suitability of PIE cells and co-cultures as models for studying this signaling pathway after RVs infection. Our results showed that PIE cells have functional TLR3, RIG-I, and MDA-5 receptors, which signal *via* IRF3 and NF-κB, inducing IFN-β and the upregulation of the ISGs MxA and RNase L (50), which are important antiviral effectors of IFN pathway.

We used PIE cells for the screening of immunobiotic LAB strains taking into consideration their ability to enhance IFN-β production upon poly(I:C) stimulation (49, 53). Thus, *L. casei* MEP221106 was selected because of its potential to impact on viral intestinal infections. *L. casei* MEP221106 had the highest capacity to improve IFN-β production in poly(I:C)-challenged PIE cells. Moreover, *in vitro* co-culture experiments showed that *L. casei* MEP221106 was able to improve not only the production of IFN-β but also the levels of other cytokines including IFN-α, TNF-α, MPC-1, and IL-6. In co-cultures of PIE cells with immune cells, we demonstrated that *L. casei* MEP221106 improved the production of inflammatory and antiviral cytokines by PPs cells when compared with control cells (49).

The PIE system was also used to screen bifidobacteria strains with anti-RVs effect (50). *Bifidobacterium infantis* MCC12 and *Bifidobacterium breve* MCC1274 were selected in the screen because they significantly upregulated IFN-β in response to poly(I:C) challenge. In addition, both MCC12 and MCC1274 strains significantly increased PIE cells resistance to RV infection (**Figure 4**), while other strains with moderate or no effect in IFN-β production did not have any influence on RVs replication (50). As a result of the enhanced IFN-β levels, there was a concomitant upregulation of the ISGs MxA and RNase L. These effectors of antiviral immunity have different mechanisms of action: RNase L degrades dsRNA and the resulting RNA fragments activate RLRs to amplify IFN production and induce apoptosis on virus infected cells (54), while MxA hijacks newly synthesized viral proteins into perinuclear complexes. Then, the upregulation of MxA, RNase L, and probably other ISGs induced by MCC12 and MCC1274 strains through IFN-β would be related to the lower RVs replication found in bifidobacteria-treated PIE cells. This is supported by the fact that IFN-β is a key factor for improving defenses against RVs since viral replication is restricted in permissive cells when they are pretreated with IFN-β (55). Accordingly, IFN-β treatment of newborn calves and piglets prior to RV infection reduces virus replication and disease severity (56).

Several cytokines are induced *via* NF-κB signaling as a result of RVs infection, including IL-8, RANTES, GM-CSF, GRO-α, MIP-1β, and IP-10 (57), as observed in both cell lines and histological intestinal samples. Secreted cytokines initiate an important primary line of host defense, but if this response lasts too long or is dysregulated, it may lead to tissue damage and epithelial barrier dysfunction. In this regard, we have reported that efficient regulation of inflammatory response induced by immunobiotic bacteria is essential to achieve full protection against pathogens (58, 59). In line with this, we also showed that bifidobacteria strains MCC12 and MCC1274 differentially modulated the production of proinflammatory mediators in RVs-infected PIE cells (**Figure 4**) (50).

Toll-like receptor negative regulators play key roles in maintaining intestinal hemostasis by regulating TLR signaling. The zinc-finger protein A20, due to its deubiquitinase and ubiquitinase E3 ligase activities, is capable to terminate TLR signaling that results in inhibition of NF-κB activation and reduction of inflammatory induced cytotoxicity (60). Saitoh et al. (61) reported that IRF3 activation is suppressed by A20. The A20 protein is able to induce the suppression of the IFN-mediated immune response and IFN-promoter-dependent transcription by physically interacting with IKK-i/IKKε and inhibiting dimerization of IRF3 following engagement of TLR3 by dsRNA. Moreover, A20 knock down results in enhanced IRF3-dependent transcription triggered by the stimulation of TLR3 or virus infection. Human monocyte-derived dendritic cells (DCs) stimulated

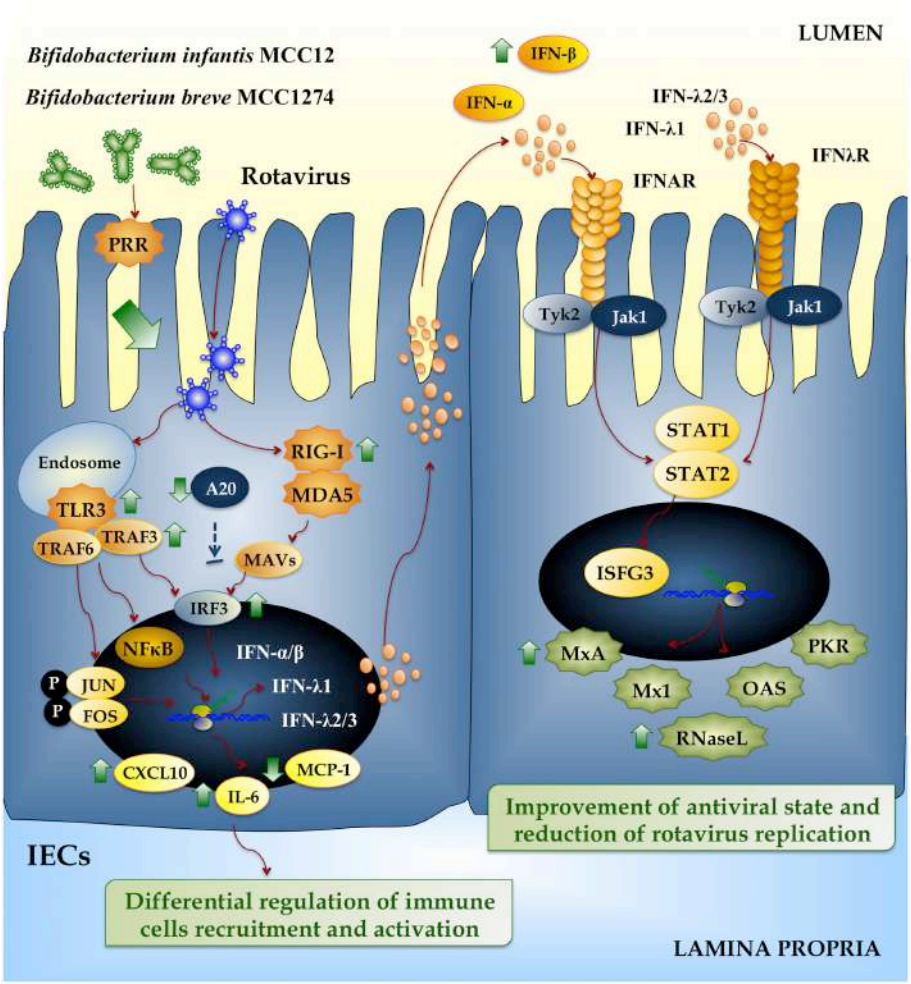

FIGURE 4 | Beneficial effects of immunobiotic bifidobacteria on the innate immune response against rotavirus in intestinal epithelial cells (IECs). Rotavirus double-strand genomic RNA activates toll-like receptor 3 (TLR3), retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), which are pattern recognition receptors (PRRs) expressed in IECs. Cellular signaling cascades mediated by interferon (IFN) regulatory factor-3 (IRF3) upregulate the expression of type I (IFN-α, IFN-β), and type III (IFNλ1, IFNλ2/3) IFN, which in turn induces the synthesis of IFN-stimulated genes with antiviral activities (MxA, Mx1, RNase L, OAS, PKR). Antiviral PRRs also activate nuclear factor κB (NF-κB) pathway and induce the secretion of proinflammatory cytokines and chemokines (IL-6, MCP-1, CXCL10). Preventive treatment of IECs with *Bifidobacterium infantis* MCC12 or *Bifidobacterium breve* MCC1274 reduce the expression of A20, increase the activation of IRF3, improve the production of the antiviral factors IFN-α, IFN-β, MxA, and RNase L, and differentially regulate the expression of IL-6, MCP-1, and CXCL10.

with poly(I:C) upregulate A20. When A20 is downregulated in DCs, they showed higher activation of NF-κB and AP-1, which resulted in increased and sustained production of IL-6, IL-10, and IL-12p70. Furthermore, DCs enhanced their T cell stimulatory capacity (62). Negative regulators involved in TLR signaling can be modulated by immunobiotic strains in human intestinal cell lines (63). In this regard, we also reported that both *B. infantis* MCC12 and *B. breve* MCC1274 significantly reduced the expression of A20 in RVs-infected PIE cells (**Figure 4**) (50), which is in line with the capacity of both strains to improve IRF3 activation and IFN-β production. In line with our findings, MacPherson et al. (64) also studied the effect of probiotics in the modulation of poly(I:C) induced inflammatory response in HT-29 cells. Stimulating HT29 cells with poly(I:C) alone increased the expression of A20, but the co-stimulation with poly(I:C) and probiotics significantly reduced A20 expression levels.

We also used these porcine *in vitro* systems to attain deeper knowledge into the mechanisms involved in the immunomodulatory effect of *L. rhamnosus* CRL1505 and concentrated our attention in the crosstalk between the immunobiotic strain and porcine IECs and APCs, in order to explain its capacity to reduce viral diarrhea episodes in children (44). Moreover, we performed comparative studies with another immunobiotic strain, *Lactobacillus plantarum* CRL1506, that is able stimulate intestinal immunity in animal models (65). Studies comparing the immunobiotic strains *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 in co-cultures of PIE cells and APCs, stimulated with poly(I:C), showed that both strains improved the production of type I IFNs in response

to poly(I:C) challenge (66). In addition, CRL1505 and CRL1506 strains modulated the expression of proinflammatory and regulatory cytokines and influenced activation and maturation of APCs (**Figure 5**). However, *L. rhamnosus* CRL1505 had a stronger effect both when applied alone or combined with a posterior poly(I:C) challenge. The improved Th1 response induced by immunobiotic lactobacilli was evidenced by the augmented expression of MHC-II, IL-1β, IL-6, and IFN-γ in DCs (66, 67). Those studies gave scientific basis for explaining the protection against intestinal viral infections achieved by *L. rhamnosus* CRL1505 in children.

The receptors, which are activated by the immunobiotics strains with antiviral capabilities MEP221106, MCC12, MCC1274, CRL1505, and CRL1506 strains in PIE cells to reduce A20, improve IRF-3 activation and increase IFN-β production remains to be uncovered. Bifidobacteria strains with a high capacity to stimulate TLR2 such as *B. longum* BB536 and *B. breve* M-16V were able to increase the expression of A20 in PIE cells and reduce TLR4-mediated inflammatory response (68, 69). On the contrary, strains with low capacity of stimulating TLR2 did not modify the expression of the ubiquitin-editing enzyme A20 in PIE cells challenged with TLR4 agonists. In our experiments, we were unable to block the increase of IFN-β induced by the lactobacilli and bifidobacteria by using anti-TLR2 or anti-TLR9 antibodies, suggesting that other receptor(s) are involved in the immunobiotic activity (66). Further studies are needed in order to find the PRRs involved in the recognition of lactobacilli and bifidobacteria leading to A20 and IFN-β modulation in PIE cells.

Recently, we confirmed *in vivo* the differential antiviral immunomodulatory activities triggered by *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 (65). Both strains increased the production of IFNs, the CRL1505 treatment being the most effective for increasing the levels of IFN-γ. Then, our results suggest that these two lactobacilli strains have potential to be used to improve the outcome of viral gastrointestinal disease. This is also supported by the human clinical trial demonstrating the capacity of *L. rhamnosus* CRL1505 to improve the infectious disease rates in children (70). Recently, Zhang et al. (71) proposed the activation of innate immunity with flagellin as a preventive and therapeutic strategy against RVs infection. They demonstrated that intraperitoneal flagellin injection reduced severity and shedding of RVs RNA in acute and chronic infected mice *via* TLR5/NLRC4 activation, which resulted in secretion of IL-22 and IL-18 by different effector cells. Although the mechanism of action of flagellin administration is substantially different to the

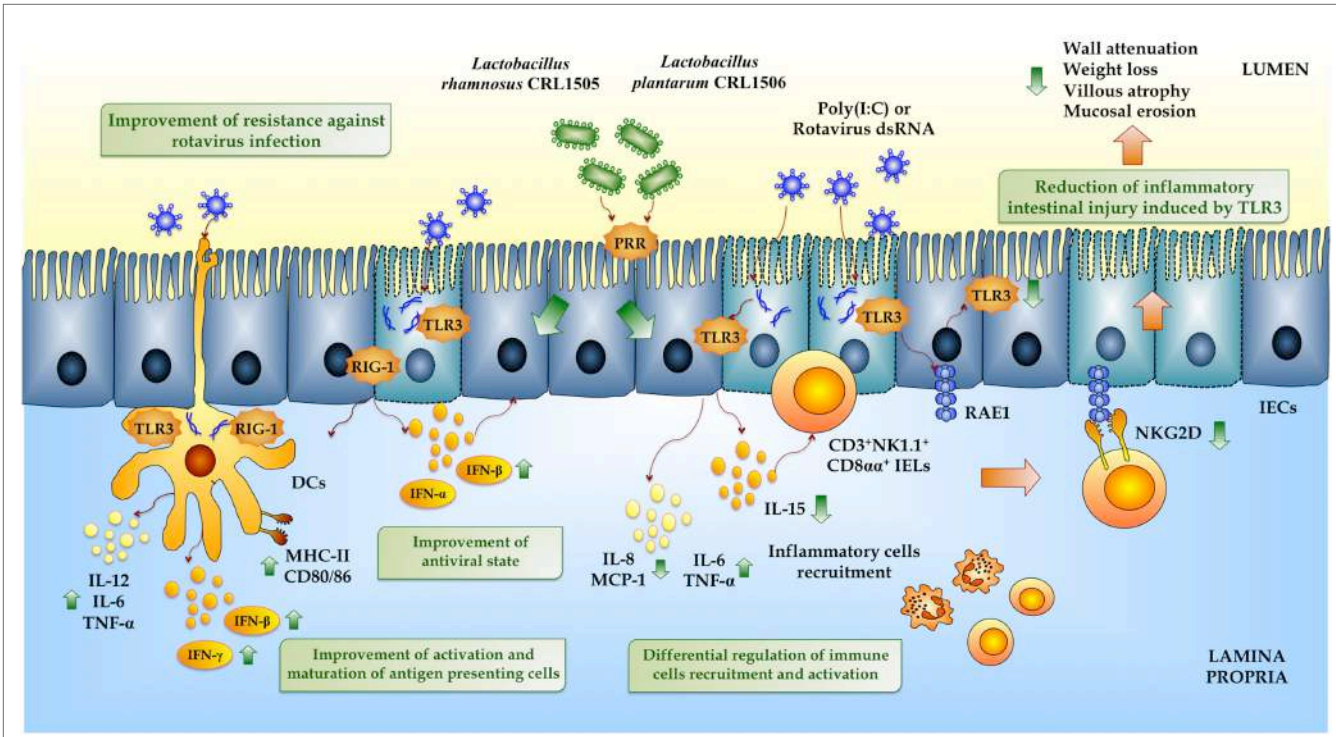

FIGURE 5 | **Beneficial effects of immunobiotic lactobacilli on the innate immune response against rotavirus in intestinal mucosa**. Rotavirus double-strand genomic RNA or poly(I:C) activate toll-like receptor 3 (TLR3), retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), which are pattern recognition receptors (PRRs) expressed in intestinal epithelial cells (IECs) and dendritic cells (DCs). Activation of antiviral PRRs increases the production of IFN-α, IFN-β, IFN-γ, and proinflammatory cytokines and chemokines (TNF-α, IL-6, IL-8, IL-12, MCP-1), which improves the antiviral state in IECs, induces the recruitment and activation of immune cells and the maturation of DCs. In addition, both purified rotavirus genomic dsRNA and poly(I:C) activate TLR3 in IECs increasing the expression of IL-15 and retinoic acid early inducible-1 (RAE1). IL-15 produced by IECs induces the recruitment of $CD3^+NK1.1^+CD8\alpha\alpha^+$ intraepithelial lymphocytes (IELs), which mediates epithelial destruction and mucosal injury by the NKG2D receptor expressed on these cells that is able to recognize RAE1. Preventive treatments with *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 improve the production of type I IFN and IFN-γ in the intestinal mucosa enhancing the antiviral state and differentially regulate the expression of inflammatory cytokines and chemokines reducing the intestinal damage, especially associated with the TLR3–IECs–IELs interaction.

mechanisms elicited by immunobiotics, both approaches rely on the principle of combating viral infection by enhancing innate immune defenses.

Lactobacillus rhamnosus CRL1505 and *L. plantarum* CRL1506 also reduced TLR3-induced small intestinal injury by regulation of proinflammatory cytokines production and IECs–IELs interaction (65) (**Figure 5**). IECs and IELs are the first line of defense against pathogens including viruses, and their interaction is essential for maintaining an appropriate immunological homeostasis. IECs produce a variety of cytokines and chemokines, including IL-6, IL-7, IL-8, IL-15, TNF-α, TGF-β, and GM-CSF. IL-15 functions as a mediator of TLR3-induced small intestinal injury (24). Abnormal TLR3 signaling results in elevated levels of IL-15, which regulates IECs apoptosis by activating perforin-mediated killing by $CD3^+NK1.1^+$ IELs (70). Moreover, IL-15 is able to enhance the cytotoxic activity of human IELs (72). Blocking the α receptor of IL-15 partially protected mice from poly(I:C)-induced small intestinal injury, including less villous atrophy, and mucosal erosion (24). Autologous ligands released by cells stress and infection are recognized by the NKG2D receptor in NK cells. NKG2D ligands expression is downregulated by gut microbiota, as demonstrated in germ-free mice, which had increased surface expression of these ligands (73). RAE1, a high affinity NKG2D ligand, which is minimally detected in normal cells, is upregulated upon TLR3 activation. In fact, blockade of NKG2D–RAE1 interaction avoids the cytotoxic effect of IELs on IECs and prevents acute small intestinal injury in mice challenged with dsRNA (27). Therefore, TLR3 signaling stimulates IECs to express IL-15 and RAE1 and induces $CD3^+NK1.1^+CD8\alpha\alpha^+$ IELs to express NKG2D through IEC-derived IL-15. In our hands, poly(I:C) treatment of mice increased intestinal injury in a IL-15- and $CD8\alpha\alpha^+NKG2D^+$-dependent manner (65). Poly(I:C) induced inflammatory-mediated intestinal tissue damage through the increase of $CD3^+NK1.1^+$ and $CD8\alpha\alpha^+NKG2D^+$ cells as well as proinflammatory mediators (TNF-α, IL-1β, IFN-γ, IL-15, RAE1, IL-8). Mice pretreated with immunobiotic lactobacilli before TLR3 activation responded with reduced levels of TNF-α, IL-15, RAE1, $CD3^+NK1.1^+$, $CD3^+CD8\alpha\alpha^+$, and $CD8\alpha\alpha^+NKG2D^+$ cells (**Figure 5**). The beneficial effect of these lactobacilli improved mice health as reflected by a significant reduction of body weight loss and intestinal tissue damage after poly(I:C) challenge (65).

It is well known that commensal bacteria in the gut are able to modulate IELs function. Furthermore, IELs are significantly reduced in germ-free mice (74, 75) underlying gut microbiota importance in the maintenance of IELs. These specialized lymphocytes are very important players in mucosal protection; they seem to occupy a unique temporal niche from which they are able to detect and limit bacterial penetration already in the first hours after pathogen attack (76). Ismail et al. (76) showed that IEL antibacterial response depends on bacterial stimulation in a MyD88-dependent signaling. Later, Jiang et al. (77) investigated the role of NOD2 signaling in the maintenance of IELs and found that NOD2 maintained IELs *via* recognition of gut microbiota. They demonstrated that stimulation of IEL requires activation of PRRs signaling in neighboring IECs (76, 77).

Therefore, it was shown that commensal bacteria establish a regulatory milieu in a healthy gut, with increased expression of immuno-inhibitory cytokines such as TGF-β and IL-10, which in turn downregulate NKG2D ligand surface expression (78, 79). This is in line with our findings for the immunomodulatory strains *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506, which reduced expression of RAE-1 and increased levels of intestinal IL-10. Whether the immunomodulatory effects of *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 are induced by direct action on the IECs (indirectly on IELs) and/or a direct effect on IELs is an open question, which we propose to address in the near future.

CONCLUSION

The detailed characterization of the cellular and molecular mechanisms underlying the intestinal innate defense against RV infection achieved in the past years has opened new ways for developing strategies to preventing and treating this viral induced diarrhea. In this sense, the use of immunobiotic bacteria to beneficially modulate IFN and inflammatory signaling pathways in IECs and immune cells is an attractive target for preventive or therapeutic intervention against RVs infection. Furthermore, the advances in the knowledge of the molecular crosstalk between immunobiotics and the gut innate immune system have provided light into the microorganism-sensing signals that allow these beneficial microorganisms to improve intestinal immune responses. This new molecular information might be helpful to improve the development of functional foods and/or pharmabiotics using immunobiotics aimed to reduce mortality and severity of RVs disease.

AUTHOR CONTRIBUTIONS

JV, MV-P, and HK designed, wrote, and revised the review article.

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REVIEW
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Unraveling the Differences between Gram-Positive and Gram-Negative Probiotics in Modulating Protective Immunity to Enteric Infections

*Sukumar Kandasamy, Anastasia N. Vlasova, David D. Fischer, Kuldeep S. Chattha†, Lulu Shao†, Anand Kumar†, Stephanie N. Langel, Abdul Rauf, Huang-Chi Huang, Gireesh Rajashekara and Linda J. Saif**

Food Animal Health Research Program (FAHRP), Veterinary Preventive Medicine Department, The Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH, USA

The role of intestinal microbiota and probiotics in prevention and treatment of infectious diseases, including diarrheal diseases in children and animal models, is increasingly recognized. Intestinal commensals play a major role in development of the immune system in neonates and in shaping host immune responses to pathogens. Lactobacilli spp. and *Escherichia coli* Nissle 1917 are two probiotics that are commonly used in children to treat various medical conditions including human rotavirus diarrhea and inflammatory bowel disease. Although the health benefits of probiotics have been confirmed, the specific effects of these established Gram-positive (G+) and Gram-negative (G–) probiotics in modulating immunity against pathogens and disease are largely undefined. In this review, we discuss the differences between G+ and G– probiotics/commensals in modulating the dynamics of selected infectious diseases and host immunity. These probiotics modulate the pathogenesis of infectious diseases and protective immunity against pathogens in a species- and strain-specific manner. Collectively, it appears that the selected G– probiotic is more effective than the various tested G+ probiotics in enhancing protective immunity against rotavirus in the gnotobiotic piglet model.

Keywords: rotavirus, probiotics, *Escherichia coli* Nissle, *Lactobacillus*, immunity, children, diarrhea, gnotobiotic piglet disease model

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Haruki Kitazawa, Tohoku University, Japan
Maria Guadalupe Vizoso Pinto, National University of Tucumán, Argentina

***Correspondence:**
Linda J. Saif
saif.2@osu.edu

†Present address:
Kuldeep S. Chattha, Canadian Food Inspection Agency, Lethbridge, AB, Canada;
Lulu Shao, University of Pittsburgh, Hillman Cancer Center, Pittsburgh, PA, USA;
Anand Kumar, Genomics and Systems Biology, Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA

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INTRODUCTION

Intestinal commensals constitute more than 1,000 species of bacteria. These commensals are involved in nutrient metabolism, development, and functioning of the gastrointestinal (GI) immune system and protection of the host from pathogens (1–3). Colonization of the GI tract is a gradual process in which *Escherichia coli* and other enterobacteria colonize the intestinal tract early after birth, followed by the subsequent establishment of anaerobes (4). The intestinal microbiota of children only becomes adult-like at 2–3 years of age (5). Perturbation of the intestinal microbiota, or dysbiosis, is associated with various diseases such as inflammatory bowel disease (6) and also affects the efficacy of various vaccines in children (7). Probiotics are widely used to restore gut homeostasis in various medical conditions in humans (8–10) and treat diarrheal diseases in children.

Diarrheal disease is one of the leading cause of deaths in children and it accounts for the death an estimated of 700,000 children annually worldwide (11). Specifically, rotavirus (RV) is a major cause of gastroenteritis in children worldwide. The protective efficacy of available RV vaccines is variable between regions and it is lowest in developing countries such as Southern Asia (50.0%) and sub-Saharan Africa (46.1%) (12). Additionally, lack of access to adequate health-care facilities to manage diarrhea is also associated with higher morbidity and mortality in children in low-income settings. Thus, enhancing vaccine efficacy, along with developing economical approaches to reduce the severity of RV diarrhea are effective strategies to ameliorate severe RV disease. Probiotics and intestinal commensals, crucial interacting partners of the gut immune system (13), are increasingly being considered for treatment of various enteric infections including human retrovirus (HRV) diarrhea (14), human norovirus gastroenteritis (15), antibiotic-associated diarrhea (16), and also to modulate protective antiviral immunity (17).

The beneficial effects of probiotics in reducing the severity of RV diarrhea and modulating viral immunity were observed in randomized clinical studies (18) and experimental studies in animal models (19) (**Table 1**). The Gram-positive (G+) *Lactobacillus* spp. were widely used to treat or prevent RV diarrhea in children. Specifically, prophylactic supplementation of *Lactobacillus rhamnosus* GG (LGG) to children significantly reduced the incidence of HRV disease (20). In our studies, gnotobiotic (Gn) piglets were used to study HRV pathogenesis due to their susceptibility to HRV infection and also the greater anatomic and physiological and immunological similarities between pigs and humans. Dual colonization of Gn piglets with G + LGG and *Bifidobacterium lactis* Bb12 resulted in a significant reduction in both fecal HRV shedding titers and diarrhea severity (21). Further, *Lactobacillus* strains have significant effects in reducing diarrhea severity in children affected by enteric diseases (22).

The effectiveness of probiotics in preventing or treating a disease is dependent on several factors such as class or strains of probiotics, the dosage of probiotics, and heterogeneity of study subjects (40, 41). Several past studies showed strain-specific differences of probiotics in modulating host immune responses (42). Thus, comparative analysis of the health benefits of different classes of probiotics is essential to tailor an effective regimen of probiotic treatment for a disease condition. Specifically variations in microbe-associated molecular patterns between G+ and Gram-negative (G−) bacteria have been attributed to differential induction of innate immunity in a host (43, 44). However, limited studies have been conducted to decipher if differences exist between G+ and G− probiotics in modulating host responses to infectious diseases. In our recent studies (31, 32), we compared the beneficial effects of G+ and G− probiotics in modulating virulent HRV infection as well as host immunity. Specifically, LGG was selected as a G+ probiotic because of its well-documented effects in reducing the severity of RV diarrhea in children (24). For the G− probiotic, we selected *Escherichia coli* Nissle 1917 (EcN) due to its proven effects in attenuating inflammatory disorders and modulating immunity in humans (45). In this review, we focused on the comparisons of the health benefits of G+ and G− probiotics in modulating microbial infections and immunity.

EFFECTS OF G+ VERSUS G− PROBIOTICS ON ENTERIC INFECTIONS AND DIARRHEA

Probiotics have been successfully used to prevent or treat enteric infections in children and animals (**Table 1**). One notable finding is the difference between G+ and G− probiotics in modulating host immunity against microbial diseases. In one study (31), the comparative efficacy of LGG and EcN probiotics in ameliorating HRV disease was assessed in Gn piglets. The EcN colonized piglets had reduced diarrhea severity and also lower mean peak virus shedding titers compared with LGG or uncolonized piglets post-virulent human RV (VirHRV) challenge (31, 32). Both EcN and LGG showed similar colonization patterns as indicated by comparable fecal shedding of each bacterium and also detection of similar levels of each probiotic bacteria in various sections of GI tract. Similarly, EcN supplementation to children with enteric infections resulted in reduced duration of diarrhea (26). Further, supplementation of EcN to infants for the first 5 days immediately after birth resulted in persistence of the probiotic for 6 months as indicated by fecal shedding of EcN (29). Similar to the higher beneficial effects of EcN than LGG on ameliorating HRV infection, higher protective effects against *Salmonella* were observed in EcN compared with *Bifidobacterium choerinum*-supplemented Gn piglets (30). The higher protective effect of EcN against *Salmonella* was associated with increased expression of ZO-1 and occludin in ileal epithelial cells and decreased inflammatory TNF-α cytokine levels in the EcN colonized Gn piglets (30). Consistent with these findings, higher TNF-α levels were induced by G+ commensals as compared with G− commensals using *in vitro* mononuclear cultures (43). EcN supplementation also attenuated lipopolysaccharides (LPS) or trinitrobenzene sulfonic acid-induced inflammatory conditions in a mouse model (46). In summary, the higher ability of G− compared with G+ probiotics in reducing the levels of inflammatory mediators during enteric infections may be major contributing factor in reducing diarrhea severity.

G+ and G− Probiotic Impacts on Modulation of B Cell Responses

Microbial colonization of the GI tract has a significant effect on the maturation of neonatal immune system (47). Consistent with this observation, administration of EcN enhanced serum EcN-specific IgA antibody and polyclonal IgM antibody responses in infants as compared with placebo group (28). Also, mono EcN or dual EcN + LGG colonization significantly increased serum total IgA and IgG responses compared with LGG colonized or uncolonized piglets (31) (**Figure 1**). Similar to systemic immunoglobulin responses, EcN colonization resulted in higher small intestinal total IgA responses compared with LGG colonization in Gn pigs. Thus, EcN had more potent immunostimulatory effects than LGG in terms of inducing mucosal and systemic B cell responses. The underlying mechanism for differential induction of antibody responses by G+ and G− bacteria might be due to variation in IgA inducing factors such as IL-10 cytokine. In fact, G−, but not G+ probiotics, induced higher IL-10 responses in

TABLE 1 | Effects of G+ and G− probiotics on diarrheal diseases and immunity in children and animal models.

Gram-positive probiotic/ commensal bacteria	Gram-negative probiotic/ commensal bacteria	Humans/ animal model/*in vitro* study	Indication	Conclusion(s)	Reference
L. rhamnosus GG (6×10^9 CFU/dose)	None	Children	Prophylaxis against diarrheal diseases	Significant reduction in incidence of HRV disease in LGG-supplemented group	(20)
L. rhamnosus GG ($10^{10–11}$ CFU)	None	Children	Effect of LGG on immune responses to HRV in children	LGG significantly enhanced RV-specific IgA antibody responses	(23)
L. rhamnosus GG (10^{10} CFU)	None	Children	Treating diarrhea	Reduced duration of diarrhea	(24)
Lactobacillus paracasei strain ST11 (10^{10} CFU)	None	Children	Treating diarrhea	Reduced severity of non-rotavirus induced diarrhea but no effect on rotavirus diarrhea	(25)
None	*Escherichia coli* Nissle 1917 (EcN) (10^8 CFU)	Children	Treat acute diarrhea in children	Reduced duration of diarrhea by 2.3 days	(26)
None	EcN (3×10^8 CFU)	Infants	To assess effects on total IgA responses in infants	Increased serum and stool IgA responses	(27)
None	EcN (10^8 CFU)	Infants	Assess impact on cellular and humoral immunity in infants	Probiotic increased both cellular proliferative and serum total IgA responses	(28)
None	EcN (10^8 CFU)	Infants	Prophylactic administration against bacterial pathogens	Significant reduction in bacterial pathogens in fecal samples	(29)
Bifidobacterium choerinum (5×10^8 CFU/ml)	EcN (5×10^8 CFU/ml)	Gn piglets	Protection against *Salmonella enterica* serovar Typhimurium infection	EcN conferred higher protection against disease than *Bifidobacterium choerinum*	(30)
L. rhamnosus GG (10^5 CFU/ml)	EcN (10^5 CFU/ml)	Gn piglets	Compare G+ and G− bacteria effect on HRV infection and immunity	EcN was more effective than LGG in ameliorating HRV disease and enhancing total IgA and NK cell responses	(31, 32)
L. rhamnosus GG (10^5 CFU/ml), *Bifidobacterium lactis* Bb12 (105 CFU/ml)	None	Gn piglets	To study effects on HRV disease	Reduced fecal virus shedding and diarrhea severity in probiotic colonized piglets	(21)
Enterococcus faecium NCIMB 10415 ($4.2–4.3 \times 10^6$/g CFU)	None	Sows and their offspring	Effect on fecal shedding of enteric viruses	Reduced fecal shedding of rotavirus and increased rotavirus specific IgA responses. No effect on hepatitis E virus, encephalomyocarditis virus, and norovirus shedding in feces	(33)
None	EcN (10^{10} CFU/ml)	Pigs	To prevent enterotoxigenic *Escherichia coli* induced diarrhea	Ameliorated clinical signs of diarrhea	(34)
None	EcN (10^8 CFU/ml)	Neonatal calf	Prevention and treatment of diarrhea	Reduction in incidence of diarrheal diseases in prophylactic group. Ameliorated severity of diarrhea in calves with enteric diseases	(35)
Lactobacillus acidophilus A9 (10^8/ml CFU)	*Escherichia coli* 13-7 (10^6/ml CFU)	Mice	Compare G+ and G− bacteria effect on cytokine responses in mice	*E. coli* 13-7 induced higher IL-12 cytokine compared to *L. acidophilus* A9	(36)
None	EcN ($1.5–2 \times 10^8$ CFU)	Mice	Assess impact on intestinal barrier function in acute dextran sodium sulfate-induced colitis	Strengthened intestinal barrier function	(37)
Lactobacillus casei Shirota	EcN	*In vitro*	Investigate effects on innate immunity	Higher IL-10 and IL-12 induction by EcN than *L. casei* Shirota	(38)
L. plantarum, L. rhamnosus, L. paracasei ssp. paracasei	*Escherichia coli* O6:K13:H1, *Escherichia coli* MS101	*In vitro*	Compare G+ and G− bacteria effect on cytokine responses of monocytes	Lactobacilli-induced higher level of IL-12	(39)

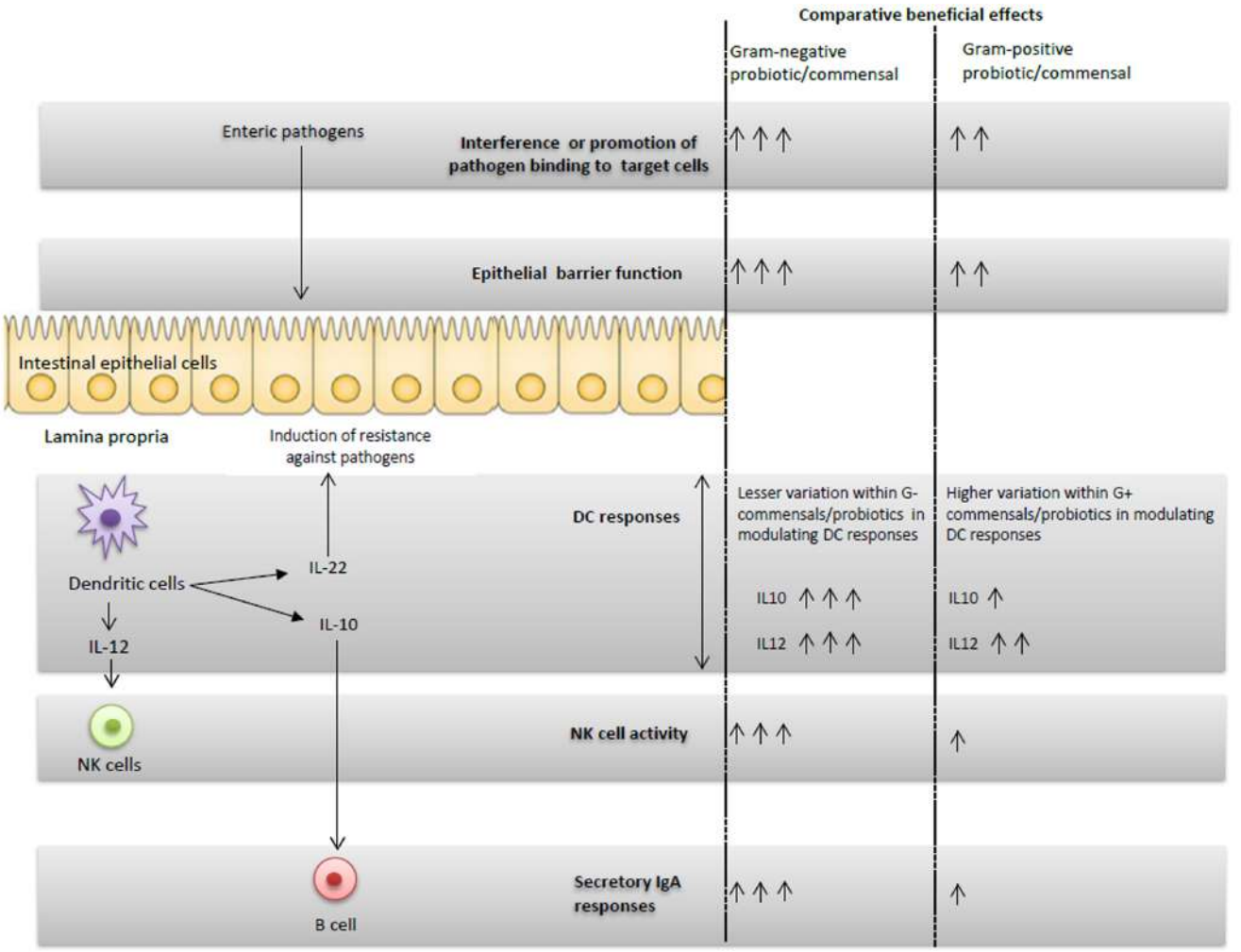

FIGURE 1 | **Schematic representation of the G+ and G− probiotics-induced immunomodulatory effects and proposed potential immune interactions**.

prior studies (31, 48, 49). IL-10 is one of the cytokines that mediates the induction of IgA antibody responses at mucosal sites through enhancing antibody class switching (50). Differences in the microbe-associated molecular patterns between the probiotics might be a potential reason for the differential induction of IL-10 by G+ and G− bacteria. Indeed, both the LPS portion of EcN and whole EcN lysate were identified as potent inducers of IL-10 production in peripheral blood mononuclear cells (51). Further, induction of total IgA responses is at least partially mediated by IL-10 *in vitro* (31). These studies demonstrate that modulation of the cytokine milieu, such as enhanced IL-10 levels, might be a potential mechanism to account for the higher antibody responses observed in G− compared with G+ probiotics groups.

It is also well established that strain-dependent variations in immunomodulatory properties are observed within G+ probiotics (52). Thus, individual probiotic strains within G+ or G− probiotic classes may differ in modulating antibody responses. Consequently, screening of the beneficial effects of individual probiotics is essential to elucidate their impacts on antibody responses.

Impact of Innate Immunity on IgA Responses to G+ versus G− Probiotics

Innate immunity plays an integral role in priming the adaptive immune responses. Thus, probiotics may induce specific changes in innate immunity that may be involved in synergistically enhancing IgA responses. Dual colonization of a G− and G+ probiotic enhanced serum total IgA responses in Gn piglets compared with mono-colonization of the probiotics (31). Thus, G+ and G− bacteria synergistically enhanced the systemic total IgA responses. In fact, combinations of G+ and G− probiotics had additive effects on induction of maturation markers in DCs as well as levels of IL-10 cytokines (53). Thus, considering the known function of DCs in induction of IgA responses (54, 55), the positive effects of combinations of G+ and G− bacteria in modulating DCs may play a role in enhancing IgA responses. Additionally, a previous

study (56) also showed that LPS, a TLR4 ligand, synergistically interacted with TLR1/2 ligands which in turn enhanced class-switch recombination in B cells. Thus, synergistic interactions of microbe-associated molecular patterns from G− and G+ probiotics might also play a role in enhancing antibody responses. Apart from DCs, it appears that intestinal epithelial cells also respond differently in terms of producing IgA mediators such as TGF-β and thymic stromal lymphopoietin (TSLP). Specifically, G− commensals induce higher production of TGF-β and TSLP as compared with G+ commensals (57). Further, higher frequencies of splenic $TLR9^+$ mononuclear cells were detected in EcN + LGG colonized compared with the monocolonized EcN or LGG Gn piglets (32). TLR9 recognizes CpG DNA and LGG has a high GC percentage in its genomic DNA (58). Thus, we speculate that higher systemic TLR9 expression in EcN + LGG compared with EcN or LGG monocolonized piglets might be a contributing factor in enhancing immunoglobulin responses as reported in several earlier studies (59, 60).

One unanswered question is the involvement of total IgA levels in modulating immunopathology during microbial infections. Previous studies have shown the involvement of IgA in moderating inflammatory responses through modulating dendritic cells and regulatory T cell functions (61, 62). Further, secretory IgA-commensal complexes were shown to reduce inflammatory responses in intestinal epithelial cells (63). Thus, the role of secretory IgA in mitigating infection-induced inflammatory responses is intriguing and requires further investigation.

Differential Effects of G+ versus G− Probiotics in Modulating Innate Immunity

Probiotics may elicit their beneficial effects against pathogens through modulating innate immunity. A role for innate immunity in mediating host defenses against enteric diseases including RV infection has been elucidated in recent studies (64–67). Specifically, functions of dendritic cells are modulated by various probiotics. It appears that DC populations in the intestine can be modulated by intestinal commensals. This concept is supported by results of an investigation in which depletion of intestinal microbiota resulted in a reduction in DCs numbers in mucosal compartments as well as impaired resistance against influenza virus infection in mice (68). Additionally, G− commensal bacteria have higher immunostimulatory effects on DCs as compared with G+ commensals (69). For example, G− EcN increased frequencies of total plasmacytoid dendritic cells (pDCs) and activated pDCs, more than the G+ LGG probiotic in Gn piglets (32). Also, G− commensals were highly potent in the induction of maturation markers in DCs as compared with G+ commensals (53). Importantly, greater variation was observed among G+ commensals in modulating DC responses, compared with less variation among G− commensals (53). Thus, the distinct ability of G− bacteria such as EcN in modulating frequencies and functions of DCs may have beneficial impacts on induction of protective immunity against pathogens.

In our recently published study (32), we observed higher NK cytotoxic function and increased frequencies of pDCs in EcN colonized compared with LGG colonized or uncolonized piglets. The enhanced NK cell activity coincided with higher serum IL-12 levels *in vivo* in EcN colonized piglets (**Figure 1**) and also DC production of IL-12 *in vitro* (32). Similar to our studies, treatment of murine bone marrow-derived DCs (BMDCs) with EcN resulted in induction of IL-12 and IL-10 cytokines and induction of activation markers in BMDCs (70). In the same study, EcN administration reduced the development of allergen-specific Th2 responses (70). Thus, our results showed that NK cell function can be modulated by probiotics, and more importantly, only G− EcN but not G+ LGG, enhanced NK cell function. These findings were further corroborated by an earlier study in which the germ-free condition impaired the priming of NK cell function by microbial ligands (71). Further, the reduced NK cell function in microbiota-depleted mice was correlated with higher mouse cytomegalovirus titers post-viral challenge (71). A recent study (72) also showed the potential role of the outer membrane vesicles from EcN in induction of IL-22 cytokine responses. IL-22, along with IFN-λ, has been shown to effectively reduce RV replication in a mouse model (66). These results underscore not only the importance of intestinal commensals in regulating innate immunity against viral infections, but also the differential abilities of distinct known G+ or G− probiotics in regulating innate immune cells.

Interactions between Commensals and Viruses That Alter Their Pathogenesis

Direct interactions between viruses and bacteria are being increasingly investigated in recent studies (73–75). Specifically, direct binding of commensal microbiota is associated with either increased or decreased viral infections (76). The ability of mouse mammary tumor virus to bind with LPS was associated with increased virus pathogenicity (77). Similarly, poliovirus stability and viral attachment to target host cells were also enhanced by interaction with bacterial LPS or peptidoglycan (78). Further, EcN binds to HRV *ex vivo* but no such interaction was found between LGG and HRV (31). Also, in this study, prior treatment of epithelial cells with EcN, but not LGG, resulted in a significant reduction in the epithelial attachment of HRV *in vitro*. Further studies are required to elucidate the potential role of physical interactions between EcN and viruses in terms of altering the course of viral infection and pathogenicity. Expression of histo-blood group antigens (HBGA) was observed in some G− intestinal commensal bacteria (79) and certain of those HBGA-expressing bacteria were shown to enhance (73) enteric viral infection. Considering the direct interactions between the commensals and pathogens, any disturbances in microbiota compositions may lead to altered susceptibility or resistance to a particular enteric pathogen. Thus, further studies are required to assess whether any difference exists between G+ and G− bacteria in binding properties with various enteric viruses and the impact on the course of viral pathogenicity.

CONCLUSION

Comparison of the beneficial effects of G+ and G− probiotics and intestinal commensals indicated that the selected G− probiotic

had higher beneficial effects in inducing protective immunity against enteric pathogens such as HRV as compared with the selected G+ probiotics in humans and animal models. In our simplified *in vivo* Gn piglet model system, it appears that the induced beneficial effects of G− EcN against HRV disease may be accomplished by the integrated interaction of DCs, NK cells, and immunoglobulins as well as direct binding of EcN to virus (**Figure 1**). Most of the initial studies showed that G− probiotics have higher immunostimulatory effects and better protective effects against HRV as compared with G+ probiotics. It remains to be determined whether these findings can be generalized to all G− commensals. Further, the potential ability of different G+ and G− probiotics to alter the composition as well as functionalities of the intestinal microbiota, and the consequences of these changes on microbial infections and vaccines is unclear. Identification of the essential components of probiotics that induce the beneficial effects against pathogens may also be useful in identifying probiotics or their products as novel adjuvants for vaccines.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial direct contribution to the work.

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Transcriptomic Analysis of the Innate Antiviral Immune Response in Porcine Intestinal Epithelial Cells: Influence of Immunobiotic Lactobacilli

Leonardo Albarracin[1,2,3†], Hisakazu Kobayashi[3,4†], Hikaru Iida[3,4], Nana Sato[3,4], Tomonori Nochi[5,6], Hisashi Aso[5,6], Susana Salva[1,2], Susana Alvarez[1,2], Haruki Kitazawa[3,4] and Julio Villena[1,2,3]**

[1] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [2] Immunobiotics Research Group, Tucuman, Argentina, [3] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [4] Livestock Immunology Unit, International Education and Research Center for Food Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [5] Cell Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [6] Infection Immunology Unit, International Education and Research Center for Food Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

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Edited by:
Juarez Antonio Simões Quaresma, Federal University of Pará, Brazil

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Kieran G. Meade, Teagasc – The Irish Agriculture and Food Development Authority, Ireland
Haitao Guo, University of North Carolina at Chapel Hill, USA

***Correspondence:**
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp;
Julio Villena
jcvillena@cerela.org.ar

†These authors have contributed equally to this work.

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Lactobacillus rhamnosus CRL1505 and *Lactobacillus plantarum* CRL1506 are immunobiotic strains able to increase protection against viral intestinal infections as demonstrated in animal models and humans. To gain insight into the host–immunobiotic interaction, the transcriptomic response of porcine intestinal epithelial (PIE) cells to the challenge with viral molecular associated pattern poly(I:C) and the changes in the transcriptomic profile induced by the immunobiotics strains CRL1505 and CRL1506 were investigated in this work. By using microarray technology and reverse transcription PCR, we obtained a global overview of the immune genes involved in the innate antiviral immune response in PIE cells. Stimulation of PIE cells with poly(I:C) significantly increased the expression of *IFN*-α and *IFN*-β, several interferon-stimulated genes, cytokines, chemokines, adhesion molecules, and genes involved in prostaglandin biosynthesis. It was also determined that lactobacilli differently modulated immune gene expression in poly(I:C)-challenged PIE cells. Most notable changes were found in antiviral factors (*IFN*-α, *IFN*-β, *NPLR3*, *OAS1*, *OASL*, *MX2*, and *RNASEL*) and cytokines/chemokines (*IL-1*β, *IL-6*, *CCL4*, *CCL5*, and *CXCL10*) that were significantly increased in lactobacilli-treated PIE cells. Immunobiotics reduced the expression of *IL-15* and *RAE1* genes that mediate poly(I:C) inflammatory damage. In addition, lactobacilli treatments increased the expression *PLA2G4A*, *PTGES*, and *PTGS2* that are involved in prostaglandin E2 biosynthesis. *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 showed quantitative and qualitative differences in their capacities to modulate the innate antiviral immune response in PIE cells, which would explain the higher capacity of the CRL1505 strain when compared to CRL1506 to protect against viral infection and inflammatory damage *in vivo*. These results provided valuable information for the deeper understanding of the host–immunobiotic interaction and their

effect on antiviral immunity. The comprehensive transcriptomic analyses successfully identified a group of genes (*IFN*-β, *RIG1*, *RNASEL*, *MX2*, *A20*, *IL27*, *CXCL5*, *CCL4*, *PTGES*, and *PTGER4*), which can be used as prospective biomarkers for the screening of new antiviral immunobiotics in PIE cells and for the development of novel functional food and feeds, which may help to prevent viral infections.

Keywords: intestinal epithelial cells, immunotranscriptomic response, TLR3, *Lactobacillus rhamnosus* CRL1505, *Lactobacillus plantarum* CRL1506, antiviral response

INTRODUCTION

In the past decade, research has demonstrated that beneficial microbes with the capacity to modulate the mucosal immune system (immunobiotics) are a potential alternative to enhance resistance against viral infections. Immunobiotic lactic acid bacteria (LAB) are able to provide protection against viral infections by modulating innate and adaptive antiviral immunity. Several reports have shown that immunobiotic LAB improve protection against enteric viral infections and shorten the duration of diarrhea, reduce the number of episodes, diminish virus shedding, normalize gut permeability, and increase the production of virus-specific antibodies (1–3). Moreover, it was demonstrated that some immunobiotic strains, when orally administered, are able to increase respiratory defenses and reduce the susceptibility to respiratory viral infections improving virus clearance and diminishing inflammatory-mediated lung tissue damage (4–7).

In developing countries, viral mucosal infections such as bronchitis and diarrhea are the most common infectious diseases in children (8–10). The use of immunobiotics to improve the outcome of those viral infections has been proposed. In this regard, in a randomized controlled trial conducted by Villena et al. (4), the immunobiotic strain *Lactobacillus rhamnosus* CRL1505 (administered in a yogurt formulation) improved mucosal immunity and reduced the incidence and severity of intestinal and respiratory infection in children. The incidence of infectious events was reduced from 66% in the placebo group to 34% in the group that received the probiotic yogurt. Furthermore, there was also a significant reduction in the occurrence of indicators of disease severity such as fever and the need for antibiotic treatment in children receiving the probiotic yogurt (4). Studies in mice models have proved that orally administered *L. rhamnosus* CRL1505 improves antiviral immune responses in the intestinal mucosa (local effect) (5, 11) and the respiratory tract (distal effect) (6, 7). Of interest, it was demonstrated that these immunomodulatory capacities are strain specific since other immunobiotic strains such as *Lactobacillus plantarum* CRL1506 exert only local affects after oral administration (5–7, 11).

The interactions of intestinal epithelial cells (IECs) with luminal antigens and immune cells play a central role in determining the type of immune response triggered by microorganisms in the intestinal mucosa (12, 13). Therefore, by using a previously established porcine intestinal epithelial (PIE) cells that is able to respond to the dsRNA synthetic analog poly(I:C) and are permissive to rotavirus (14, 15), we aimed to evaluate the similarities and differences in the innate antiviral immune response induced by *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506. We hypothesized that transcriptomic analyses using microarray technology in PIE cells could provide valuable information to gain insights in the mechanisms involved in the capacity of immunobiotics to modulate the innate antiviral immune response in the gastrointestinal tract and could provide some clues about their ability to stimulate immunity in distal mucosal sites such as the respiratory tract. Therefore, the aim of this study was to investigate the transcriptomic response of PIE cells to the challenge with viral molecular associated pattern poly(I:C) and the changes in that immunotranscriptomic profiles induced by the immunobiotics strains with antiviral capabilities *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506. We obtained a global overview of the immune genes involved in the innate antiviral immune response in PIE cells that include type I interferons (IFNs), several IFN-stimulated genes (ISGs), cytokines, chemokines, adhesion molecules, and genes involved in prostaglandin biosynthesis. It was also determined that lactobacilli differently modulated immune gene expression in poly(I:C)-challenged PIE cells by increasing the expression of antiviral factors and cytokines/chemokines and reducing genes involved in poly(I:C)-mediated inflammatory damage. Moreover, the study allowed us to identify a group of genes that could be used as biomarkers for the screening of new antiviral immunobiotics in PIE cells.

MATERIALS AND METHODS

PIE Cells

PIE cells are intestinal non-transformed cultured cells originally derived from intestinal epithelia isolated from an unsuckled neonatal swine (16). When PIE cells are cultured, they assume a monolayer with a cobblestone and epithelial-like morphology and with close contact between cells (14, 16, 17). PIE cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 100 U/ml streptomycin, and 100 mg/ml penicillin at 37°C in an atmosphere of 5% CO_2. PIE cells grow rapidly and are well adapted to culture conditions even without transformation or immortalization (17–19).

Microorganisms

Lactobacillus rhamnosus CRL1505 and *L. plantarum* CRL1506 belong to CERELA Culture Collection and were originally isolated from goat milk (19). These strains were grown in Man-Rogosa-Sharpe broth at 37°C. For immunomodulatory assays, overnight

cultures were harvested by centrifugation, washed three times with sterile PBS, counted in a Petroff-Hausser counting chamber, and resuspended in DMEM until use.

Immunomodulatory Effect of Lactobacilli in PIE Cells

Evaluation of the immunomodulatory activity of *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 was performed using PIE cells as described previously (19). PIE cells were seeded at 3×10^4 cells per well in 12-well type I collagen-coated plates (Sumitomo Bakelite Co., Tokyo, Japan) and cultured for 3 days. After changing medium, lactobacilli (5×10^8 cells/ml) were added, and 48 h later, each well was washed vigorously with medium at least three times to eliminate all stimulants. Then cells were stimulated with poly(I:C) (60 μg/ml) for 3, 6, 12, or 24 h for reverse transcription (RT)-PCR studies or for 12 h for microarray studies.

Microarray Analysis

Total RNA was isolated from lactobacilli-treated and control PIE cells using PureLink RNA Mini Kit (Life Technologies Inc., Gaithersburg, MD, USA) and treated with DNase. RNA integrity of all samples were evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), using the RNA 6000 Nano Kit (20). Complementary DNA synthesis was performed using 200 ng of RNA. Hybridization with Porcine (V2) Gene Expression Microarray (Agilent Technologies) was performed at Hokkaido System Science Co. Scanning and digitization of Microarray were done by Agilent Technologies Microarray Scanner and Agilent Feature Extraction 10.7.3.1, respectively.

Data normalization and expression analysis were performed using GeneSpring software version 13.1 (Agilent Technologies). Significant genes up and downregulated in test samples [those stimulated with poly(I:C) or lactobacilli plus poly(I:C)] with respect to control samples [without poly(I:C) stimulation] were identified. Genes with significant changes in transcript abundance were selected on the basis of two criteria: a *t*-test *P* value of less than 0.05, which was considered statistically significant, and a cutoff in transcript abundance of at least twofold. Statistical analysis was conducted using the Limma package from BioConductor in R software (version 3.2.5). Results were expressed as log2 scale (log2 ratio). Genes whose expressions were log2 > 1 and $P < 0.05$ were annotated using PANTHER 11.1 (pantherdb.org). Genes were further analyzed according to Gene Ontology (GO) classification. Microarray data were submitted to NCBI-GEO under the accession number GSE93225.

Quantitative Expression Analysis by Two-Step Real-time Quantitative PCR (qPCR)

Two-step real-time qPCR was performed to characterize the expression of selected genes in PIE cells. Total RNA was isolated from each PIE cell sample using TRIzol reagent (Invitrogen). All cDNAs were synthesized using a Quantitect RT kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time qPCR was carried out using a 7300 real-time PCR system (Applied Biosystems, Warrington, UK) and the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase with 6-carboxyl-X-rhodamine (Invitrogen). The primers used in this study were described before (19, 20). The PCR cycling conditions were 2 min at 50°C, followed by 2 min at 95°C, and then 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The reaction mixtures contained 5 μl of sample cDNA and 15 μl of master mix, which included the sense and antisense primers. According to the minimum information for publication of quantitative real-time PCR experiments guidelines, β-actin was used as a housekeeping gene because of its high stability across porcine various tissues (14, 15, 20). Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples. The quality of the RNA in all experiments was checked by Agilent 2100 Bioanalyzer, and all samples were determined to be suitable for the qPCR assay considering values of A260/A280 and A260/A230 over 2.0 and the RIN value over 9.0.

Statistical Analysis

Statistical analyses were performed using GLM and REG procedures available in the SAS computer program (SAS, 1994). Comparisons between mean values were carried out using one-way ANOVA and Fisher's least significant difference test. For these analyses, *P* values <0.05 were considered significant.

RESULTS

Immunotranscriptomic Changes in PIE Cells after Poly(I:C) Challenge

The transcriptomic response of PIE cells to the challenge with poly(I:C) was first investigated. Microarray analysis was performed in PIE cells 12 h after the stimulation with poly(I:C). When these cells were compared with unchallenged PIE cells, it was found that there were 5,140 transcripts (representing 1,178 unique genes) and 3,359 transcripts (representing 788 unique genes) upregulated and downregulated, respectively (**Figures 1A,B**).

Of these differentially regulated genes, 165 were assigned to immune-related functions according to GO database (**Figures 1C,D**; Table S1 in Supplementary Material). Changes in the immunotranscriptome response in PIE cells after poly(I:C) stimulation included genes in the following GO Biological Process pathways: "immune system process," "regulation of defense response," "cell adhesion," "innate immune response," "regulation of viral process," "cellular response to interferon-gamma," and several pathways related to immune cells migration and chemotaxis (**Figure 1C**).

The most remarkable changes in PIE cells after stimulation with poly(I:C) were found in expression type I IFNs and antiviral factors, cytokines, chemokines, adhesion molecules, and prostaglandins.

A significant increase in the expression of *IFN*-β and *IFN*-α was observed in poly(I:C)-challenged PIE cells with fold changes (log_2 ratio) of 4.3 and 3.5, respectively (Table S1 in Supplementary Material). Increased expression of the IFN-induced antiviral factors *OAS1* (11.2), *OASL* (10.7), *IFIT1* (9.9), *IFIT3* (9.1), *IFIT2* (8.3), *MX1* (7.9), *MX2* (6.3), *OAS2* (6.3), *IFIT5* (3.0), *RNASEL* (2.2), and *RNASE4* (1.9) was also observed. In addition, a significant

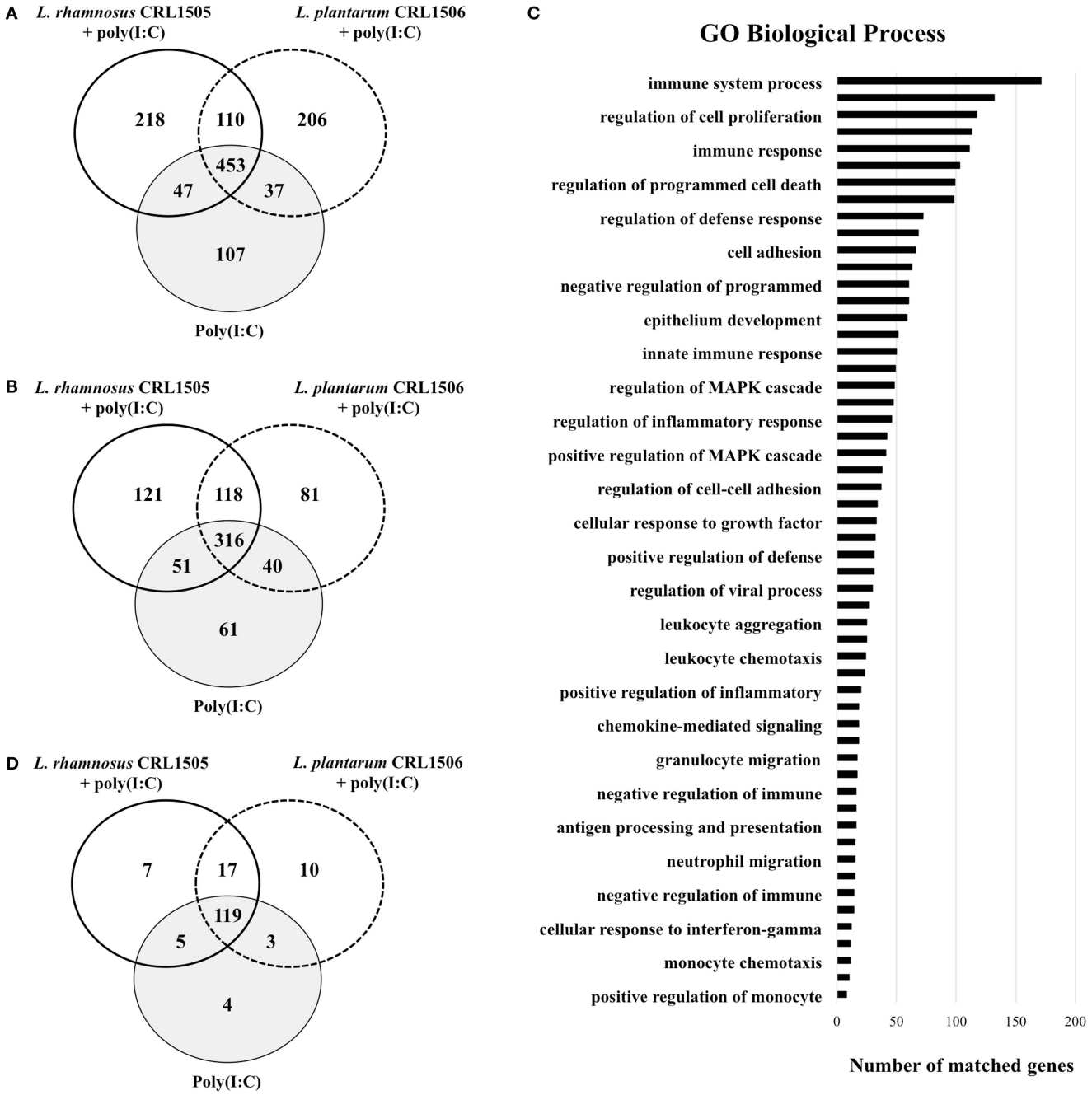

FIGURE 1 | Differentially regulated genes in porcine intestinal epithelial (PIE) cells treated with immunobiotic *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 and challenged with the viral molecular associated pattern poly(I:C). Non-lactobacilli-treated PIE cells challenged with poly(I:C) were used as controls. The changes in gene expression were evaluated by comparing the three mentioned groups with unchallenged PIE cells. Venn diagrams showing the number of differentially upregulated **(A)** and downregulated **(B)** genes for each experimental group. Number of matched genes categorized according to Gene Ontology (GO) database **(C)**. Venn diagram showing the number of differentially regulated genes that are known to have immune-related functions for each experimental group **(D)**.

upregulation of the transcriptional regulators *IRF7* (4.6), *STAT1* (4.3), *IRF1* (3.9), *IRF9* (2.5), and *STAT2* (2.2) were found in poly(I:C)-challenged PIE cells (Table S1 in Supplementary Material).

The stimulation of PIE cells with poly(I:C) significantly increased the expression of the inflammatory cytokines *IL-1*α (4.1), *IL-6* (4.0), and *IL-15* (1.8) (Table S1 in Supplementary Material). There was also a 3.9-fold increase in the expression

of the sensor of the inflammasome polymeric complex *NPLR3*. Chemokines involved in monocyte and T lymphocyte recruitment and activation such as *CXCL10* (13.2), *CCL5* (8.4), *CXCL9* (8.1), *CCL4* (7.8), *CCL20* (5.9), *CCL23* (5.1), *CCL28* (2.9), *CCL8* (2.3), and *CCL2* (2.0) were increased after poly(I:C) stimulation. In addition, we observed a significant upregulation of the chemotactic factors for neutrophils *CXCL5* (2.5), *CXCL11* (10.3), and *CXCL8* (1.2) (Table S1 in Supplementary Material). Moreover, *CSF1* (2.9) and *CFS2* (2.9) that are factors able to stimulate the growth and differentiation of hematopoietic precursor cells from granulocytes and macrophages were also increased.

An upregulation of genes for adhesion molecules in PIE cells after stimulation with poly(I:C) was observed, including *SELE* (5.3), *VCAM-1* (4.0), *SELL* (2.6), *ICAM-1* (2.2), *EPCAM* (1.8), and *SELP* (1.8) (Table S1 in Supplementary Material). There was also a sevenfold increase in the expression of *LGALS9* (galectin 9), which is involved in epithelial–lymphocytes interaction.

The microarray analysis revealed increases in the expression of several genes related to prostaglandins biosynthesis in poly(I:C)-challenged PIE cells including *PTGS2* (5.0), *PTGIR* (3.6), *PTGIS* (1.6), *PTGER4* (1.6), and *PLA2G4A* (1.2). In addition, factors belonging to the complement system were upregulated including *C1R* (7.5), *C1S* (5.7), *C3* (2.9), and *CFB* (3.9) (Table S1 in Supplementary Material).

Changes in the expression of some pattern recognition receptors (PRRs) were detected including *TLR2* (1.6), and *PGLYRP2* (8.1). In addition, we detected changes in the viral innate immune receptors *TLR3* (2.8), *DDX58* (8.9) also known as retinoic acid inducible gene-I (*RIG-I*), *IFIH1* (5.4) also known as melanoma differentiation associated gene-5 (*MDA-5*), and *PKR* (3.2) (Table S1 in Supplementary Material). We also detected increases in the expression of serum amyloid A2 (*SAA2*) (8.6).

qPCR Analysis of Selected Genes in PIE Cells after Poly(I:C) Challenge

To further evaluate gene expression changes induced by poly(I:C) in PIE cells, qPCR was performed. From the 165 immune and immune-related genes differentially regulated by poly(I:C) (**Figure 1D**; Table S1 in Supplementary Material), we selected 39 belonging to IFN and IFN-induced antiviral factors, cytokines, chemokines, adhesion molecules, prostaglandins, *SAA2, A20, GZMA, LYZ,* and trefoil factor 1 (*TFF1*) to be studied by qPCR. We confirmed that the direction of the changes in gene expression was in agreement with results obtained in the microarray analysis in all the studied genes.

We detected a significant increase in the expression of *IFN*-β and *IFN*-α that reached a maximum value on hour 12 after poly(I:C) stimulation (**Figure 2**). *IRF3*, *RNASEL, MX1*, and *MX2* showed a peak on hour 12 after poly(I:C) challenge (**Figure 2**). Similarly, we observed increases in expression of *OAS1* and *OASL* with peaks at hour 24 and in *OAS2* with peaks between hours 6 and 12 after the poly(I:C) stimulation.

A significant increase in expression of *CCL4*, *CCL20*, *CXCL2*, and *CXCL5* with peaks on hour 3 after poly(I:C) challenge was also detected (**Figure 3**). Similarly, we observed increases in expression of *CCL8* and *CXCL10* with peaks at hour 6 and in *CCL11* and *CCL5* with peaks at hours 12 and 24, respectively. In addition, expression of *CCL23* increased from hour 3 and stayed in the same level between hours 6 and 24 after stimulation of PIE cells (**Figure 3**). *CXCL14* expression was significantly reduced after poly(I:C) challenge and returned to basal levels at hour 24. Poly(I:C) also increased the expression of the adhesion molecules *SELE*, *SELL*, *ICAM-1*, and *EPCAM* (Figure S1 in Supplementary Material).

Increased expression of *IL-1*β, *IL-5,* and *IL-15* was observed in poly(I:C)-challenged PIE cells (**Figure 4**) showing all of them their highest values after 6 h of stimulation. Amphiregulin (*AREG*) was also increased after poly(I:C) challenge with a peak at hour 24. On the contrary, *IL-9* expression was significantly reduced between hours 6 and 12 and returned to the basal levels at hour 24 (**Figure 4**).

We detected a significant increase in the expression of *PTGS2*, *PTGIR*, *PLA2G4A*, *PTGES*, and *PTGER4* (**Figure 4**). In addition, a slight increase in *PTGIS* was observed at hour 3, and a significant downregulation occurred at hour 24. A decrease in *PTGFRN* between hours 3 and 12 was also observed (**Figure 4**).

Finally, we observed upregulation of *GZMA*, *LYZ*, *TFF1*, and *SAA2* with peaks at hours 3, 6, 12, and 24, respectively (Figure S2 in Supplementary Material).

Modulation of Poly(I:C)-Induced Immunotranscriptome Changes in PIE Cells by Immunobiotics

Next, we analyzed microarray data to evaluate the effect of the immunobiotic strains *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 on the immunotranscriptomic response of PIE cells after the challenge with poly(I:C). For that purpose, PIE cells were stimulated with *L. rhamnosus* CRL1505 or *L. plantarum* CRL1506 and then challenged with poly(I:C). Comparative analysis of microarray profiles indicated that both CRL1505 and CRL1506 strains differentially modulated the expression of several genes related to the innate antiviral immune response in PIE cells after poly(I:C) stimulation (Table S2 in Supplementary Material).

The Venn diagram analysis was used to find genes that were uniquely and commonly modulated between lactobacilli-treated and control PIE cells (**Figure 1D**). Of the 165 differentially expressed genes in the Venn diagram analysis, 4 (*PPARA*, *TFF1*, *STAT3*, and *DUOX1*) were unique to the poly(I:C) challenge. Seven (*TNFRSF11B*, *C5*, *LOC100127164*, *PIK3R5*, *IL27*, *IL17RC*, and *IBSP*) and 10 (*NFIA*, *CADM4*, *CDH24*, *CCR7*, *IL1RAPL2*, *TNFAIP8L2*, *DPEP1*, *CDH19*, *BPIFA1*, and *TNFSF18*) unique genes were found in the CRL1505 stimulation plus poly(I:C) challenge and the CRL1506 stimulation plus poly(I:C) challenge groups, respectively. In addition, five genes (*RNASE6*, *PROC*, *VTN*, *CCL28*, and *PLG*) were common to CRL1505 treatment plus poly(I:C) and control, whereas three genes (*IL23RA*, *ITGA1*, and *IL20RB*) were common to CRL1506 treatment plus poly(I:C) and control. It was also observed that 119 genes were common to all the 3 treatments (**Figure 1D**). The cluster analysis in Figure S3 in Supplementary Material depicts the transcriptomic patterns of differentially modulated genes between lactobacilli-treated and control PIE cells. The treatment with CRL1505 plus poly(I:C)

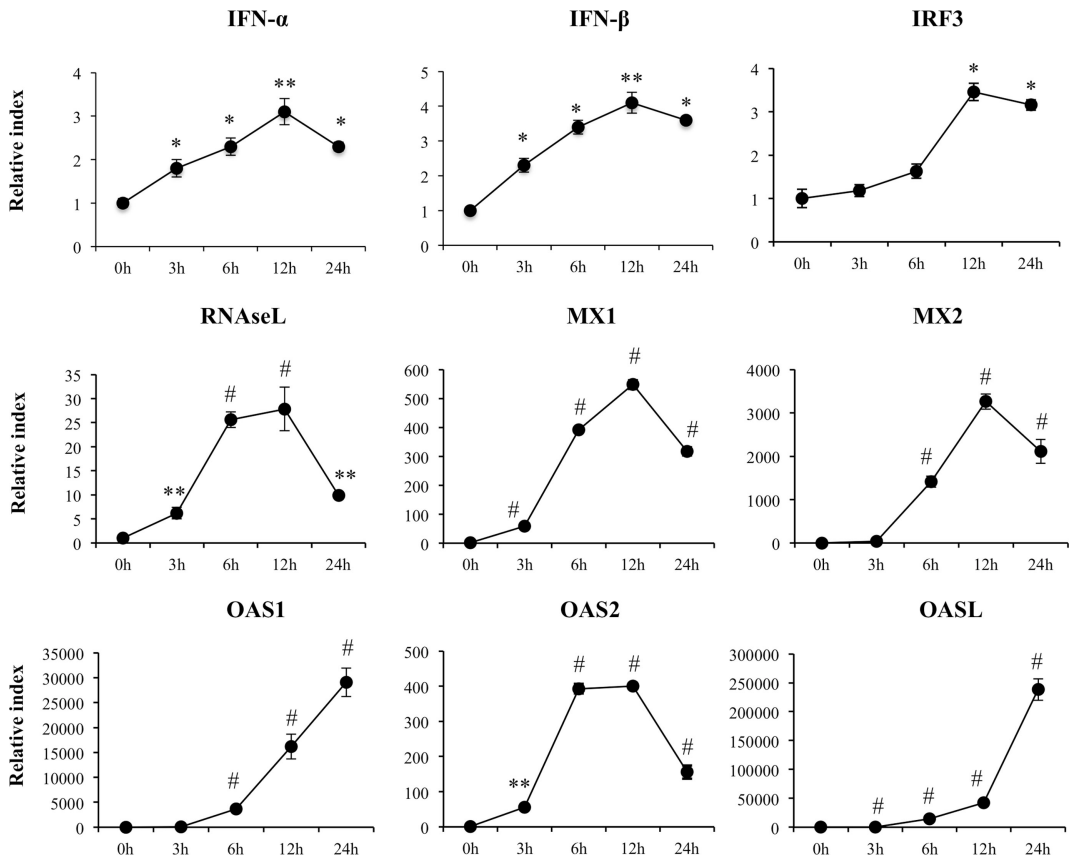

FIGURE 2 | Expression of type I interferons (*IFN*-β and *IFN*-α), IFN regulatory factor 3, and IFN-induced antiviral genes in porcine intestinal epithelial (PIE) cells after the challenge with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. The results represent data from three independent experiments. Symbols indicate significant differences when compared to unchallenged control PIE cells (time 0 h) (*$P < 0.05$, **$P < 0.01$, #$P < 0.001$).

clustered closer to the treatment with CRL1506 plus poly(I:C) and both clustered separated from the control.

Closer examination of gene expression revealed differences in several genes sheared by immunobiotic-treated PIE cells and controls (Table S1 in Supplementary Material). Most remarkable differences were found in the genes belonging to IFN and IFN-induced antiviral factors, cytokines, chemokines, and adhesion molecules. Both lactobacilli treatment significantly increased *IFN*-β, *IFN*-α, *TLR3*, *OAS1*, *OASL*, *MX2*, *RNASEL*, *RNASE4*, and *STAT5A* when compared to controls. In addition, stimulation of PIE cells with *L. rhamnosus* CRL1505 plus poly(I:C) significantly increased the expression levels of *IFIT1*, *IFITM1*, *DDX58/RIG1*, *IFIH1/MDA5*, *IRF7*, *STAT1*, *NLRP3*, *IRF1*, *STAT2*, and *IRF2* when compared with PIE cells stimulated only with poly(I:C).

Although expression of *IL1A*, *IL6*, *IL8*, *AREG*, *CXCL10*, *CCL5*, *CCL4*, *CCL20*, *CCL23*, *CSF2*, *CCL3L1*, and *SELL* was upregulated in lactobacilli-treated PIE cells after the challenge with poly(I:C), the increases were significantly higher when compared to control PIE cells without lactobacilli treatment (Table S1 in Supplementary Material). *L. rhamnosus* CRL1505 plus poly(I:C) also increased the expression levels of *VEGFA*, *IL17RC*, *CXCL11*, *CCRL2*, *CXCL5*, *CXCL2*, *SELE*, *CDHR4*, and *EPCAM* when compared with PIE cells stimulated only with poly(I:C), an effect that was not observed with CRL1506 treatment. Interestingly, *IL27* was upregulated only in PIE cells receiving the CRL1505 strain plus poly(I:C). In addition, the expression levels of *IL15* and *RAE1* were reduced by lactobacilli treatments.

We also observed an increased expression of *PLA2G4A*, *PTGES*, and *PTGS2* genes in lactobacilli-treated PIE cells after the challenge with poly(I:C) when compared to the control cells, whereas *PTGER4* and *PTGER2* were diminished in lactobacillus-treated cells (Table S1 in Supplementary Material). *L. rhamnosus* CRL1505 plus poly(I:C) also increased the expression levels of *PTGIR*.

Expression of *TLR6*, *MYD88*, *NCOA1*, and *NFKB1* was significantly higher in lactobacilli-treated PIE cells after the challenge with poly(I:C) when compared to controls. In addition, the transcripts of other immune and immune-related genes including *GZMH*, *TFF1*, *LYZ*, *C1R*, *CFB*, *PLG*, *CFD*, *SAA2*, and *NOS2* were higher in lactobacilli-treated PIE cells than controls (Table S1 in Supplementary Material). Stimulation of PIE cells with *L. rhamnosus* CRL1505 plus poly(I:C) significantly increased the expression levels of *C1S*, *C3*, and *PLAU* when compared with PIE cells stimulated only with poly(I:C).

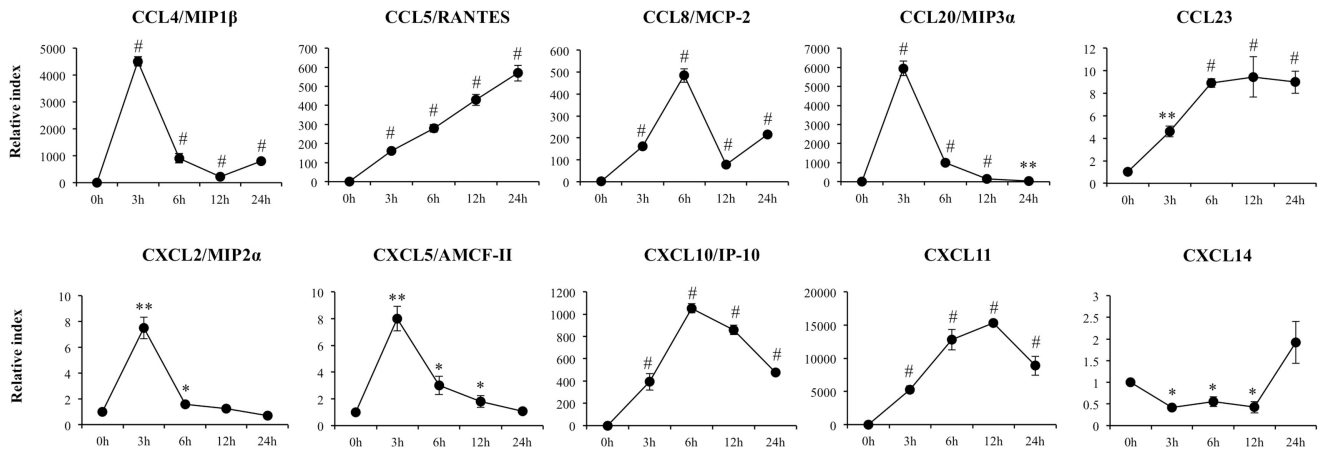

FIGURE 3 | Expression of chemokines genes in porcine intestinal epithelial (PIE) cells after the challenge with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. The results represent data from three independent experiments. Symbols indicate significant differences when compared to unchallenged control PIE cells (time 0 h) (*$P < 0.05$, **$P < 0.01$, #$P < 0.001$).

qPCR Analysis of Selected Genes in PIE Cells after Stimulation with Immunobiotics and Poly(I:C) Challenge

To confirm the changes induced by *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 in the immunotranscriptome response of poly(I:C)-challenged PIE cells, qPCR was performed on selected genes. Genes with or without significant differences between lactobacilli-treated and non-treated PIE cells were chosen. The transcriptional changes evaluated by qPCR indicated a similar overall trend in the transcription.

Both *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 induced a significantly higher expression of *IFN*-α and *IFN*-β when compared with control poly(I:C)-challenged PIE cells (**Figure 5**). In addition, *IRF3* and the IFN-induced antiviral factors *RNASEL*, *MX2*, *OAS1*, and *OASL* were significantly higher in lactobacilli-treated PIE cells than in controls. Furthermore, *MX2* expression was higher in PIE cells treated with CRL1505 strain than those treated with CRL1506. Expression of *MX1* and *OAS2* in lactobacilli-treated PIE cells was not different from the control PIE cells after the challenge with poly(I:C) (**Figure 5**).

Expression of *CCL8* and *CXCL14* in lactobacilli-treated PIE cells was not different from the control PIE cells after the challenge with poly(I:C) (**Figure 6**). In contrast, the levels of *CCL23*, *CXCL8*, and *SELL* were significantly higher in lactobacilli-treated PIE cells when compared to the controls (**Figure 6**). In addition, both lactobacilli significantly increased the expression of *CCL4*, *CCL5*, *CCL20*, and *CXCL10*; however, values in *L. rhamnosus* CRL1505-treated PIE cells were higher than in cells treated with *L. plantarum* CRL1506. Only *L. rhamnosus* CRL1505 was able to increase the expression of *CXCL2*, *CXCL5*, *CXCL11*, *EPCAM*, *ICAM-1*, and *SELE* when compared to control PIE cells (**Figure 6**).

In agreement with the results from our microarray analysis, both lactobacilli strains were able to increase the expression of *IL-1*β, *IL-6*, and *AREG* and reduce the expression of *IL-15* and *PTGER4*, with no significant differences between them (**Figure 7**). Moreover, no differences in *TGF*-β or *PTGIS* were found between lactobacilli-treated and control PIE cells. Both lactobacilli significantly increased the expression of *PLA2G4A*, *PTGES*, and *PTGS2*; however, values in *L. rhamnosus* CRL1505-treated PIE cells were higher than in cells treated with *L. plantarum* CRL1506. In addition, only *L. rhamnosus* CRL1505 was able to significantly increase the expression of *IL-9* and *PTGIR* when compared to control PIE cells (**Figure 7**).

Expression of *TLR2* and *PGLYRP2* in CRL1505- or CRL1506-tretaed PIE cells was not different from the control PIE cells after the challenge with poly(I:C). In contrast, expression levels of *RIG1*, *TLR3*, and *TLR6* (**Figure 8**) were significantly higher in lactobacilli-treated PIE cells when compared to the controls. We also observed that *A20* (*TNFAIP3*) was reduced in lactobacilli-treated PIE cells when compared to the controls (**Figure 8**). *SAA2*, *GZMA*, *LYZ*, *TFF1*, and *C1R* were significantly upregulated in lactobacilli-treated PIE cells when compared to the controls (Figure S4 in Supplementary Material). Only *L. rhamnosus* CRL1505 was able to significantly increase the expression of *C3* when compared to control PIE cells, whereas both lactobacilli reduced the expression of *CFB* (Figure S4 in Supplementary Material).

DISCUSSION

It is known that IECs senses viral dsRNA through PRRs including TLR3, RIG-I, and MDA-5. After the recognition of dsRNA by those receptors, cellular signaling cascades are activated to react against viral infection. Antiviral PRRs activation leads to the production of cytokines, chemokines, IFNs, and IFN-regulated gene products that play a key role in establishing an antiviral state for virus clearance and restriction of spread (21).

High-throughput microarray technology has been employed for screening genes involved in the immune responses to enteric virus or poly(I:C) (22, 23). By using a human colon epithelial cell line (HT29 cells), Bagchi et al. (22) evaluated the

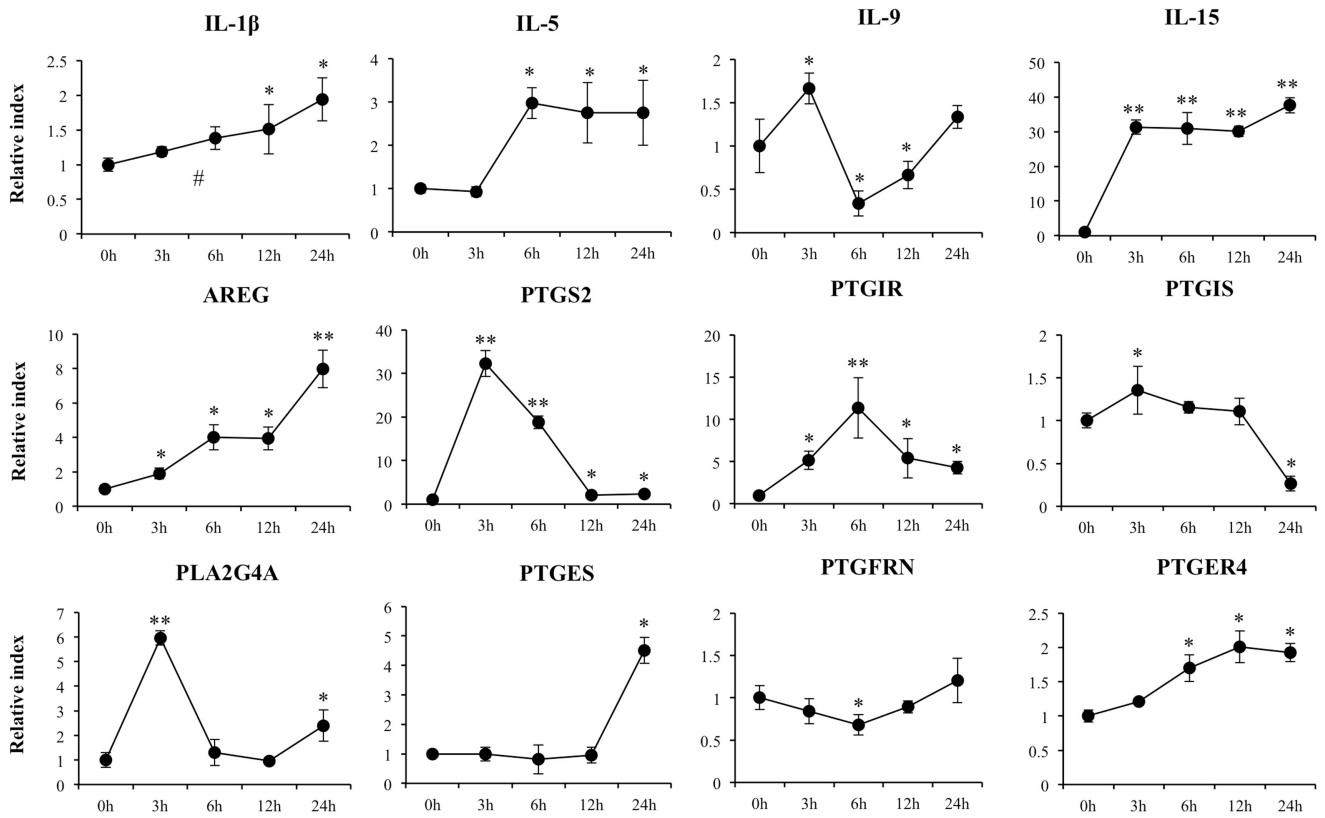

FIGURE 4 | Expression of cytokines and genes involved in prostaglandins biosynthesis in porcine intestinal epithelial (PIE) cells after the challenge with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. The results represent data from three independent experiments. Symbols indicate significant differences when compared to unchallenged control PIE cells (time 0 h) (*$P < 0.05$, **$P < 0.01$, $^{\#}P < 0.001$).

immunotranscriptomic response of IECs to the challenge with different rotavirus strains. Microarray data revealed a set of commonly differentially regulated genes for the three rotaviruses used in that work. Of interest, several IFN inducible genes (*OAS1*, *MX1*, *IL18*, *IITP3*, *TAP1*, and *RSAD2*) as well as several cytokines and chemokines (*CCL5*, *CXCL10*, *CXCL11*, *IL8*, and *CCL15*) were upregulated by rotavirus infection. Later, it was observed that the stimulation of HT29 cells with poly(I:C) enhanced the expression of several genes associated with the dsRNA recognition by PRRs including antiviral factors (*IRF1*, *ISG20*, *IFIT2*, *OASL*, and *STAT5*), and proinflammatory cytokines (*CSF1*, *CSF2*, *IL29*, *TNF*-α, *CXCL11*, and *CLCF1*) (23). Those transcriptomic studies indicated that poly(I:C) and rotavirus induce similar innate antiviral immunotranscriptomic responses in IECs.

Previously, the response of PIE cells to poly(I:C) challenge was evaluated, and it was found that MCP-1, IL-8, TNF-α, IL-6, and both IFN-α and IFN-β were upregulated in PIE cells after stimulation (14). The suitability of PIE cells as a model for studying immune signaling pathways after rotavirus infection was also evaluated. Our results showed that PIE cells have functional TLR3, RIG-I, and MDA-5 receptors, which are able to detect rotavirus infection and enhance the expression of IFN-β and the ISGs MxA and RNase L (15), which are important antiviral effectors of IFN pathway. In this study, we corroborated and deepen those findings by using microarray technology and qPCR. We demonstrated that stimulation with poly(I:C) significantly alters gene expression profiles of PIE cells. Of the transcripts differentially modulated by poly(I:C), several were assigned to immune-related functions. Our results showed that the activation of IRF3 and NF-kB pathways in PIE cells by poly(I:C) increased the expression of IFN-α and IFN-β, several ISGs (*OAS1*, *OASL*, *IFIT1*, *IFIT3*, *IFIT2*, *MX1*, *MX2*, *OAS2*, *IFIT5*, *RNASEL*, and *RNASE4*), cytokines (*IL-1*β, *IL-5*, and *IL-15*), and chemokines (*CCL4*, *CCL20*, *CXCL2*, *CXCL5*, *CCL8*, *CXCL10*, *CCL11*, *CCL5*, and *CCL23*). Moreover, some adhesion molecules were also significantly upregulated in PIE cells after poly(I:C) stimulation including *SELE*, *SELL*, *ICAM-1*, and *EPCAM*. In addition, we also observed a significant upregulation of the dsRNA detection sensors *TLR3*, *RIG1*, and *MDA5*. This is in agreement with studies in HT29 cells showing that RIG1 was upregulated by rotavirus infection (22).

These results are in line with the transcriptomics studies mentioned before and indicate that PIE cells are able to mount a complex innate antiviral immune response involving changes needed to induce a mucosal antiviral state and promote the recruitment of inflammatory cells to the intestinal tissue, which are intended to eliminate the viral pathogen (**Figure 9A**). These features also exhibit that PIE cells are an excellent laboratory tool

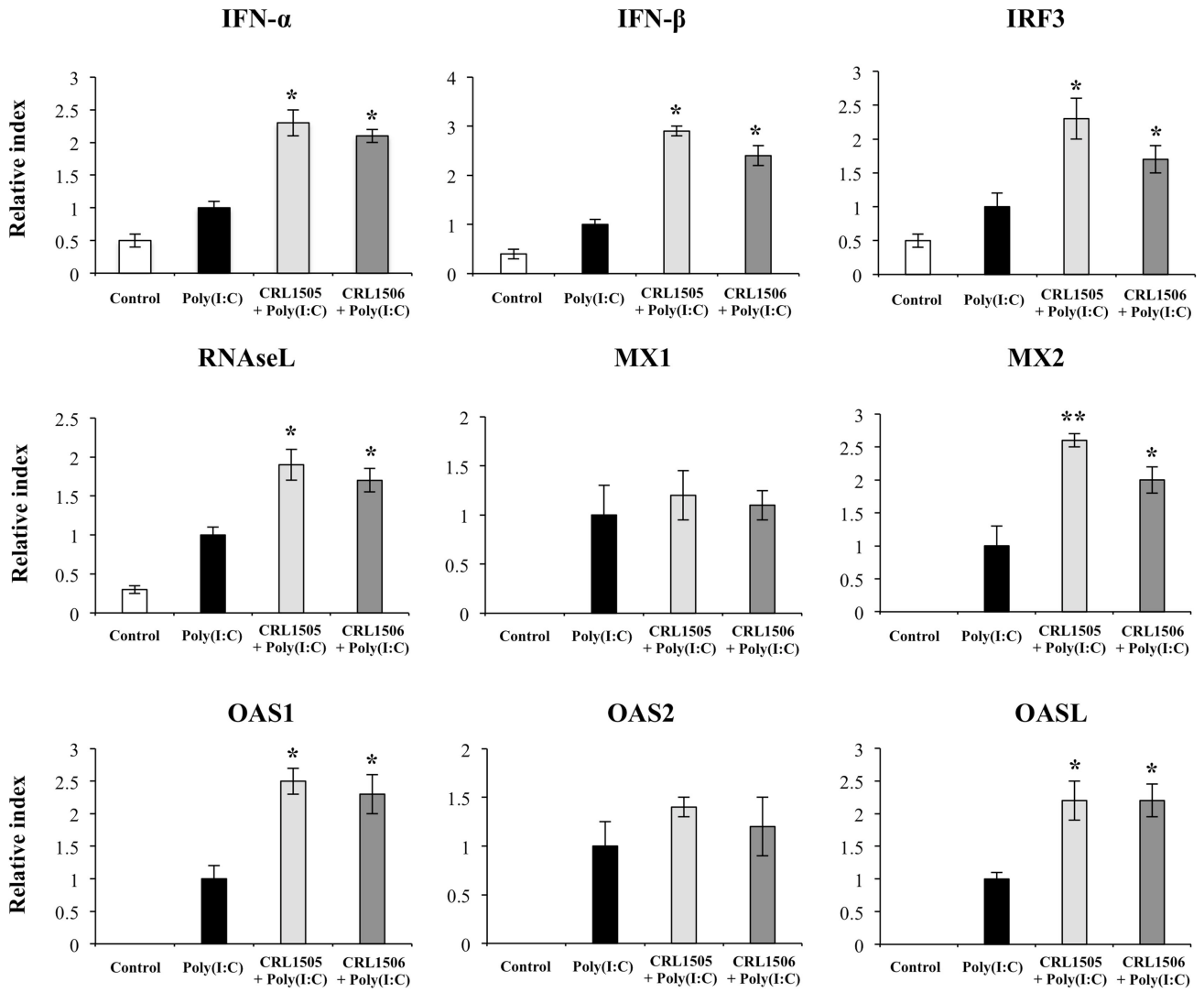

FIGURE 5 | Expression of type I interferons (*IFN*-β and *IFN*-α), IFN regulatory factor 3, and IFN-induced antiviral genes in porcine intestinal epithelial (PIE) cells treated with immunobiotic *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 and challenged with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. Non-lactobacilli-treated PIE cells with or without poly(I:C) challenge were used as controls. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*$P < 0.05$, **$P < 0.01$).

to study treatments able to favorably modulate the innate antiviral response.

Several studies have shown that immunobiotics are able to beneficially modulate PRRs-mediated inflammatory response in the gut by regulating the functions of IECs (24). In this regard, our previous studies demonstrated that the immunobiotic strains *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 differentially regulated the expression of IFN-α, IFN-β, MCP-1, IL-8, and IL-6 in PIE cells after TLR3 activation (19). In line with those previous findings, we described here that the treatment of PIE cells with lactobacilli resulted in differential expression of several immune genes in response to the poly(I:C) challenge (**Figure 9B**), which include not only antiviral factors and cytokines as previously demonstrated but also adhesion molecules, complement factors, enzymes involved in prostaglandin biosynthesis, and PRRs. Most notable changes were found in *IFN*-α, *IFN*-β, *NPLR3*, *OAS1*, *OASL*, *MX2*, *RNASEL*, and *RNASE4* that were significantly increased in lactobacilli-treated PIE cells when compared to the controls. It is known that RNAse L, OAS, MX, and NPLR3 are important factors for the protection of the intestinal mucosa against rotavirus infection (25–27). This finding is of interest because it confirms our previous *in vitro* (19) and *in vivo* (11) studies demonstrating the antiviral capacity of *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506.

In addition, it was observed that *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 differentially regulated the expression of

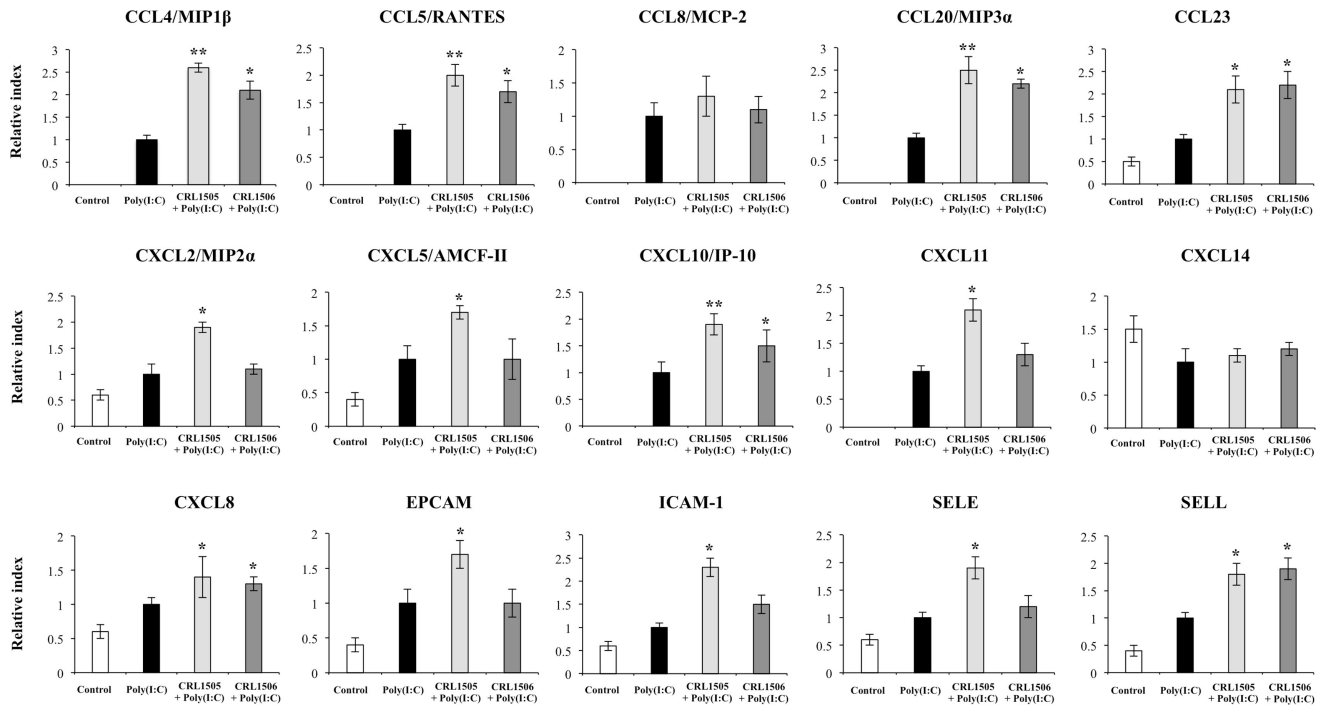

FIGURE 6 | Expression of chemokines and adhesion molecules genes in porcine intestinal epithelial (PIE) cells treated with immunobiotic *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 and challenged with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. Non-lactobacilli-treated PIE cells with or without poly(I:C) challenge were used as controls. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*$P < 0.05$, **$P < 0.01$).

cytokines, chemokines, and adhesion molecules (**Figure 9B**). Expression levels of *IL-1*β, *IL-6*, *SELL*, *CCL4*, *CCL5*, *CCL20*, *CCL23*, *CXCL8*, and *CXCL10* were higher in lactobacilli-treated PIE cells than controls. In addition, *ICAM1*, *EPCAM*, *CXCL2*, *CXCL5*, and *CXCL11* were increased in CRL1505-tretated PIE cells. This pattern of cytokines/chemokines and adhesion molecules gene expression induced by lactobacilli would allow us to predict an improved recruitment and activation of immune cells to the gut mucosa, which could beneficially influence the elimination of the virus. It is also necessary to consider that in several viral infections, the excessive recruitment of inflammatory cells and/or their deregulated activation may contribute to the damage of the infected tissue rather than the resolution of the infection. It was reported that poly(I:C), when administered intraperitoneally to mice, mimics the local intestinal immune response elicited by an enteric viral infection (28, 29). Both purified dsRNA from rotavirus and poly(I:C) are able to induce severe mucosal damage in the gut *via* TLR3 activation including villous atrophy, mucosal erosion, and gut wall attenuation (28). It was demonstrated that TLR3 activation in IECs by poly(I:C) or rotavirus genomic dsRNA induce the expression of IL-15 and retinoic acid early inducible-1 (RAE1), which mediate epithelial destruction and mucosal injury by interacting with the NKG2D receptor expressed on $CD3^+NK1.1^+CD8\alpha\alpha^+$ intraepithelial lymphocytes (IELs) (30). Here, we found a significant reduction in the expression of *IL-15* and *RAE1* in PIE cells treated with lactobacilli. This is in line with our previous work that showed that mice pretreated with immunobiotic lactobacilli responded with reduced levels of TNF-α, IL-15, RAE1, and $CD3^+NK1.1^+$ $CD8\alpha\alpha^+$ IELs after TLR3 activation with poly(I:C) (11). Those changes significantly diminished the inflammatory damage of the intestinal mucosa.

Our transcriptomic study indicates that other regulatory mechanisms would be improved by lactobacilli to limit the inflammatory damage during intestinal viral infection. A significant upregulation of *AREG* and *TFF1* expression was observed in lactobacilli-treated PIE cells when compared to controls. Recently, it was demonstrated that the mucosal surfaces of lung and intestine are protected from detrimental inflammation by group 2 innate lymphoid cells (ILC2s). Monticelli et al. (31) showed that following activation with IL-33, ILC2s in the gut increased the expression of *AREG*, limited intestinal inflammation, and decreased disease severity in mice treated with dextran sodium sulfate. Moreover, it was reported that the number of ILC2s increased in the respiratory tract after infection influenza virus and that depletion of those cells induced impaired airway remodeling and altered lung epithelial integrity, diminishing lung function. Notably, these defects were restored by administration of AREG (32). On the other hand, TFF1 is a stable secretory protein expressed in gastrointestinal mucosa that stabilize the mucus layer and affect healing of the epithelium. By using TFF1-knockout mice, it was showed that this factor plays a

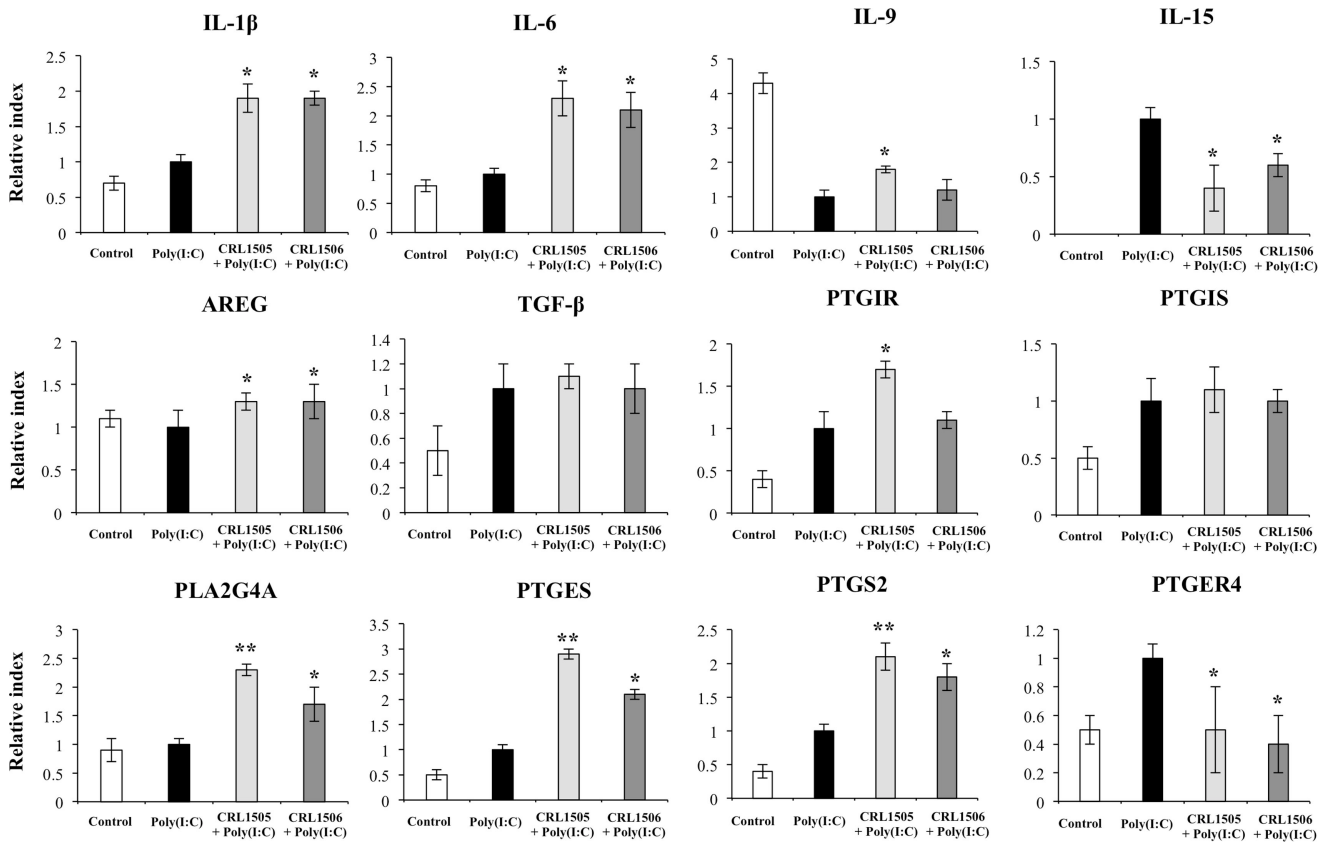

FIGURE 7 | Expression of cytokines and genes involved in prostaglandins biosynthesis in porcine intestinal epithelial (PIE) cells treated with immunobiotic *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 and challenged with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. Non-lactobacilli-treated PIE cells with or without poly(I:C) challenge were used as controls. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*$P < 0.05$, **$P < 0.01$).

critical role in maintaining mucosal integrity and regulating the pro-inflammatory response to gastrointestinal pathogens (33). Moreover, a recombinant *Lactococcus lactis* strain, genetically modified to secrete human TFF1, was able to reduce the severity of mucosal damage in an animal model of oral mucositis (34).

We also observed that poly(I:C) stimulation induced transcriptomic changes in several genes involved in the biosynthesis of prostaglandins and that lactobacilli treatments increased the expression of several of those genes including *PLA2G4A*, *PTGES*, and *PTGS2*. Upregulation of *PLA2G4A* and *PTGES* indicates that PIE cells treated with lactobacilli would increase their production of prostaglandin E2 (PGE2). It has been reported that PGE2 regulates immune function in several ways that are able to affect viral pathogenesis. Production of pro-inflammatory cytokines and chemokines by immune cells are inhibited in presence of PGE2, whereas IL-10 is enhanced (35, 36) indicating that PGE2 could have a role in the protective activity of *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 against inflammatory damage. It was also reported that PGE2 inhibits type I IFN production in epithelial and immune cells, thereby causing an increase in virus replication (37). Interestingly, the expression of PGE2 receptors (*PTGER4* and *PTGER2*) was downregulated in PIE cells treated with the immunobiotic strains indicating that cells were protected from this effect of PGE2.

Whether the capacity of *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 to differentially modulate AREG, TFF1, and prostaglandins production is involved in their beneficial effects on intestinal or respiratory viral infections *in vivo* is an open question, which we propose to address in the near future.

The zinc-finger protein A20 is capable to terminate TLR signaling, which results in inhibition of NF-κB activation and reduction of inflammatory-induced cytotoxicity (38). Saitoh et al. (39) reported that IRF3 activation is suppressed by A20. The A20 protein is able to induce the suppression of the IFN-mediated immune response and IFN-promoter-dependent transcription following engagement of TLR3 by dsRNA. A20 knock down results in enhanced IRF3-dependent transcription triggered by the stimulation of TLR3 or virus infection. Furthermore, it was reported that A20 was upregulated by different rotavirus strains in HT29 cells. Interestingly, the same work demonstrated that the knock down of A20 in IECs by siRNA significantly reduced virus titers indicating that A20 is required for rotavirus infection (39). We have reported previously that two immunobiotic bacteria with antiviral capabilities, *Bifidobacterium infantis* MCC12 and

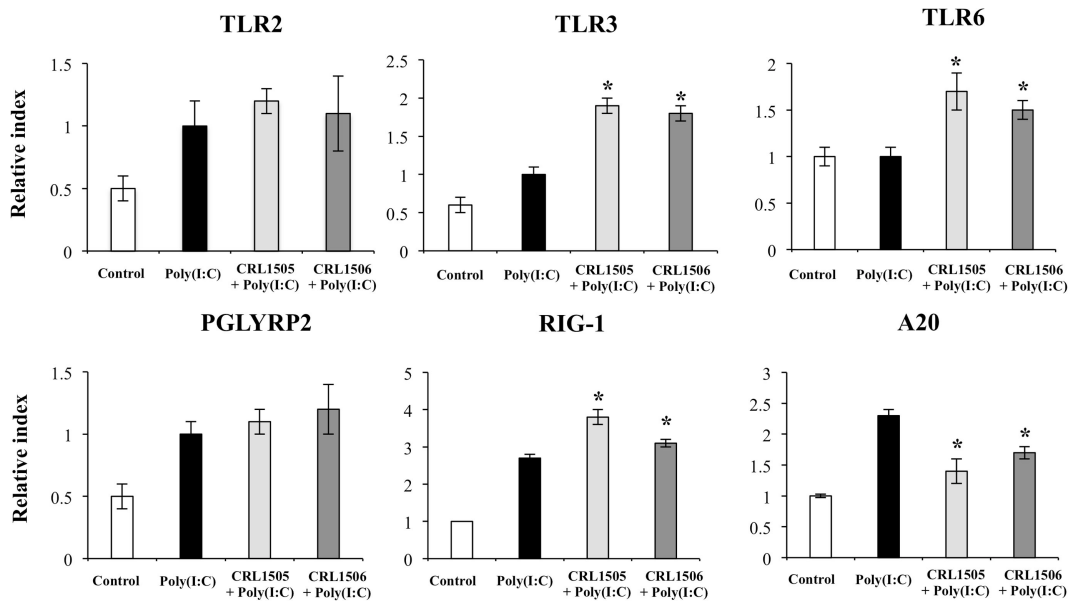

FIGURE 8 | Expression of pattern recognition receptors and A20 genes in porcine intestinal epithelial (PIE) cells treated with immunobiotic *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 and challenged with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. Non-lactobacilli-treated PIE cells with or without poly(I:C) challenge were used as controls. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*$P < 0.05$).

Bifidobacterium breve MCC1274, significantly reduced the expression of A20 in rotavirus-infected PIE cells (15), which was in line with the capacity of both strains to improve IRF3 activation and IFN-β production. In line with our findings, MacPherson et al. (23) showed that the stimulation of HT29 cells with poly(I:C) alone increased the expression of A20, but the co-stimulation with poly(I:C) and probiotics significantly reduced A20 expression levels. Although our microarray analysis did not show differences between lactobacilli-treated and control PIE cells when the *A20* (*TNFAIP3*) transcript was evaluated, qPCR analysis showed a significant reduction of A20 expression in immunobiotic-treated cells. Therefore, the reduction of A20 in IECs could be a key effect for the antiviral capabilities of immunobiotics.

Lactobacillus rhamnosus CRL1505 and *L. plantarum* CRL1506 showed quantitative and qualitative differences in their capacities to modulate the innate antiviral immune response in PIE cells. Higher expression levels of the antiviral factors *MX2* and *IFIT2* were found in CRL1505-treated PIE cells when compared to CRL1506-treated cells. Moreover, some antiviral factors were upregulated only with *L. rhamnosus* CRL1505 treatment including *IFIT1*, *IFIT3*, *RIG-1*, *MDA5*, *NLRP3*, and *MSX1*. As mentioned before, *RIG-1*, *MDA5*, and *NLRP3* are important factors in the protection against gastrointestinal virus such as rotavirus. In addition, *MSX1* (also known as *HOX7*) was recently identified as an important modulator of RIG-1-mediated signaling pathway with the ability to induce the activation of the TBK1 kinase and IRF3, increasing the expression of antiviral genes and improving innate antiviral responses (40). Furthermore, *L. rhamnosus* CRL1505 differentially regulated the expression of proinflamamtory and anti-inflammatory factors in poly(I:C)-challenged PIE cells. Higher expression of *CCL4*, *CCL5*, *CCL20*, and *CXCL10* were found in CRL1505-treated PIE cells when compared to CRL1506-treated cells, whereas *CXCL2*, *CXCL5*, and *CXCL11* were upregulated only with *L. rhamnosus* CRL1505 treatment, indicating a higher capacity of this strain to induce recruitment of immune cells. It also seems that the CRL1505 strain would have a higher ability to improve the regulation of the inflammatory response. We observed higher expression of *PLA2G4A* and *PTGES* that would enhance the production of the anti-inflammatory PGE2. Of interest, microarray analysis showed an increase in the expression of IL-27 in *L. rhamnosus* CRL1505 treatment, an effect that was not observed in the other experimental groups. IL-27 is a member of IL-12 family of cytokines that is produced mainly by myeloid cell populations, including macrophages, inflammatory monocytes, and dendritic cells, but its production has been reported in endothelial cells and epithelial cells as well (41). This cytokine has important roles in the early regulation of Th1 differentiation and the suppression of cellular activation and production of proinflammatory cytokines (42). It was demonstrated that IL-27 induces IL-10 production from both mouse and human $CD4^+$ and $CD8^+$ T cells and NK cells (43). Moreover, some recent studies reported a role for this cytokine in restricting virus replication (42). These effects would explain the higher capacity of the CRL1505 strain when compared to CRL1506 to protect against viral infection and inflammatory damage (5, 11, 19).

In conclusion, the genome-wide transcriptional profiling performed in this work allowed us to obtain a global overview of the expression patterns of immune and immune-related genes involved in the response of PIE cells to poly(I:C) stimulation. This study also confirmed that *L. rhamnosus* CRL1505 and *L.*

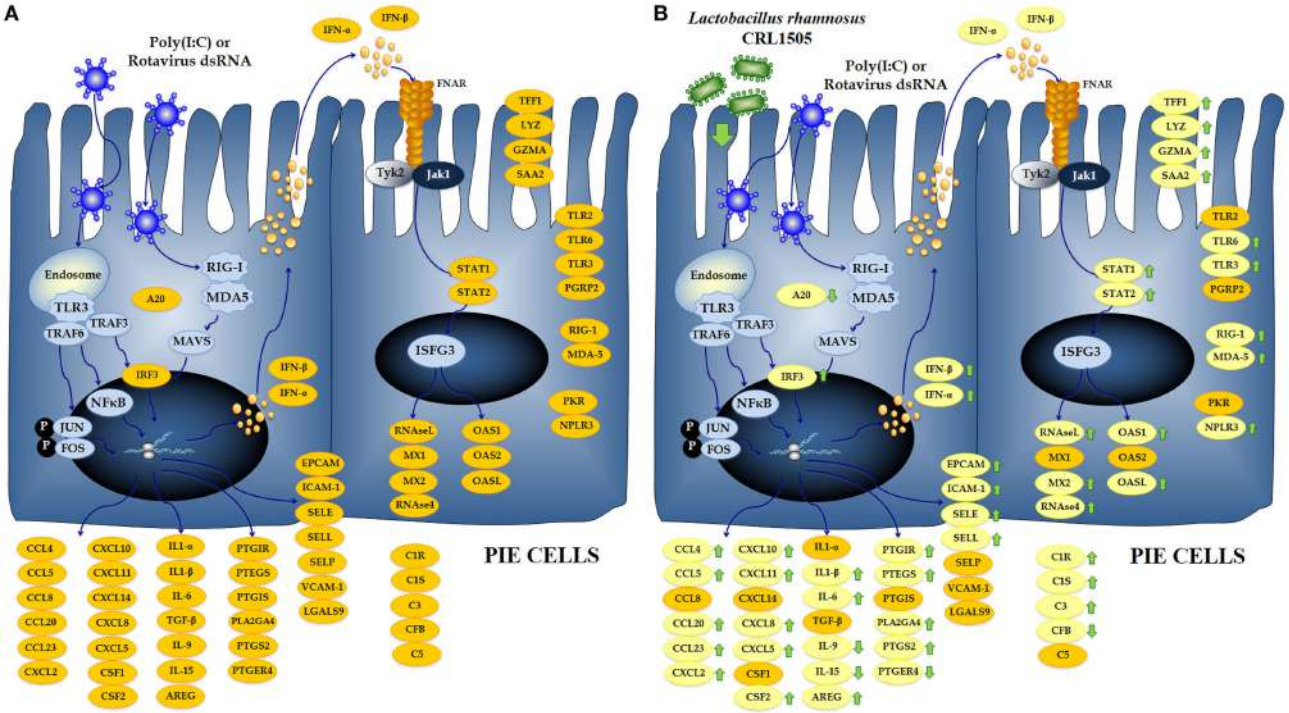

FIGURE 9 | Global overview of the signaling pathways and immune genes differentially regulated in porcine intestinal epithelial (PIE) cells after the challenge with the viral molecular associated pattern poly(I:C) (A). Global overview of the signaling pathways and immune genes differentially regulated in PIE cells treated with *Lactobacillus rhamnosus* CRL1505 and challenged with poly(I:C) **(B)**.

plantarum CRL1506 differently modulate gene expression in poly(I:C)-challenged PIE cells inducing changes that could help to explain the antiviral activities observed in animal models and clinical trials. These results provided clues for the better understanding the mechanism underlying host–immunobiotic interaction.

The main outcome from the study is that our transcriptomic analysis successfully identified a group of genes (*IFN*-β, *RIG1*, *RNASEL*, *MX2*, *A20*, *IL27*, *CXCL5*, *CCL4*, *PTGES*, and *PTGER4*), which can be used as prospective biomarkers for the screening of new antiviral immunobiotics in PIE cells. Classically, the selection of potential immunobiotic strains is performed by studying few biomarkers *in vitro*, and in many cases, the selected strains do not exhibit the same immunomodulatory activity when they are evaluated later in *in vivo* models. Our preliminary studies indicate that the set of biomarkers found in this work allows an efficient *in vitro* selection of new strains with antiviral activity in PIE cells, which present antiviral activity when they are evaluated later in animal models. This efficient selection of immunobiotics could improve the development of novel functional food and feeds, which may help to prevent viral infections.

AUTHOR CONTRIBUTIONS

HA, SA, JV, and HaK designed the study and manuscript writing. LA, HisK, HI, and NS did the laboratory work in the expression and statistical analysis. LA and JV participated in the data analysis of microarray. SS, TN, JV, and HaK contributed to data analysis and interpretation. All the authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00057/full#supplementary-material.

FIGURE S1 | Expression of adhesion molecules genes in porcine intestinal epithelial (PIE) cells after the challenge with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. The results represent data from three independent experiments. Symbols indicate significant differences when compared to unchallenged control PIE cells (time 0 h) (*$P < 0.05$, **$P < 0.01$).

FIGURE S2 | Expression of trefoil factor 1, lysozyme, granzyme, and A20 genes in porcine intestinal epithelial (PIE) cells after the challenge with the viral molecular associated pattern poly(I:C), analyzed by

quantitative PCR. The results represent data from three independent experiments. Symbols indicate significant differences when compared to unchallenged control PIE cells (time 0 h) (*$P < 0.05$, **$P < 0.01$, #$P < 0.001$).

FIGURE S3 | Heat map analysis of the differentially regulated genes in porcine intestinal epithelial (PIE) cells treated with immunobiotic *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 and challenged with the viral molecular associated pattern poly(I:C). Non-lactobacilli-treated PIE cells challenged with poly(I:C) were used as controls.

FIGURE S4 | Expression of trefoil factor 1, lysozyme, granzyme, serum amyloid A2, and complement system factors genes in porcine intestinal epithelial (PIE) cells treated with immunobiotic *Lactobacillus rhamnosus* CRL1505 or *Lactobacillus plantarum* CRL1506 and challenged with the viral molecular associated pattern poly(I:C), analyzed by quantitative PCR. Non-lactobacilli-treated PIE cells with or without poly(I:C) challenge were used as controls. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to poly(I:C)-challenged control PIE cells (*P < 0.05).

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Visualization of Probiotic-Mediated Ca^{2+} Signaling in Intestinal Epithelial Cells *In Vivo*

Takahiro Adachi[1], Shigeru Kakuta[2], Yoshiko Aihara[3], Tomonori Kamiya[4], Yohei Watanabe[4], Naomi Osakabe[5], Naoki Hazato[5], Atsushi Miyawaki[6], Soichiro Yoshikawa[7], Takako Usami[8], Hajime Karasuyama[7], Hiromi Kimoto-Nira[9], Kazuhiro Hirayama[10]* and Noriko M. Tsuji[4]**

[1] Department of Immunology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan, [2] Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan, [3] Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, Kobe, Japan, [4] Biomedical Research Institute, National Institute for Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, [5] Department of Bio-science and Engineering, Shibaura Institute of Technology, Saitama, Japan, [6] Laboratory for Cell Function and Dynamics, Advanced Technology Development Group, Brain Science Institute, RIKEN, Saitama, Japan, [7] Department of Immune Regulation, Tokyo Medical and Dental University, Tokyo, Japan, [8] Laboratory of Recombinant Animals, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan, [9] NARO Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan, [10] Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

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Haruki Kitazawa, Tohoku University, Japan

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***Correspondence:**
Takahiro Adachi
tadachi.imm@mri.tmd.ac.jp;
Kazuhiro Hirayama
akazu@mail.ecc.u-tokyo.ac.jp;
Noriko M. Tsuji
nm-tsuji@aist.go.jp

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Probiotics, such as lactic acid bacteria (LAB) and *Bacillus subtilis* var. *natto*, have been shown to modulate immune responses. It is important to understand how probiotic bacteria impact intestinal epithelial cells (IECs), because IECs are the first line of defense at the mucosal surface barrier and their activities substantially affect the gut microenvironment and immunity. However, to date, their precise mechanism remains unknown due to a lack of analytical systems available for live animal models. Recently, we generated a conditional Ca^{2+} biosensor Yellow Cameleon (YC3.60) transgenic mouse line and established 5D (*x*, *y*, *z*, time, and Ca^{2+}) intravital imaging systems of lymphoid tissues including those in Peyer's patches and bone marrow. In the present study, we further advance our intravital imaging system for intestinal tracts to visualize IEC responses against orally administrated food compounds in real time. Using this system, heat-killed *B. subtilis natto*, a probiotic TTCC012 strain, is shown to directly induce Ca^{2+} signaling in IECs in mice housed under specific pathogen-free conditions. In contrast, this activation is not observed in the *Lactococcus lactis* strain C60; however, when we generate germ-free YC3.60 mice and observe the LAB stimulation of IECs in the absence of gut microbiota, C60 is capable of inducing Ca^{2+} signaling. This is the first study to successfully visualize the direct effect of probiotics on IECs in live animals. These data strongly suggest that probiotic strains stimulate IECs under physiological conditions and that their activity is affected by the microenvironment of the small intestine, such as commensal bacteria.

Keywords: probiotic, Ca^{2+} signaling, intestinal epithelial cell, intravital imaging, *Lactococcus*, *Bacillus subtilis*, small intestine, germ-free mouse

INTRODUCTION

Food compounds are digested and absorbed through the gastrointestinal tract for nutrition, and probiotic bacteria and polysaccharides affect immunological homeostasis in the gut (1–6). Fermentative lactic acid bacteria (LAB) are aerobic and abundant in the environment and are very often contained in the average diet, consequently composing a major part of small intestinal commensal flora (7–9). LAB, therefore, affect the maturation of host immune cells and intestinal immune homeostasis under normal steady-state conditions (10–12). Oral administration of some LAB strains has been shown to stimulate innate immunity at mucosal sites and to potentiate systemic immune responses against pathogenic bacteria or viruses (13–17). In addition to resident LAB, orally administrated LAB, although inactive, have a substantial effect on the regulation of immunity. We recently described an anti-inflammatory mechanism exclusive to LAB strains. Most LAB strains contain large amounts of double-stranded RNA and are sensed by the endosomal toll-like receptor (TLR) 3 on intestinal dendritic cells to produce interferon-β. This innate sensing procedure contributes to anti-inflammatory and protective immune responses both locally and systemically; therefore, both live and inactive LAB can be utilized as effective probiotics (12). Functional maturation of the immune system is largely dependent on mucosal biological events, and our findings suggest a co-evolutional process through a long-term mutualism between LAB and the immune system. We have demonstrated that *Lactococci* tolerates bile acids and low pH and adheres to human enterocyte-like Caco-2 cells (18). We have not, however, determined the mechanism of interaction between LAB and intestinal epithelial cells (IECs).

Intestinal epithelial cells communicate with commensal microbes and probiotics and potentiate immune responses *via* cytokines and antigen delivery (19). Probiotics trigger signaling pathways in IECs, such as NF-κB and MAP kinase, which affect the immune response and integrity of the mucosal surface barrier. However, it is difficult to monitor their biological events in real time *in vivo.* This issue became an obstacle in our initial study on the interaction between probiotics and IECs. Thus, it would be of great value to develop a reliable analytical system for intravital imaging of IECs.

Calcium ions (Ca^{2+}) are universal second messengers performing multiple functions in most cells. In the immune system, stimulation of immunological receptors, including B-cell antigen and cytokine receptors, induces intracellular Ca^{2+} mobilization concomitant with other signaling events such as phosphorylation of cellular substrates (20–24). To visualize Ca^{2+} signaling *in vivo*, we generated a conditional Föster/fluorescent resonance energy transfer (FRET)-based calcium biosensor Yellow Cameleon 3.60 (YC3.60) transgenic mice (25). YC3.60 is a double-chromophore indicator that employs FRET between a cyan fluorescent protein (CFP) and a circularly permuted variant of the yellow fluorescent protein (YFP) Venus (26). Ca^{2+} signaling can be monitored by measuring the ratio of YFP to CFP (YFP/CFP). FRET-based ratiometric indicators including YC3.60 can be corrected for unequal sensor expression and motion-derived changes in fluorescent intensity. Therefore, ratiometric sensors, such as YC3.60, are suitable for *in vivo* whole-body imaging in mice. Accordingly, we have recently established 5D (*x*, *y*, *z*, time, and Ca^{2+} signal) live imaging of immunological tissues including those in bone marrow and Peyer's patches (25).

Here, we applied our system to detect probiotic-mediated Ca^{2+} signaling in IECs *in vivo* and found differences between the two types of Gram-positive probiotic bacteria, *Lactococcus lactis* and *Bacillus subtilis* var. *natto.* Our results suggest, for the first time, that probiotic strains stimulate small IECs *via* intravital observations; in addition, these results facilitate the understanding of probiotic-mediated immunoregulatory mechanisms.

MATERIALS AND METHODS

Mice

The conditional YC3.60 expression transgenic mouse line has been previously described (25). The floxed YC3.60 reporter ($YC3.60^{flox}$) mouse line was crossed with a CD19-Cre mouse line (27), which resulted in $CD19^+$ cell-specific YC3.60 expression in $YC3.60^{flox}$/CD19-Cre mice due to the loss of the loxP-flanked neomycin cassette. The $YC3.60^{flox}$ mouse line was crossed with a CAG-Cre (28) mouse line, which expresses the Cre gene ubiquitously. These mice were maintained in our animal facility under specific pathogen-free (SPF) conditions in accordance with the guidelines of the Tokyo Medical and Dental University for animal care. These procedures have been approved by the Committee of the Tokyo Medical and Dental University for animal care.

Germ-free BALB/cA mice were bred at the Laboratory of Veterinary Public Health, the University of Tokyo, and were used as foster mothers. Germ-free animals were kept in flexible vinyl isolators in a room at 24°C, relative humidity of 60%, and 12 h periods of light and dark, and were fed a CMF-pelleted diet (Oriental Yeast Co., Tokyo, Japan) sterilized by γ-irradiation at a dose of 50 kGy. For the generation of germ-free mice with ubiquitous YC3.60 expression, *in vitro* fertilization and cesarean operation were performed as described below. Female mice with ubiquitous YC3.60 expression were superovulated by an intraperitoneal injection of 7.5 IU eCG followed by 7.5 IU hCG at an interval of 48 h. Eggs were collected from sacrificed female mice and fertilized with the sperm of male mice with ubiquitous YC3.60 expression in HTF medium (ARK Resource, Kumamoto, Japan). After overnight culture in the KSOM medium (ARK Resource), two-cell embryos were transferred into the oviducts of pseudopregnant female ICR mice. The estimated delivery date was controlled by a subcutaneous injection of Progehorrmon (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan). The surrogate mothers were sacrificed at the fetal age of 19.5 days by cervical dislocation, and the uterus was aseptically removed with clamps at the top of each uterine horn and the base of the uterus close to the cervix. The uterus was introduced into an isolator for operation through a germicidal trap with 2% peracetic acid solution kept at 37°C. The uterus was cut with scissors, and pups were removed. Their breathing was stimulated, and they were cleaned with dry gauze. After the pups started breathing normally, they were transferred to the isolator with their foster mothers. The germ status was checked once a month. These procedures were

approved by the Committee for Care of Laboratory Animals in the Graduate School of Agricultural and Life Sciences at the University of Tokyo.

Probiotic Bacteria

Lactococcus lactis subsp. *cremoris* C60 (29) was cultured in MRS broth (BD Difco) for 20 h at 30°C (late-log phase) at the National Institute of Advanced Industrial Science and Technology (AIST). The bacteria were harvested, washed two times, and resuspended in sterile saline. The suspensions were then heated for 30 min at 70°C (heat-killed) and were stored at −80°C. Heat-killed *B. subtilis* var. *natto* TTCC12 (late-log phase) were kindly provided from Takano Foods Co. Ltd. and were stored at −80°C.

Flow Cytometry

Calcium ions mobilization was analyzed using flow cytometry. Ca^{2+} mobilization in YC3.60-expressing cells was analyzed by flow cytometry using CyAn ADP™ (Beckman Coulter) as previously described (25). Antibodies with the following specificity of CD19-Alexa647 and B220-Alexa647 (BioLegend) were used.

Intravital and *In Vitro* Microscope

Intestinal epithelial cells from anesthetized mice were imaged. Small intestinal tracts were surgically opened lengthwise, placed on a cover glass, and immobilized on a microscope stage. For image acquisition, a Nikon A1 laser-scanning confocal microscope with a 20× objective and NIS-Elements AR software was used as previously described (25). We used a dichronic mirrors (DM457/514) and two bandpass emission filters (482/35 for CFP, 540/30 for YFP). YFP/CFP ratio was obtained by excitation at 458 nm. Images of purified spleen cells in PBS were also obtained as above. Acquired images were analyzed with NIS-Elements software (Nikon).

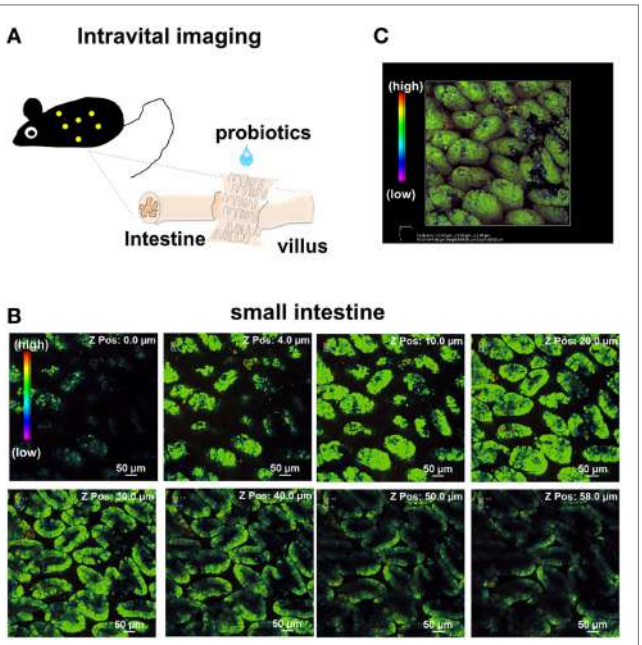

FIGURE 1 | Structure of intestinal villi in a mouse under ubiquitous YC3.60 expression. **(A)** Schematic method of intravital imaging of intestinal epithelial cells (IECs). **(B)** *Z*-stack analysis of epithelial cells in the intestinal tract. Intravital imaging of small intestinal villi in the jejunum was performed using confocal lazar microscopy. Ratiometric images (yellow fluorescent protein/cyan fluorescent protein at excitation of 458 nm) are shown. *Z*-stack images of 2-µm intervals up to a depth of 58 µm were obtained. Only representative images are shown. A rainbow parameter indicates relative Ca^{2+} concentration; scale bar, 50 µm. **(C)** 3D structures of small IECs with intracellular Ca^{2+} concentrations. 3D images based on *Z*-stack images **(B)** were obtained using NIS-Elements software. Shown are representative results from three mice.

RESULTS

Establishment of *In Vivo* Ca^{2+} Signaling Detection System in Intestinal Gut Epithelial Cells

We previously established an intravital imaging system of Ca^{2+} signaling in lymphoid tissues, such as in Peyer's patches, spleen, and bone marrow (25). To visualize Ca^{2+} signaling in IECs, we surgically opened the small intestinal tract of the mice with ubiquitous YC3.60 expression, fixed a cover glass on it, and placed it on the stage of the confocal microscope (**Figure 1A**). Images of the villi in the middle of the small intestine of the mice with ubiquitous YC3.60 expression are shown in **Figure 1B**. Images of over 50 µm from top of the villi to the basal were obtained. Reconstructed 3D structures showed that almost the entire length of the small intestinal villi could be visualized (**Figure 1C**). There were no salient differences in intracellular Ca^{2+} concentration among the total epithelial cells, and they included heterogeneous minor subpopulations, such as goblet cells, enteroendocrine cells, and tuft cells (30, 31).

Intravital imaging of the IECs showed sporadic but relatively minute Ca^{2+} signaling in some regions under steady-state conditions (**Figure 2A**). Less than 1% IECs exhibited spontaneous Ca^{2+} signaling (**Figure 2B**). To determine if the perceptive Ca^{2+} signaling response is observed in this system, we first tested ionomycin, as a positive control, on the stimuli. Upon the addition of ionomycin, transient Ca^{2+} elevation was observed in many IECs (**Figures 2C,D**). Thus, a system was established to detect *in vivo* real-time Ca^{2+} signaling of IECs.

Effect of Probiotics on Ca^{2+} Signaling in the IECs of the Mice with Ubiquitous YC3.60 Expression *In Vivo*

Lactococcus lactis (18, 29) regulate immune responses by inducing cytokines in dendritic cells and *B. subtilis natto* regulate gut flora and immunity (32–34). We tested whether these probiotics induce Ca^{2+} signaling in IECs. Intravital imaging of IECs exhibited Ca^{2+} signaling upon *B. subtilis natto* treatment (**Figure 3A**; Video S1 in Supplementary Material). *Bacillus subtilis natto* induced gradual and sustained elevation of intracellular Ca^{2+} concentration in most cells (**Figures 3A,B**). **Figure 3C** shows that intracellular Ca^{2+} concentration in IECs was strikingly increased after adding *B. subtilis natto*. Thus, the kinetics of *B. subtilis natto*-mediated Ca^{2+} signaling in IECs is clearly distinct from that

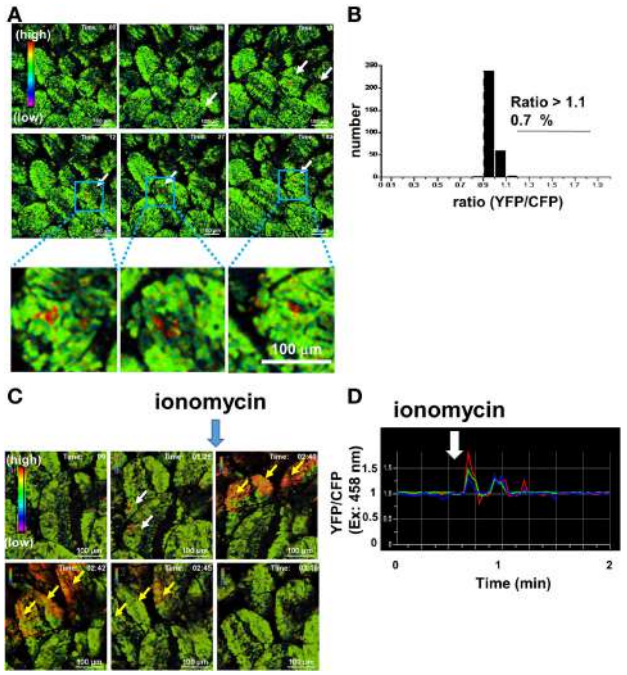

FIGURE 2 | Intravital Ca^{2+} signaling images mediated by ionomycin in the intestinal tract of a mouse under ubiquitous YC3.60 expression. **(A)** Representative Ca^{2+} signaling images in the intestinal tract of a mouse under ubiquitous YC3.60 expression without any stimulation. Ratiometric images [yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP) at excitation of 458 nm] are shown. The rainbow parameter indicates relative Ca^{2+} concentration. **(B)** Distribution of time-integrated intracellular Ca^{2+} concentrations of randomly selected regions. $N = 10$; frame = 30. **(C)** Representative Ca^{2+} signaling images in the intestinal tract of a mouse under ubiquitous YC3.60 expression with ionomycin. Ratiometric images (YFP/CFP at excitation of 458 nm) are shown. Five micromolars of ionomycin in PBS were added at the indicated time point. A rainbow parameter indicates relative Ca^{2+} concentration; scale bar, 100 μm; frame = 85. **(D)** Ratiometric intensities (YFP/CFP at excitation of 458 nm) of indicated regions, represented by yellow arrows in **(C)** ($n = 3$), were measured for 2 min at 2-s intervals. Spontaneous Ca^{2+} signals are indicated by white arrows. Shown are representative results from two mice.

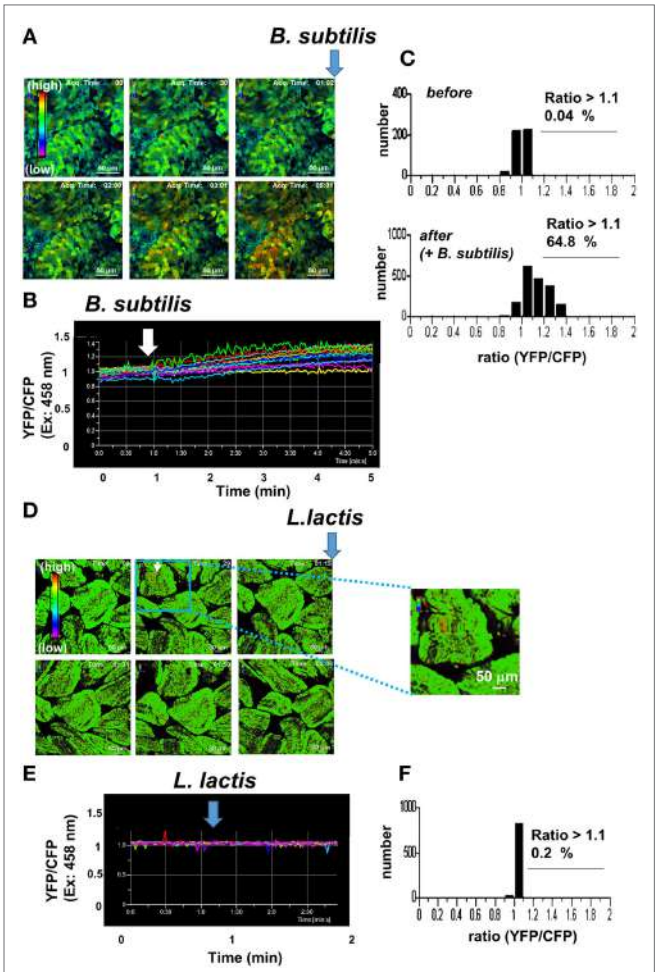

FIGURE 3 | Intravital Ca^{2+} signaling images mediated by probiotics in the intestinal tract of a mouse under ubiquitous YC3.60 expression. **(A)** Representative Ca^{2+} signaling images in the intestinal tract of a mouse under ubiquitous YC3.60 expression and specific pathogen-free (SPF) conditions. Ratiometric images [yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP) at excitation of 458 nm] are shown. The 0.1 ml of *Bacillus subtilis natto* in PBS (10^9 cells/ml) was added at the indicated time point. A rainbow parameter indicates relative Ca^{2+} concentration. **(B)** Time course for fluorescence intensities of YFP/CFP on excitation at 458 nm. Randomly selected regions ($n = 10$) were measured. Scale bar, 50 μm; frame = 151. Spontaneous Ca^{2+} signals are indicated by arrows. **(C)** Distribution of time-integrated intracellular Ca^{2+} concentrations of randomly selected regions before (upper panel) and after (lower panel) stimulation; $n = 10$. **(D)** Representative Ca^{2+} signaling images in the intestinal tract of a mouse under ubiquitous YC3.60 expression and SPF conditions. Ratiometric images (YFP/CFP at excitation of 458 nm) are shown. The 0.1 ml of *Lactococcus lactis* in PBS (10^9 cells/ml) was added at the indicated time point. **(E)** Time course for fluorescence intensities of YFP/CFP on excitation at 458 nm; frame = 85. **(F)** Distribution of time-integrated intracellular Ca^{2+} concentrations of randomly selected regions; $n = 10$. Shown are representative results from three mice.

observed under steady-state conditions (**Figure 2A**). One LAB strain, *L. lactis* C60, did not induce Ca^{2+} signaling in IECs except for spontaneous signals (**Figures 3D–F**). This result is surprising as both *B. subtilis* and LAB are Gram-positive bacteria and well-known probiotics; yet the responses of IECs in SPF mice were distinct in inducing Ca^{2+} signaling. LAB compose a major part of small intestinal commensal flora, and therefore, chronic exposure to the bacteria species may have induced hyporesponsiveness of IECs against LAB.

LAB Induces Ca^{2+} Signaling in IECs under Germ-Free Conditions

As *Lactococcus* is a related genus of *Enterococcus*, a constituent of gut-resident LAB in the small intestine (7), it may constantly stimulate IECs under steady-state conditions. IECs may refrain from responding to heat-killed *L. lactis* C60 and induce Ca^{2+} signaling due to chronic microbial stimuli by *Enterococci*. We attempted to clarify whether small intestinal microenvironments, especially gut commensal flora, modulate the responsiveness of IECs against LAB.

To this end, we generated germ-free YC3.60 mice to determine whether *L. lactis* can induce Ca^{2+} signaling in IECs in the absence of gut microbiota. As shown in **Figure 4** and Video S2 in Supplementary Material, *L. lactis* C60 induced sustained intracellular Ca^{2+} elevation as *B. subtilis natto*, indicating that *L. lactis* C60 can stimulate IECs directly in the absence of gut microbiota. Many IECs were stimulated by adding *L. lactis* under germ-free conditions (**Figure 4B**). Furthermore, the IECs in germ-free mice exhibited sporadic Ca^{2+} signals under steady-state conditions regardless of *L. lactis* stimulation (**Figures 4A,C**). The frequency of sporadic Ca^{2+} signals in germ-free mice (**Figure 4B**, left panel) is higher than that in the SPF mice (**Figure 2A**).

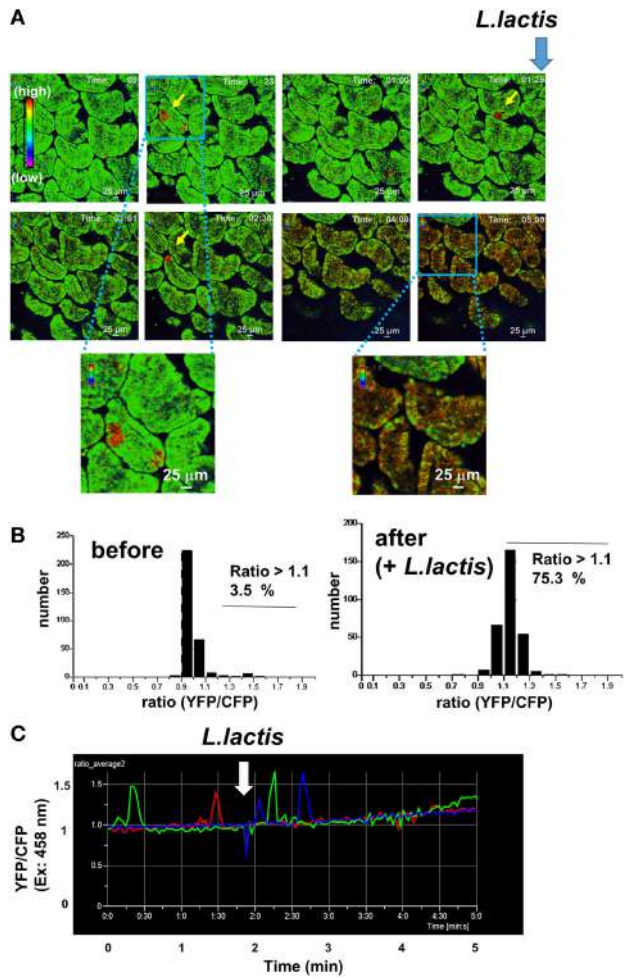

FIGURE 4 | Intravital Ca^{2+} signaling images mediated by *Lactococcus lactis* in the intestinal tract of a mouse under ubiquitous YC3.60 expression. **(A)** Representative Ca^{2+} signaling images in the intestinal tract of a mouse under ubiquitous YC3.60 expression and germ-free conditions. Ratiometric images [yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP) at excitation of 458 nm] are shown. The 0.1 ml of *L. lactis* in PBS (10^9 cells/ml) was added at the indicated time point. A rainbow parameter indicates relative Ca^{2+} concentration. Scale bar, 25 μm; frame = 145. Spontaneous Ca^{2+} signals are indicated by arrows. **(B)** Distribution of time-integrated intracellular Ca^{2+} concentrations of randomly selected regions before (left) and after (right) stimulation. **(C)** Time course for fluorescence intensities of YFP/CFP on excitation at 458 nm in the indicated region is represented by the yellow arrow in **(A)**; $n = 3$. Shown are representative results from three mice.

L. Lactis Induces Ca^{2+} Signaling in B Cells *In Vitro*

Probiotics directly stimulate various immune cells such as dendritic cells, macrophages, NK cells, and T cells (11, 35). We prepared primary B cells from the spleens of YC3.60^{flox}/CD19-Cre mice and stimulated with *B. subtilis natto* (**Figures 5A,B**) or *L. lactis* (**Figures 5C,D**). Upon stimulation, both *B. subtilis natto* and *L. lactis* induced Ca^{2+} mobilization in primary B cells (**Figure 5**), confirming their direct stimulation of B cells. Time-lapse observation of single cells was useful to clarify heterogeneity in the kinetics of Ca^{2+} signaling (**Figures 5B,D**).

DISCUSSION

Here, we establish a real-time visualization system of Ca^{2+} signaling in small IECs *in vivo* to monitor food signals. By using this system, we show that *B. subtilis natto* triggers Ca^{2+} signaling in IECs and that *L. lactis* evokes Ca^{2+} signaling in gut tissue under germ-free conditions but not under SPF conditions. These results suggest that gut microbiota regulate their responses against orally administered probiotics.

In this study, we successfully visualize probiotic-mediated Ca^{2+} signaling in IECs based on the 5D (x, y, z, time, and Ca^{2+}) live imaging system in YC3.60 mice (25). A calcium biosensor YC3.60 is a double-chromophore indicator that employs FRET between CFP and YFP mutants (Venus) (26). Ca^{2+} signaling can be monitored by measuring YFP/CFP when CFP is excited. Motion-induced artifacts or unequal biosensor expression are corrected by an internal control in the denominator of the ratio. Thus, the ratiometric sensor YC3.60 is suitable for *in vivo* imaging of the gut, which exhibits vigorous motion with peristalsis in addition to beating and breathing. Moreover, 5D live imaging of tissues enables time-lapse monitoring of dynamic Ca^{2+} signaling in single cells, cell–cell interactions, and other segments of interests. Response of these tissues is quantified and integrated over the desired time span as shown in **Figures 2B**, **3C,F** and **4B**.

Probiotic *B. subtilis natto*, but not *L. lactis*, triggers Ca^{2+} signaling in the gut epithelium in SPF mice, although both of them are capable of stimulating spleen B cells from mice with the same microbial conditions. Since *L. lactis* C60 stimulates IECs in the absence of gut microbiota in germ-free mice, the gut microbiota may shape the responsiveness of IECs against LAB.

Intestinal epithelial cells express a series of pattern recognition receptors (PRRs) including TLRs, nucleotide-binding sites, leucine-rich repeat-containing receptors, and retinoic acid-inducible gene-I-like receptors (36). Bacterial components stimulate these PRRs and regulate IRFs, NF-κB, and/or the MAP kinase signaling pathway. One study showed that IECs from germ-free mice show a reduction in TLR expression (37). Another study reported that immunobiotic strains regulate the expression and activity of TLRs in IECs (38). These findings suggest that microbial conditions shape the homeostatic regulation of functional PRRs. Since

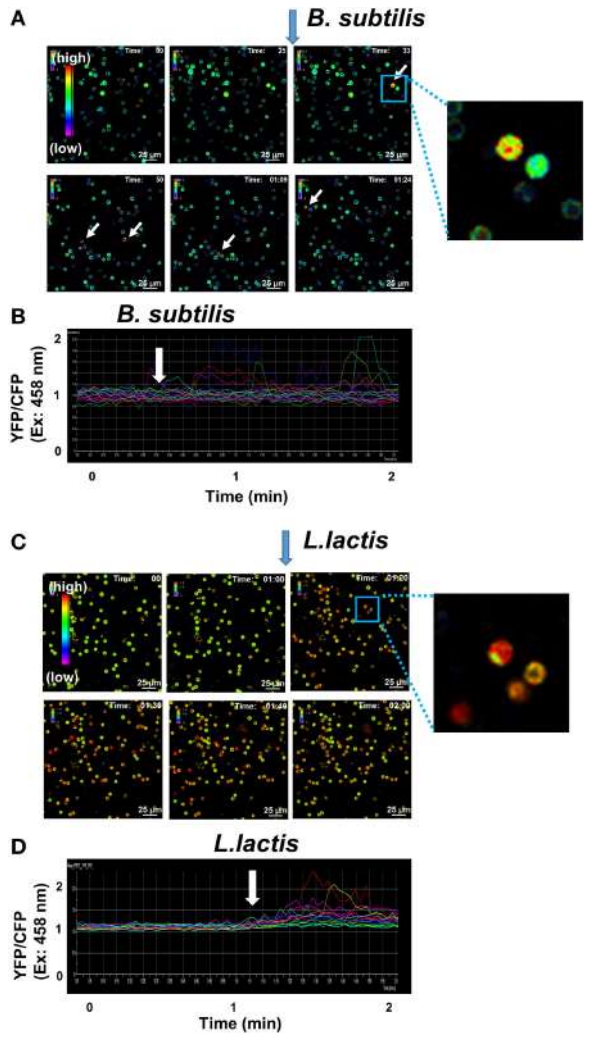

FIGURE 5 | Ca^{2+} signaling images mediated by *probiotics* in B cells *in vitro*. **(A)** Representative Ca^{2+} signaling images in spleen B cells form CD19-Cre/YC3.60 mice. Ratiometric images [YFP/cyan fluorescent protein (CFP) at excitation of 458 nm] are shown. Ten microliters of *Bacillus subtilis natto* in PBS (10^9 cells/ml) were added to the cell culture (0.2 ml) at the indicated time point. A rainbow parameter indicates relative Ca^{2+} concentration. **(B)** The time course for fluorescence intensities of YFP/CFP on excitation at 458 nm in the cells (n = 15). Scale bar, 25 μm; frame = 56. **(C)** Representative Ca^{2+} signaling images in spleen B cells of CD19-Cre/YC3.60 expression in mice. Ratiometric images (YFP/CFP at excitation of 458 nm) are shown. Ten microliters of *Lactococcus lactis* in PBS (10^9 cells/ml) were added to the cell culture at the indicated time point. A rainbow parameter indicates relative Ca^{2+} concentration. **(D)** The time course for fluorescence intensities of YFP/CFP on excitation at 458 nm in the cells (n = 15). Scale bar, 25 μm; frame = 61. Shown are representative results of three experiments.

Lactococcus is similar to *Enterococcus*, a major member of small intestinal commensal LAB, probably IECs, at least in part, is hyporesponsive to this symbiotic genus of bacteria. We hypothesize that due to this hyporesponsiveness, further stimulation with *L. lactis* does not induce visible Ca^{2+} signaling despite the expression of PRRs on the IECs. As reported, probiotic strains tolerate IECs (38, 39); such causal relationships may also explain the frequent and sporadic Ca^{2+} signaling observed in IECs of germ-free mice under steady-state conditions (**Figure 4**). The molecular mechanisms underlying these observations, however, remain unclear.

We find that IECs exhibit sporadic transient Ca^{2+} signaling, although we do not know the precise mechanism of this action. The signals appear to be more striking in the germ-free mice than in the SPF mice, suggesting that these signals are mediated by the gut microbiota or endocrine systems (40).

B cells also express PRRs and are directly stimulated by microbial components (41). Although *L. lactis* C60 fails to induce Ca^{2+} signaling in IECs under SPF conditions, it strongly induces Ca^{2+} signaling in spleen B cells from SPF mice. In contrast, *B. subtilis natto* induces Ca^{2+} signaling in both IECs *in vivo* and B cells *in vitro*. It is not known whether the different reactivity against two types of probiotics can be attributed to the difference in the methods between the intravital and *in vitro* assay, to the skewed influence of stimuli including commensal flora and diet on local and systemic immune cells, or to the outcome of strain difference of probiotics. Additional studies are required to evaluate these results further.

Understanding the function of IECs is important to evaluate immune responses, since stimulated IECs produce cytokines and/or chemokines (11, 23). Goblet cells and M cells deliver antigens to dendritic cells (42, 43). Beneficial probiotic signaling may be transferred to immune cells through these mechanisms, resulting in the regulation of immune tolerance or response. In this study, we show that an intravital imaging system using YC3.60 mice allows for the detection of real-time activation of IECs by probiotics. This system is proven here to be a powerful method for not only clarifying the effects of probiotics on epithelial cell–immune cell communication with stoichiometries but also detecting a subtle disorder before pathological onset (25) and developing preventive and therapeutic strategies with probiotics.

ETHICS STATEMENT

YC3.60 mice were maintained in our animal facility under SPF conditions in accordance with the guidelines of the Tokyo Medical and Dental University for animal care. IECs of anesthetized mice were imaged. Small intestinal tracts were surgically opened, immobilized on a microscope stage, and maintained. Then, images were obtained by a confocal laser microscopy. These procedures have been approved by the Committee of the Tokyo Medical and Dental University for animal care. Germ-free BALB/cA mice used as foster mothers were bred at the Laboratory of Veterinary Public Health, the University of Tokyo. All the germ-free animals were kept in flexible vinyl isolators in a room with 24°C, relative humidity of 60% and 12-h periods of light and dark and fed CMF-pelleted diet (Oriental Yeast Co., Tokyo, Japan) sterilized by γ-irradiation at dose of 50 kGy. For the generation of germ-free ubiquitous YC3.60 expression mice, *in vitro* fertilization and caesarean operation were performed as described below. Female ubiquitous YC3.60 expression mice were superovulated by intraperitoneal injection of 7.5 IU eCG followed by 7.5 IU hCG at an interval of 48 h. Eggs were collected

from sacrificed female mice and fertilized with sperm of male ubiquitous YC3.60 expression mice in HTF medium (ARK Resource, Kumamoto, Japan). After over night culture in KSOM medium (ARK Resource), two-cell embryos were transferred into oviduct of pseudopregnant female ICR mice. Estimated delivery date was controlled by subcutaneous injection of Progehorrmon (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan). The surrogate mothers were sacrificed at the fetal age of 21st day by cervical dislocation and "uterine package" was aseptically removed with clamps at the top of each uterine horn and the base of the uterus close to the cervix. The "uterine package" was introduced into isolator for operation through germicidal trap with 2% peracetic acid solution kept at 37°C. Then, "uterine package" was cut open with scissors and pups were taken out. The pups were stimulated breathing while cleaning them with dry gauze. After the pups started breathing normally, pups were transferred to the isolator with foster mothers. The germ-free status was check once a month. These procedures have been approved by the Committee for Care of Laboratory Animals in the Graduate School of Agricultural and Life Sciences at the University of Tokyo. There is no additional consideration.

AUTHOR CONTRIBUTIONS

KH, NT, YA, NO, AM, SY, HK, and TA designed the research; TA and NT wrote the manuscript; KH, SK, YA, TU, NH, TK, YW, HK-N, SY, and TA performed the experiments, analyzed the data, and prepared the figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00601/full#supplementary-material.

VIDEO S1 | Intravital Ca^{2+} signaling images mediated by *Bacillus subtilis natto* in the intestinal tract of a mouse under ubiquitous YC3.60 expression and specific pathogen-free conditions. *B. subtilis natto* in PBS (10^9 cells/ml) in PBS was added at the time point of 1 min 30 s. A rainbow parameter indicates relative Ca^{2+} concentration. The time course for fluorescence intensity of yellow fluorescent protein/cyan fluorescent protein on excitation at 458 nm was performed. Scale bar, 50 μm; frame = 151.

VIDEO S2 | Intravital Ca^{2+} signaling images mediated by *Lactococcus lactis* in the intestinal tract of a ubiquitous YC3.60 expression mouse under germ-free conditions. *L. lactis* in PBS (10^9 cells/ml) was added at the time point of 1 min 50 s. A rainbow parameter indicates relative Ca^{2+} concentration. The time course for fluorescence intensity of yellow fluorescent protein/cyan fluorescent protein on excitation at 458 nm was performed. Scale bar, 25 μm; frame = 145.

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REVIEW
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Respiratory Antiviral Immunity and Immunobiotics: Beneficial Effects on Inflammation-Coagulation Interaction during Influenza Virus Infection

Hortensia Zelaya[1,2], Susana Alvarez[1,2,3], Haruki Kitazawa[4,5] and Julio Villena[1,3,4]**

[1] Immunobiotics Research Group, Tucuman, Argentina, [2] Institute of Applied Biochemistry, National University of Tucuman, Tucuman, Argentina, [3] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [4] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [5] Livestock Immunology Unit, International Education and Research Center for Food and Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

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***Correspondence:**
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp;
Julio Villena
jcvillena@cerela.org.ar

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Influenza virus (IFV) is a major respiratory pathogen of global importance, and the cause of a high degree of morbidity and mortality, especially in high-risk populations such as infants, elderly, and immunocompromised hosts. Given its high capacity to change antigenically, acquired immunity is often not effective to limit IFV infection and therefore vaccination must be constantly redesigned to achieve effective protection. Improvement of respiratory and systemic innate immune mechanisms has been proposed to reduce the incidence and severity of IFV disease. In the last decade, several research works have demonstrated that microbes with the capacity to modulate the mucosal immune system (immunobiotics) are a potential alternative to beneficially modulate the outcome of IFV infection. This review provides an update of the current status on the modulation of respiratory immunity by orally and nasally administered immunobiotics, and their beneficial impact on IFV clearance and inflammatory-mediated lung tissue damage. In particular, we describe the research of our group that investigated the influence of immunobiotics on inflammation–coagulation interactions during IFV infection. Studies have clearly demonstrated that hostile inflammation is accompanied by dysfunctional coagulation in respiratory IFV disease, and our investigations have proved that some immunobiotic strains are able to reduce viral disease severity through their capacity to modulate the immune-coagulative responses in the respiratory tract.

Keywords: immunobiotics, influenza virus, inflammation, coagulation, respiratory immunity

INTRODUCTION

Influenza virus (IFV) is a member of the *Orthomyxoviridae* family that contains a negative-sense, single-stranded, segmented RNA genome protected by a capsid of viral ribonucleoproteins. This virus is categorized into subtypes based on the expression of hemagglutinin (HA) and neuraminidase on the surface of the viral envelope.

Influenza is a highly contagious viral infection that has a substantial impact on global health and IFV is a major respiratory pathogen that causes a high degree of morbidity and mortality, especially in high-risk populations such as infants, elderly, and immunocompromised hosts. Given the high capacity of IFV to change antigenically, acquired immunity is often not effective to limit infection and therefore vaccination must be constantly redesigned to achieve protection. Improvement of respiratory and systemic innate immune mechanisms has been proposed to reduce the incidence and severity of IFV disease.

In the last decade, several research works have demonstrated that microbes with the capacity to modulate the mucosal immune system (immunobiotics) are a potential alternative to beneficially modulate the outcome of IFV infection. This review provides an update of the current status on the modulation of respiratory immunity by orally and nasally administered immunobiotics, and their beneficial impact on IFV clearance and inflammatory-mediated lung tissue damage. In particular, we describe the research of our group that investigated the influence of immunobiotics on inflammation–coagulation interactions during IFV infection. Studies have clearly demonstrated that hostile inflammation is accompanied by dysfunctional coagulation in respiratory IFV disease, and our investigations have proved that some immunobiotic strains are able to reduce viral disease severity through their capacity to modulate the immune-coagulative responses in the respiratory tract.

RESPIRATORY IMMUNE RESPONSE AND IFV

The first barrier that protects the host against IFV infection is the respiratory epithelium through its capacity to recognize the viral attack. When IFV successfully overcomes the respiratory barrier constituted by the mucus layer and the ciliar movement, it mediates its attachment and internalization into respiratory epithelial cells to start its replication (1). During the viral attack, several pathogen-associated molecular patterns (PAMPs) are exposed and recognized by pattern-recognition receptors (PRRs) expressed in respiratory cells (**Figure 1**). It is now well established that the most important PRRs involved in the recognition of IFV are the Toll-like receptor (TLR)-3 and TLR7 and the RNA recognition protein RIG-1 (2). TLR3 is expressed in endosomes and is able to recognize viral double-stranded RNA (dsRNA) that is produced during viral replication; while endosomal TLR7 and cytoplasmic RIG-I recognize single-stranded RNA (ssRNA). RIG-I signals through mitochondrial antiviral signaling protein. The PAMPs–PPRs interaction leads to the activation of several signaling pathways that induce the activation of nuclear factor κB (NF-κB) and interferon (IFN) regulatory factor 3 (IRF3) and the production of type I and III IFNs and inflammatory cytokines (2).

Type I IFNs, especially IFN-β, produced and released during the earlier stages of IFV infection are key to develop an antiviral state in the respiratory tract. It was reported that human bronchial epithelial cells release preformed IFN-β in response to IFV challenge inducing a protective role (3). IFNs produced by infected cells are able to act in a paracrine or autocrine manner activating their receptors (IFNAR) and increasing the expression of hundreds of genes that counteract viral replication. Functional genomic studies have identified several of the IFN-induced factors that have important roles in controlling IFV replication (2) including the IFN-inducible transmembrane proteins 1, 2, and 3 (4), MX1 proteins (5), and 2′,5′-oligoadenylate synthetase (OAS)-RNAaseL system (6).

Proinflammatory cytokines and chemokines produced as a result of TLR3 and RIG-I activation during IFV infection are also important for the generation of the respiratory antiviral innate immune response. Infection of epithelial cells by IFV increases the expression of TNF-α, IL-6, IL-8, CCL2 (MIP-1), CCL5 (RANTES), CCL3 (MIP-1α), and CXCL10 (IP-10) (7). The production of these cytokines is complemented by activity of inflammasomes that induce the activation of caspase-1 and promote the generation of the active forms of IL-1β and IL-18 (**Figure 1**). IFV has been shown to activate mainly the NLRP3 inflammasome which is essential for the protection against the virus since several studies demonstrated that mice lacking NLRP3 or caspase-1 have decreased IL-1β and IL-18 secretion and increased mortality after IFV challenge (8–10).

The proinflammatory cytokines and chemokines are responsible for the activation of resident immune cells such as innate lymphoid cells, alveolar macrophages, and dendritic cells (DCs) as well as for the recruitment of neutrophils, macrophages, and lymphocytes into the respiratory tract (2, 7) (**Figure 2**). Respiratory cells infected with IFV express HA on their surface that is important for its recognition by NK cells (11). It was established that HA expressed by the infected cells is recognized by NKp44 and PKp46 receptors of NK cells that then mediated the lysis of IFV-infected cells (12). Macrophages activated during IFV infection produce IFNs, IL-6, TNF-α, and nitric oxide synthase that amplify the inflammatory response. In addition, macrophages limit the viral spread by the elimination of apoptotic-infected cells and through phagocyte-mediated opsonophagocytosis of IFV (7). The production of proinflammatory cytokines during the generation of the respiratory innate immune response against IFV also conditions the adaptive immune response, which includes the production of virus-specific systemic and mucosal antibodies as well as the induction of specific T cell responses (13).

After exposure to IFV there is an activation of antibody responses in the respiratory tract. Upper airway exposure results primarily in an IgA response while the contact of IFV with the deep lung induces an increased production of pathogen-specific IgG (14). Following exposure to IFV in the airways there is an antigen uptake and processing by DCs, activation of $CD4^+$ Th cells, and generation of IgA-producing plasma cells that populate airway lamina propria. Secretory IgA has a non-inflammatory protective function since these antibodies can bind to virus without activating complement or stimulating the release of inflammatory mediators by innate immune cells (14, 15). IgA prevents IFV from adhering to the epithelial surface by inducing viral agglutination, and masking adhesion epitopes. In the deep lung, when IFV reach the alveolar space, there is a differentiation and expansion of antibody-secreting plasma cells that are committed to the production of IgG. Induction of

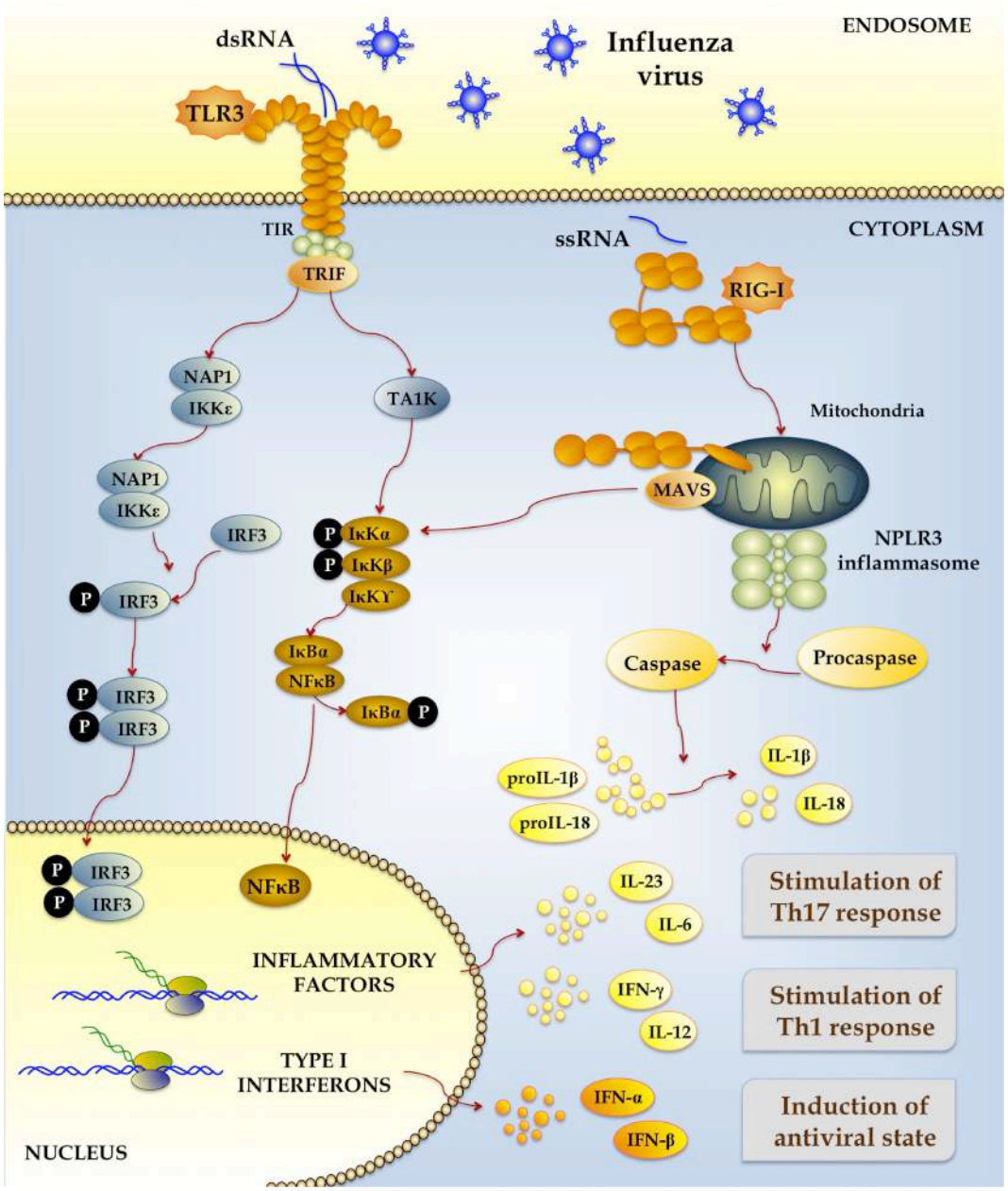

FIGURE 1 | Signaling pathways activated by the recognition of influenza virus-associated molecular patterns by pattern-recognition receptors expressed in respiratory epithelial cells and immune cells.

neutralizing respiratory and serum IgG antibodies is a key event in the defense against influenza infection since IgG prevents systemic spread (16). Influenza infection in the lungs also activates the cellular adaptive immune response by stimulating the production of IFN-γ by Th1 cells that effectively activate $CD8^+$ T cells and macrophages, which clear virus and infected cells from the lungs (17). Therefore, during uncomplicated influenza, adaptive immune response ultimately results in clearance of IFV from the lungs through the activity of virus-specific antibodies and $CD4^+$ and $CD8^+$ T cells.

ROLE OF MICROBIOTA ON IFV INFECTION

The gut microbiome, which is defined as the collective group of microorganisms and their associated genes within the intestinal tract, is considered as a key player in the modulation of host intestinal immune responses (18, 19). In fact, the impact of gut commensal bacteria on the innate and adaptive immune responses to enteric pathogens has been recognized conclusively (20–22). However, the effect of gut microbiome on the immune

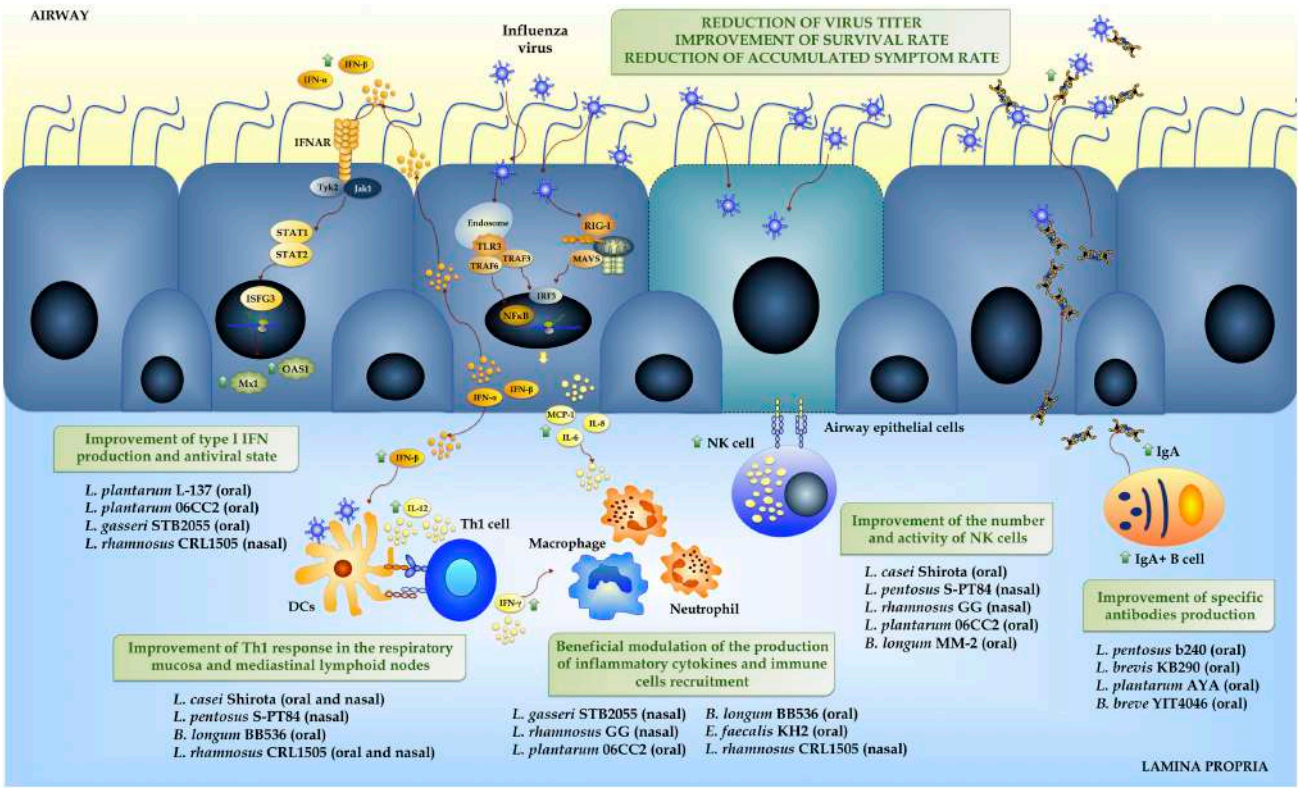

FIGURE 2 | Innate immune response against influenza virus in the respiratory mucosa mediated by the recognition of viral-associated molecular patterns by pattern-recognition receptors expressed in respiratory epithelial cells. Beneficial effects of immunobiotics administration on the resistance and immune response against Influenza Virus in the respiratory mucosa.

responses in distal mucosal sites and its impact in the outcome of respiratory infections has recently been exposed. In this regard, some studies have demonstrated an important role for intestinal microbiota in maintaining respiratory antiviral immunity against IFV (23, 24).

Iwasaki and colleagues observed that commensal bacteria within the gut, especially gram-positive bacterial populations, had an important role in supporting an appropriate immune response to IFV infection in the respiratory tract (23). The work demonstrated that oral antibiotic treatments impaired the resistance of mice to the intranasal infection with IFV as noted by the elevated lung viral titers when compared to non-antibiotic-treated animals. Results indicated that gut gram-positive bacteria provided protection by triggering an adequate inflammatory response through inflammasomes activation. In antibiotic-treated mice, synthesis of pro-IL-1β, pro-IL-18, and NLRP3 was impaired even at the steady state. In addition, depletion of gram-positive bacterial populations in the gut resulted in an alteration of the distribution and activation of respiratory DCs at steady state as well as in a diminished DCs migration from the lung to the draining lymph nodes, resulting in reduced activation of $CD8^+$ and $CD4^+$ T cells after influenza challenge (23). Alteration of respiratory DCs activities also correlated with impaired expansion of influenza-specific B cells and reduced influenza-specific antibodies.

By using germ-free and antibiotic-treated mice challenged with IFV, Abt et al. (24) showed that the absence or the alteration of intestinal microbiota induced an exacerbated weight loss, a greater drop in blood oxygen saturation, increased mortality, and elevated lung viral titers indicating a weaker ability to resist influenza. Even more, germ-free and antibiotic-treated mice infected with IFV experienced higher epithelial cell necrosis, peribronchiolar inflammation, severe bronchiole epithelial degeneration, and epithelial hyperplasia when compared to conventional animals (24). Interestingly, those effects were observed when both the PR8 strain and the X31-GP33 virus, a less pathogenic strain of IFV that causes minimal mortality and morbidity in conventional mice, were used. Consistent with the work by Ichinohe et al. (23), germ-free and antibiotic-treated mice challenged with IFV had an impaired adaptive immune response as shown by the lower influenza-specific antibodies (serum IgM and IgG), fewer number of IFV-specific T cells present in lungs, as well as a reduced capacity of specific T cells to produce effector cytokines such as TNF-α, MIP-1α, IL-2, and IFN-γ (24). Moreover, authors demonstrated that the alterations of adaptive immune responses were related to defects in the early innate immune response mediated by macrophages. In fact, transcriptional profiling and computational analyses of macrophages from antibiotic-treated mice indicated a reduced expression of antiviral genes including *Ifnb*, *Tnfa*, *Il1b*, *Irf7*, *Mx1*, and *Oas1a* when compared to

conventional mice. In addition, functional assays of macrophages from antibiotic-treated mice demonstrated that those cells had a defective response to type I IFNs and an impaired capacity to limit IFV replication (24).

The cellular and molecular mechanisms through which the gut microbiome and their derived signals maintain and modulate immune responses in distal mucosal sites are poorly understood. Two possible mechanisms that are not mutually exclusive have been proposed to explain this beneficial effect of the gut microbiome. One possibility is that distal mucosal and peripheral immune cells are directly exposed to bacterial products that activate PRRs in the steady state and help to maintain the normal immune tone. There is evidence that bacterial products from gut commensals such as peptidoglycan can be absorbed and circulate throughout the host and help to modulate the normal development of immune cells (25). In line with this hypothesis, Iwasaki and colleagues speculated that bacterial products from gut commensals trigger PRRs to stimulate immune cells systemically and that factors released by those cells supported steady-state production of pro-IL-1β, pro-IL-18, and NLR proteins. This idea was sustained by their observation that intestinal injection of TLR ligands restored immune responses to IFV in antibiotic-treated mice (23). Another possibility is that commensal bacteria may indirectly influence systemic and distal mucosal immune responses through immune factors released from the intestinal mucosa including cytokines, chemokines, and grow factors.

These research works demonstrated that the gut microbiome provides signals to sustain antiviral innate immune defense mechanisms in the respiratory tract allowing robust and efficient effector responses upon challenge by viral pathogens such as IFV. Therefore, the role of the gut microbiome in regulating respiratory antiviral immunity represents an exciting area of research that could help to provide the scientific basis for the development of novel prevention strategies for lung infectious diseases. However, several questions need to be answered to identify new alternatives to improve antiviral respiratory defenses by modulating the microbiota. How the different microbial species from the gut microbiota influence the common mucosal immune system? Which PRRs are activated by the gut microbiota to functionally modulate antiviral immunity locally and in distal mucosal sites? Which cellular functions are modulated by the microbiota after PRR activation? Has the microbiota the ability to influence immune responses to other respiratory viruses? Are similar immune mechanisms activated by the microbiota in high-risk populations (infants, elderly, immunocompromised hosts) in which respiratory viral infections are more frequent and severe? Is it possible to beneficially modulate antiviral respiratory defenses by orally administering selected microorganisms with immunomodulatory capacities? Research from the last years has provided some answers for the last question.

BENEFICIAL EFFECTS OF IMMUNOBIOTICS ON IFV INFECTION

The first studies that assessed the capacity of immunobiotics to favorably modulate the immune response against IFV focused on the humoral immunity (**Table 1**). Yasui et al. (26) reported that the oral administration of *Bifidobacterium breve* YIT4064 improved the production of anti-IFV IgG antibodies in serum of IFV-infected mice. The YIT4064 strain reduced viral titers, improved the survival rate, and decreased the severity of the symptoms associated to the influenza infection. Similarly, it was shown that orally administered non-viable *Lactobacillus pentosus* b240 (27) or viable *Lactobacillus brevis* KB290 (28) were able to improve the levels of respiratory specific IgA and IgG antibodies of mice challenged with IFV. Moreover, the improved humoral response induced by these strains correlated with significant reduction of viral titers, body weight loss, and a decrease of the alterations of physical conditions induced by IFV. More recently, Kikuchi et al. (29) demonstrated a beneficial effect on the outcome to IFV infection related to an improved respiratory humoral response in *Lactobacillus plantarum* AYA-treated mice. In addition, the work proposed a mechanism for the distal immunomodulatory activity induced by orally administered immunobiotics. Authors showed that *L. plantarum* AYA fed to mice impacted in Peyer's patches (PPs) inducing an activation of antigen presenting cells (mainly $CD11b^+$ DCs) and increasing the production of IL-6. Those changes promoted an IgM-to-IgA class switch recombination, the differentiation of IgA^+ B cells into plasma cells, and improved the production of mucosal IgA in both the intestine and the respiratory tract. Those studies show that immunobiotics are capable to modulate the production of systemic and mucosal antibodies against influenza and therefore, to enhance the humoral immune response (**Figure 2**). However, the precise mechanism by which orally administered immunobiotics induce IgA production in distant mucosal sites remains unclear.

It was also demonstrated that immunobiotics are able to improve cellular immune response against IFV (**Figure 2**). In this regard, it was reported that orally administered *Lactobacillus casei* Shirota improved the outcomes of IFV infection of aged (30) and infant mice (31) by increasing systemic and respiratory NK cell activity and improving the production of IFN-γ and TNF-α by respiratory lymphocytes. Both studies also demonstrated that IFV titers were significantly reduced in aged and infant mice treated with the Shirota strain (30, 31). Similar to the mechanism proposed to explain the improvement of humoral response, it was postulated that immunobiotic *L. casei* Shirota stimulated Th1 cells and NK cells in PPs and induced a mobilization of those cells to lungs and respiratory-associated lymphoid tissues where they produced IFN-γ and enhanced the antiviral defenses. Several other studies corroborated these findings by showing similar effects for orally administered lactobacilli (32, 33). Immunobiotic *Lactobacillus* strains (*L. gasseri* TMC0356, *L. rhamnosus* GG, or *L. plantarum* 06CC2) beneficially modulated NK cells activity and Th1 response against IFV, diminished virus titers and reduced lung pathological changes (32, 33) (**Table 1**). More recently, Kawahara et al. (34) described the improvement of respiratory antiviral response by an orally administered bifidobacteria strain. It was shown that *Bifidobacterium longum* MM-2 increased respiratory NK cell activity and IFN-γ production resulting in improved clinical symptoms, reduced mortality, and decreased virus titers after IFV challenge.

TABLE 1 | Effect of immunobiotics on influenza virus (IFV) infection in mice models.

Immunobiotic strain	Viability	Administration protocol	Challenge	Mice	Immunobiotic effects	Effect on IFV infection	Reference
Effects on humoral immune response							
Bifidobacterium breve YIT4064	Non-viable	Oral *ad libitum* administration of food with 0.05% *B. breve* YIT4064 during 15 weeks before IFV challenge. Treatment was continued for 2 weeks after infection	IFV (H1N1) strain A/PR/8/34	Six-week-old male BALB/c	Improved the production of anti-IFV IgG antibodies in serum	Reduced viral titers, improved survival rate, and decreased severity of symptoms	(26)
Lactobacillus pentosus b240	Non-viable	*L. pentosus* was administered by gavage at doses of 0.4, 2, or 10 mg per mouse per day during 21 days before IFV challenge. Treatment was continued for 2 weeks after infection	IFV (H1N1) strain A/PR/8/34	Six-week-old female BALB/c	Improved levels of respiratory IgA and IgG specific antibodies	Reduced IFV titers	(27)
Lactobacillus brevis KB290	Viable	*L. brevis* was administered by gavage at a dose of 10^9 cells per mouse per day during 14 days before IFV challenge	IFV (H1N1) strain A/PR/8/34	Six to eight-week-old female BALB/c	Improved levels of IFV-specific IgA in the respiratory tract	Reduced body weight loss and decreased alterations of physical conditions	(28)
Lactobacillus plantarum AYA	Non-viable	Oral *ad libitum* administration of food with 5% of *L. plantarum* AYA (120 mg per mouse per day) during 28 days before IFV challenge	IFV (H3N2) strain X-31	Seven-week-old female BALB/c	Improved the production of IgA in the respiratory tract	Reduced body weight loss and decreased mortality	(29)
Effects on cellular immune response							
Lactobacillus casei Shirota	Non-viable	Oral *ad libitum* administration of food with 0.05% of *L. casei* Shirota during 4 weeks before IFV challenge	IFV (H1N1) strain A/PR/8/34	Fifteen-week-old female BALB/c	Improved systemic and respiratory NK cell activity and production of interferon (IFN)-γ and TNF-α by respiratory lymphocytes	Reduced IFV titers	(30)
L. casei Shirota	Viable	*L. casei* Shirota was administered by gavage at a dose of 10^9 cells per mouse 5 times/week for about 3 weeks (total, 17 times) before IFV challenge	IFV (H1N1) strain A/PR/8/34	Neonatal and infant mice	Improved systemic and respiratory NK cell activity and production of IFN-γ and TNF-α by respiratory lymphocytes	Reduced IFV titers, decreased accumulated symptom rate, and decreased mortality	(31)
Lactobacillus gasseri TMC0356	Viable	Ten milligrams of lyophilized bacteria in 200 µl of saline was administered orally per day during 14 days before IFV challenge. Treatment was continued for 5 days after infection	IFV (H1N1) strain A/PR/8/34	Five-week-old female BALB/c	Improved NK cell activity and production of IFN-γ	Reduced virus titers and diminished lung pathological changes	(32)
Lactobacillus rhamnosus GG	Viable	Ten milligrams of lyophilized bacteria in 200 µl of saline was administered orally per day during 14 days before IFV challenge. Treatment was continued for 5 days after infection	IFV (H1N1) strain A/PR/8/34	Five-week-old female BALB/c	Improved NK cell activity and production of IFN-γ	Reduced virus titers and diminished lung pathological changes	(32)
L. plantarum 06CC2	Non-viable	*L. plantarum* was administered by gavage twice daily during 2 days before IFV challenge (20 mg/mouse). Treatment was continued for 7 days after infection	IFV (H1N1) strain A/PR/8/34	Six-week-old female BALB/c	Beneficially modulated NK cells activity and improved Th1 response	Reduced virus titers and diminished lung pathological changes	(33)

(*Continued*)

TABLE 1 | Continued

Immunobiotic strain	Viability	Administration protocol	Challenge	Mice	Immunobiotic effects	Effect on IFV infection	Reference
Bifidobacterium longum MM-2	Viable	Oral administration of 2×10^9 cells per mouse per day during 14 days before IFV challenge. Treatment was continued for 2 days after infection	IFV (H1N1) strain A/PR/8/34	Six-week-old female BALB/c	Increased respiratory NK cell activity and IFN-γ production	Improved clinical symptoms, reduced mortality, and decreased virus titers	(34)
L. casei Shirota	Non-viable	Nasal administration of 20 or 200 μg per mouse per day during 3 days before IFV challenge	IFV (H1N1) strain A/PR/8/34	Ten to eleven-week-old female BALB/c	Increased levels of IL-12, IFN-γ, and TNF-α in mediastinal lymphoid nodes and lungs	Decreased virus titers and increased survival rates	(35)
L. pentosus S-PT84	Non-viable	Nasal administration of 20 or 200 μg per mouse per day during 3 days before IFV challenge	IFV (H1N1) strain A/PR/8/34	Eight to twelve-week-old female BALB/c	Increased IL-12, IFN-α, and NK cell activity in the respiratory tract. Increased levels of IL-12 and IFN-γ in mediastinal lymphoid nodes	Decreased virus titers and increased survival rates	(36)
L. rhamnosus GG	Viable	Nasal administration of 200 μg per mouse per day during 3 days before IFV challenge	IFV (H1N1) strain A/PR/8/34	Seven-week-old female BALB/c	Increased respiratory NK cell activity	Reduced IFV titers, decreased accumulated symptom rate, and increased survival rates	(37)
L. rhamnosus	Viable	Sublingual administration of 10^8 cells per mouse per day during 7 days before IFV challenge	IFV (H1N1) strain A/FMI/33	Adult female BALB/c	Improved levels of IgA specific antibodies, IL-12, and decreased levels of TNF-α and IL-6 in lungs. Increased NK cell activity in spleen. Increased CD25 expression by $CD4^+$ and $CD8^+$ in lung and mediastinal lymphoid nodes	Increase of the survival rates and decrease in the lung lesion scores	(38)
Effects on innate immune response							
L. plantarum L-137	Non-viable	Intragastric administration of 5–100 mg/kg of mouse per day during 7 days before IFV challenge. Treatment was continued for 7 days after infection	IFV (H1N1) strain A/NWS/47	Seven-week-old female C57BL/6	Improved production of type I IFNs	Reduced viral loads in lungs and improved survival	(39)
L. gasseri SBT2055	Viable	Oral administration of 10^8 or 10^9 cells per mouse per day during 7–21 days before IFV challenge	IFV (H1N1) strain A/PR/8/34	Five to seven-week-old male C57BL/6	Enhanced lung expression of the antiviral genes *Mx1* and *Oas1a* and differentially regulated inflammatory response	Enhanced survival rates, reduced lung viral titers and diminished lung inflammatory damage	(40)
L. rhamnosus CRL1505	Viable	Oral administration of 10^8 cells per mouse per day during 5 days before IFV challenge	IFV (H1N1) strain A/PR/8/34	Six-week-old male BALB/c	Differentially regulated levels and kinetics of inflammatory cells (neutrophils and macrophages) and cytokines (TNF-α, IL-6, IL-10, and type I IFNs) Diminished coagulation activation in blood and respiratory tract	Decreased IFV titers in lungs, lessened pulmonary damage, and increased survival	(41)
L. rhamnosus CRL1505	Viable and non-viable	Nasal administration of 10^8 cells per mouse per day during 2 days before IFV challenge	IFV (H1N1) strain A/PR/8/34	Six-week-old male BALB/c	Differentially regulated levels and kinetics of inflammatory cells (neutrophils and macrophages) and cytokines (TNF-α, IL-6, IL-10, and type I IFNs) Diminished coagulation activation in blood and respiratory tract	Decreased IFV titers in lungs, lessened pulmonary damage, and increased survival	(42)

(*Continued*)

TABLE 1 | Continued

Immunobiotic strain	Viability	Administration protocol	Challenge	Mice	Immunobiotic effects	Effect on IFV infection	Reference
Enterococcus faecalis KH2	Viable and non-viable	Intragastric administration of 8.5×10^{10} cell per kg of mouse per day during 7 or 12 days before IFV challenge	IFV (H1N1) strain A/WSN/33	Adult male C57BL/6	Diminished concentrations of proinflammatory factors especially MCP-1	Reduced mortality, weight loss, and lung viral titers	(43)
L. pentosus b240	Non-viable	Oral administration of 10^{10} cells per mouse per day during 21 days before IFV challenge. Oral treatment was continued for 14 days after infection	IFV (H1N1) pdm strain A/California/04/09	Six-week-old female BALB/c	Downregulated expression of the immune related genes *Cyr61*, *Egr1*, and *Fos*, and genes related to Acyl-CoA-mediated metabolism. Upregulated expression of the antiviral gene Rsad2 in the lungs	Prolonged mouse survival. No effect on virus titers and no apparent differences in the extent of lung damage	(44)

Research work has also demonstrated that nasal administration of immunobiotics is an interesting alternative to improve cellular response against influenza infection (35–37) (**Table 1**). Hori et al. (35) observed that BALB/c mice nasally treated with non-viable *L. casei* Shirota had increased levels of IL-12, IFN-γ, and TNF-α in mediastinal lymphoid nodes and lungs. This improved cellular respiratory immunity correlated with a beneficial clinical outcome to IFV challenge. Similar observations were performed with nasally administered *L. pentosus* S-PT84 (36) and *L. rhamnosus* GG (37).

Other recent studies have also demonstrated the ability of immunobiotics to improve respiratory innate antiviral defenses in the respiratory tract (**Table 1**; **Figure 2**). It was reported that orally administered non-viable *L. plantarum* L-137 improved protection against IFV by increasing type I IFN production (39). The work clearly demonstrated that the increased production of IFN-β induced by the immunobiotic strain correlated with the reduction of viral loads in lungs as well as the improved survival of infected mice. More recently, it was shown that *L. gasseri* SBT2055 enhanced survival rates and reduced lung viral titers in mice infected with IFV (40). Interestingly, authors observed that the lung expression of the antiviral genes *Mx1* and *Oas1a* was enhanced in *L. gasseri* SBT2055-treated mice and that the inflammatory response triggered by IFV was differentially regulated inducing a lower inflammatory damage (40).

Our group has also reported a beneficial regulation of the IFV-triggered inflammatory response by immunobiotics. Lung damage induced by IFV is known to be produced by virus replication as well as the uncontrolled inflammatory response that is characterized by a hypersecretion of proinflammatory cytokines, especially TNF-α, IL-1β, and IL-6 (45). The adequate production of inflammatory factors is necessary to protect against IFV infection together with an appropriate regulation with anti-inflammatory cytokines to prevent the damage of lung tissue. Thus, the proper balance of cytokines is a key factor in determining the outcome of IFV infection. In this regard, we observed that orally (41) or nasally (42) administered immunobiotic *L. rhamnosus* CRL1505 differentially regulated the levels and kinetics of inflammatory cells and cytokines in mice after IFV challenge. In our experimental model, we observed increased levels of respiratory TNF-α, IL-6, neutrophils, and macrophages in CRL1505-treated mice early after the challenge with IFV. Later, proinflammatory cytokines and infiltrated cells started to decrease in immunobiotic-treated animals in contrast to control mice, in which those parameters continued increasing. The trend toward lower inflammatory factors and cells registered later during IFV infection in *L. rhamnosus* CRL1505-treated mice correlated with a reduced severity of pulmonary damage when compared to control mice (41, 42).

Chen et al. (43) also investigated the ability of orally administered *Enterococcus faecalis* KH2 to beneficially modulate the innate immune response to influenza infection. Authors observed that KH2 strain protected C57BL/6 mice against IFV as observed by the reduced mortality, weight loss, and lung viral titers. As expected, IFV enhanced the levels of proinflammatory mediators in the respiratory tract including IL-6, TNF-α, IFN-γ, IL-1β, IL-17, and MCP-1 while the treatment with *E. faecalis*

significantly diminished the concentrations of proinflammatory factors, especially MCP-1. Considering that monocyte migration mediated by MCP-1 has been linked to several respiratory inflammatory disorders including IFV infection, authors investigated the role of MCP-1/CCR2 pathway in the immunobiotic effect of *E. faecalis* KH2. The work reported that the protective activity of the KH2 strain was abrogated when recombinant MCP-1 was administered concomitantly (43).

It is not clear how immunobiotics initiates the cross-talk with the immune system in order to modulate the respiratory antiviral immunity. It is not known exactly which PRRs are activated by immunobiotics in the intestinal or respiratory mucosa to functionally modulate antiviral immunity locally and in distal mucosal sites, respectively. Neither it has been determined with exactitude which cellular functions are modulated by immunobiotics immediately after PRR activation. Research from the last decade has demonstrated that the immunomodulatory effects of probiotic bacteria are the consequence of complex interactions between several bacterial molecules and host receptors located in different immune and non-immune cells (46, 47). It has also been shown that the immunomodulatory properties of immunobiotics are dependent on the strains. Therefore, studies carried out with certain strains cannot be easily extrapolated to other bacteria, even those of the same genus and species (48, 49). Consequently, it is still necessary to carry out deeper studies to find out the molecular mechanisms by which immunobiotics beneficially influence the respiratory antiviral immunity.

The studies mentioned before showed the potential of immunobiotics to be used for the reduction of the incidence and severity of IFV infections. However, in addition to deepening the knowledge of their mechanisms of action, several other points should be considered for the efficient application of immunobiotics in humans.

For example, it is necessary to determine the best time as well as the most appropriate route for their administration. Immunobiotics used as components of functional foods can be included in diets on a regular basis and thus help to improve respiratory defenses, especially in high-risk populations and during the seasons with the highest incidence of respiratory infections occurs. In this sense, in a randomized controlled trial we demonstrated that *L. rhamnosus* CRL1505 (administered in a yogurt formulation) improved mucosal immunity and reduced the incidence and severity of intestinal and respiratory infection in children (50). Hence, the incidence of infectious events was reduced from 66% in the placebo group to 34% in the group that received the probiotic yogurt. Furthermore, there was also a significant reduction in the occurrence of indicators of disease severity such as fever and the need for antibiotic treatment in children receiving the probiotic yogurt. This immunobiotic yogurt (YOGURITO®) has been included into official National Nutritional Programs in Argentina and is given daily to children at schools in several provinces thanks to the Government actions. Epidemiological studies in the schools receiving the immunobiotic product have shown a reduction in the incidence of infections and in the associated school absenteeism (Alvarez et al., unpublished results).

On the other hand, as mentioned earlier the nasal administration of immunobiotics is more efficient than the oral administration to enhance respiratory immunity. This route of administration poses a practical disadvantage considering that the treatments with immunobiotics showed favorable results when they were used before the infectious challenges. In this way, it would be necessary to predict the exact moment in which the viral pathogen will be in contact with the host in order to carry out the prophylactic immunobiotic treatment. This option could be used for example during a school or work outbreak in which cases of respiratory infections occur and it is desired to prevent or reduce the severity of infections in asymptomatic individuals. For an intervention of these characteristics, it would be also important to determine the exact time after the contact with the virus in which it is possible to administer immunobiotics to achieve the beneficial effect. In a recent study, Percopo et al. (51) have defined this as "the window of opportunity." The work evaluated the effect of the nasal administration of live or inactivated *L. plantarum* NCIMB 8826 in a mice model of severe respiratory infection with the pneumonia virus of mice (PVM) and found that immunobiotic treatment promoted full survival from acute PVM infection when administered within 1 day after virus challenge (51). Similar studies would be of value in IFV infection models.

Another point of interest is related to the duration of the improvement of respiratory defenses after the last immunobiotic administration. Our studies have showed that the immunomodulatory effect of some nasally administered immunobiotics persisted for at least 15 days (Villena et al., unpublished results). Other studies have also reported short-term protection after nasal treatment with different immunobiotic strains (43). Interestingly, Garcia-Crespo et al. (52) found that adult mice primed nasally with *L. plantarum* NCIMB 8826 or *Lactobacillus reuteri* F275 were completely protected against lethal PVM infection and that protection persisted for at least 5 months after the initial priming. These findings open an interesting challenge in the study of immunobiotics to improve the defenses against IFV, since it would be very useful to establish the duration of the protective effect for each strain and treatment, since in the majority of cases these long-term studies were not taken into account.

IFV infections often result in mild to moderate lung infection; however, life-threatening disease can occur. It has been demonstrated that the most severe disease outcomes are associated with secondary bacterial pneumonia caused primarily by *Staphylococcus aureus* or *Streptococcus pneumoniae* (53). Taking into account the high incidence of viral infections and the frequency of associated secondary bacterial infections which contribute to aggravate the health status of the host and reduce its chance of recovery, various approaches for preventing and treating influenza and secondary bacterial pneumonia are been investigated. A wide range of antibiotics and anti-inflammatory drugs has been tested in mice [reviewed in Ref. (54)]. It would be of interest to evaluate the potential beneficial effect of immunobiotics on these circumstances. In this regard, preliminary studies from our laboratory showed that nasally administered *L. rhamnosus* CRL1505 is able to improve survival, reduce

bacterial cell counts in lung and blood, and limit lung inflammatory damage caused by *S. pneumoniae* infection in mice produced after the infection with IFV or respiratory syncytial virus (RSV) (Villena et al., unpublished results). These results opened an interesting topic for future investigations.

Finally, it would be also of interest to investigate whether immunobiotic treatments may influence other physiological systems involved in the defenses against viral respiratory infections such as the coagulation system. Our group has made some progress in this regard, as mentioned below.

RESPIRATORY IMMUNE-COAGULATIVE RESPONSE AND IFV

Coagulation is an extremely ordered process that involves the interaction of three key components: endothelial cells (ECs), platelets, and coagulation factors. Tissue injury that activates ECs typically initiates coagulation that is characterized by the binding of platelets to activated ECs and the formation of the platelet plug. Almost simultaneously, tissue factor (TF) released by ECs result in factor X activation, which induces thrombin and the generation of fibrin strands to strengthen the platelet plug leading to a stable platelet–fibrin clot. All these processes are tightly regulated by anticoagulant and fibrinolytic mechanisms to avoid thrombotic and/or haemorrhagic complications. A key role has been attributed to ECs in the temporal and special regulation of coagulation activation. Resting ECs avoid the inappropriate plug formation by controlling platelet adhesion and activation and generating several anticoagulant factors providing a non-thrombogenic barrier (55, 56). Once activated or injured, ECs expose collagen to blood, increase platelet binding and aggregation, reduce the expression physiological anticoagulant factors, increase the expression of TF and von willebrand factor, and suppress the fibrinolytic activity (57, 58). All these changes in the hemostatic system facilitate thrombosis in the infected or inflammated tissue.

Both hemorrhagic and thrombotic complications have been described during IFV infection. Influenza is able to cause pulmonary hemorrhage and edema related to coagulopathy or induce uncontrolled thrombosis through an over-activated coagulation (**Figure 3**) (55, 58). Animal models have helped to explain the mechanisms by which IFV infection activates coagulation and key role has been attributed to TF. It was described that IFV activates coagulation by enhancing TF production, thrombin generation and fibrin deposition in C57BL/6 mice (59). In a mice model of IFV infection, it was recently shown that wild-type animals increased lung TF expression and activation of coagulation but presented alveolar hemorrhage (60). Moreover, selective deletion of TF in epithelial cells from lung significantly reduced TF expression after IFV infection and had higher alveolar hemorrhage and

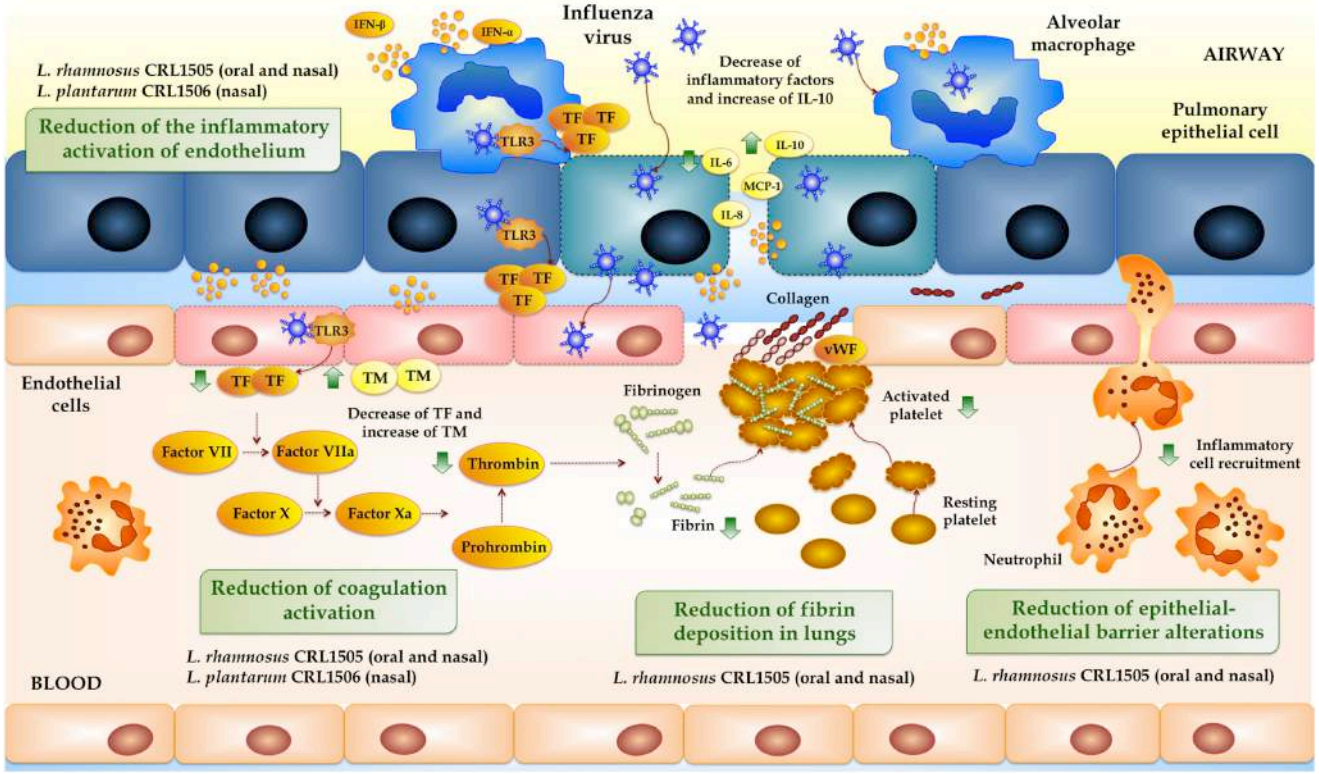

FIGURE 3 | Activation of the immuno-coagulative response by influenza virus in the respiratory mucosa. Beneficial effects of immunobiotics administration on the immuno-coagulative response triggered by toll-like receptor 3 or Influenza Virus in the respiratory mucosa.

reduced survival than controls. On the contrary, deficiency of TF in either respiratory myeloid cells or ECs did not enhanced alveolar hemorrhage or modified survival of IFV-infected mice (60). These results indicate that an appropriate modulation in the production of TF in the lung during IFV infection is necessary to maintain tissue hemostasis avoiding hemorrhage and excessive fibrin deposition. Production of TF by lung epithelial cells will be required to maintain alveolar hemostasis during IFV infection, while excessive release of TF by macrophages and ECs would contribute to pathology and lung tissue injury (59, 60).

It is considered that ECs may play an important role in the pathogenesis of IFV. Influenza infection is able to induce alveolar edema and pulmonary hemorrhage through the alteration of ECs via several mechanisms, including direct damage and loss of tight junctions and apoptosis (61). In addition, recognition of damage-associated molecular patterns such as HMGB1 or oxidized phospholipids through TLR4 activates ECs to drive lung injury (62). Direct stimulation of TLR3 by viral RNA also results in the upregulation of TF and the downregulation of thrombomodulin (TM) in ECs (63). At the same time, the inflammatory activation of ECs leads to the activation of the coagulation cascade. Inflammation caused by IFV infection increases various proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 that induce the secretion of TF by ECs and monocytes (58). In addition to their roles in coagulation, activated proteins such as thrombin, FXa, and FVIIa also enhance the inflammatory response. The inflammatory potentiating abilities of coagulation factors are mediated through their activation of protease-activated receptors (PARs) that are expressed in platelets, ECs, macrophages, and respiratory epithelial cells (58). The TF/thrombin/PAR-1 pathway has been associated to the promotion of a deleterious innate inflammatory response to IFV infection in mice (64, 65).

Therefore, both the hyper-inflammatory response and the aberrant activation of coagulation, which are potentiated with each other, are involved in severe influenza pneumonia and are key events that have to be controlled in order to reach a favorable resolution of the infectious process.

BENEFICIAL EFFECTS OF IMMUNOBIOTICS IN IFV IMMUNE-COAGULATIVE RESPONSE

Considering the importance of the coagulative response in the outcome of influenza infection and the ability of immunobiotics to beneficially influence the immune response to this respiratory pathogen, we wonder whether some immunobiotic strains would be able to beneficially modulate the immuno-coagulative response triggered by IFV. For this purpose, we performed challenge-infection experiments in mice and evaluated the influence of viable and non-viable immunobiotic *L. rhamnosus* CRL1505 strain on the respiratory immuno-coagulative response induced by IFV (41, 42).

Our data demonstrated that oral administration of *L. rhamnosus* CRL1505 to mice significantly reduced lung viral titers and tissue damage after the challenge with IFV (41). We later explored the capacity of nasally administered *L. rhamnosus* CRL1505, alive or heat killed, to reduce the influenza burden of disease (42). Those treatments induced a significant decrease in IFV titers in lungs, lessened pulmonary damage, and increased survival. Interestingly, a similar effect was achieved with the nasal administration of viable and non-viable CRL1505 strain. Moreover, the nasal route was more efficient than the oral administration to protect mice against IFV infection (41, 42). The protective effect achieved by the immunobiotic strain was related to its ability to modulate the respiratory antiviral immune response, particularly to its capacity to improve the levels of IFN-γ and IFN-β in the respiratory tract (**Figure 2**). Type I IFNs trigger the activation of the JAK-STAT pathway and increase the expression of antiviral genes. In addition, IFN-γ is produced by immune cells, especially Th1 cells, and it further improves antiviral immune response by inducing activation of NK cells and macrophages. Therefore, the modulation of type I IFNs and IFN-γ would be responsible of the reduction of viral loads in IFV-infected mice previously treated with the CRL1505 strain, similarly to other immunobiotic strains as mentioned before (**Table 1**). We demonstrated that the CRL1505 strain increased the levels of gut $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells, induce a mobilization of these lymphocytes into the lung and enhanced the respiratory production of IFN-γ and the activity of local antigen presenting cells (41, 66, 67). It was also noted that nasal administration was more effective than the oral route to increase pulmonary $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells (41, 42). The mechanism by which nasally administered viable or heat-killed *L. rhamnosus* CRL1505 improves $IFN\text{-}\gamma^+$ T cells population is not clear. However, our studies support the possibility that the immunobiotic strain *L. rhamnosus* CRL1505 impact in the nasal-associated lymphoid tissue or bronchial-associated lymphoid tissue producing an innate imprinting in antigen presenting cells that contribute to the enhanced number and activity of $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells.

Our studies also showed that immunobiotic treatments were able to beneficially modulate the activation of coagulation during respiratory viral infection, an effect that was not reported before (41, 42). Then, our studies were the first in demonstrating a beneficial modulation of the immune-coagulative response during respiratory TRL3 activation and IFV infection induced by immunobiotic microorganisms (**Figure 3**).

Although IFV is an ssRNA virus, it generates dsRNA replication intermediates that activate TLR3 and contribute to the initiation of the antiviral respiratory immune response. In fact, IFV triggers type I IFN secretion through TLR3 recognition in immune (myeloid DCs or macrophages) and non-immune (fibroblasts or pneumocytes) cells (68). Challenge-infection experiments with respiratory viruses in $TLR3^{-/-}$ mice showed that TLR3 does not modify the clearance of viral pathogens but it is relevant for the modulation of the lung inflammatory response (69, 70). It was showed that wild-type mice mount a robust inflammatory response in the lung after IFV infection and that this process is significantly diminished in $TLR3^{-/-}$ animals (70). $TLR3^{-/-}$ mice showed a longer survival when compared wild-type animals and this effect was associated with a reduction of inflammatory cells recruitment and lower levels of inflammatory factors in the respiratory tract. Other *in vivo* studies also demonstrated that TLR3 activation by poly(I:C) enhanced proinflammatory cytokines and

TABLE 2 | Effect of probiotics on influenza virus (IFV) infection in humans.

Strain	Viability	Route	Population studied	Effects	Reference
Lactobacillus fermentum CECT5716	Viable	Oral (capsule)	Randomized, double-blinded, and placebo-controlled human clinical trial in adults	Coadjuvant capability for anti-IFV vaccine. Lower incidence of influenza-like illness during 5 months after vaccination Increased proportion of NK cells, higher induction of Th1 cytokines and augmented specific T-helper and T-cytotoxic lymphocytes. Increased antigen specific IgA	(83)
Lactobacillus casei DN-114 001	Viable	Oral (fermented dairy drink Actimel®)	Randomized, multicentre, double-blind, and controlled studies in elderly population over 70 years of age	Coadjuvant capability for anti-influenza vaccine. Improved IFV-specific antibody titers after vaccination	(84)
Lactobacillus GG		Oral (capsule)	Randomized, double-blind, and placebo-controlled pilot study in adults	Coadjuvant capability for anti-IFV vaccine. Increased protective titer 28 days after vaccination for the H3N2 strain	(85)
Lactobacillus plantarum L-137	Non-viable (heat killed)	Oral (capsule)	Randomized, double-blind, and placebo-controlled pilot study in adults	Improved levels of interferon (IFN)-β before vaccination	(86)
Bifidobacterium animalis ssp. *lactis* BB-12w and *Lactobacillus paracasei* ssp. *paracasei* 431w	Viable	Oral (capsule and acidified dairy drink)	Randomized, double-blind, placebo-controlled, and parallel-group study in adults	Coadjuvant capability for anti-IFV vaccine. Improved vaccine-specific secretory IgA in saliva. Significant higher levels of vaccine-specific plasma IgG, IgG1, and IgG3	(87)
Lactobacillus rhamnosus strains GG and LC705	Viable	Macrophage stimulation	Human primary macrophages	Quantitative different IL-1β and type I IFN gene expression levels in macrophages. Diminished IFV replication and production of viral proteins in macrophages	(88)
Lactobacillus pentosus b240	Non-viable (heat killed)	Oral (tablet)	Randomized, double-blind, and placebo-controlled trial in elderly population over 65 years of age	Significant reduction of the incidence rate of the common cold	(89)
L. paracasei MoLac-1	Non-viable (heat killed)	Oral (jelly)	Randomized, double-blind, and placebo-controlled trial in elderly nursing home resident volunteers	Coadjuvant capability for anti-IFV vaccine. Improvement of hemagglutination inhibition titers against all different types of influenza antigens analyzed. Improvement in antibody titers against A/H3N2	(90)
L. rhamnosus GG	Viable	Oral (supplemented milk)	Randomized, double-blinded, and placebo-controlled in children of 2–6 years of age	Probiotic intervention did not reduce significantly the occurrence of the examined respiratory viruses, but the children that received the *GG* strain had fewer days with respiratory symptoms	(91)
Lactobacillus brevis KB290	Viable	Oral (fermented drink)	Open-label, parallel-group trial in children of 6–12 years of age	Reduced incidence of IFV infection in schoolchildren	(92)
L. rhamnosus GG	Viable	Oral (added to breast milk or formula)	Randomized, double-blind, and placebo-controlled trial in infants between the first and third days of life	Significant reduction in the incidence of viral respiratory tract infections	(93)
Lactococcus lactis ssp. *lactis* JCM5805	Viable	Oral (fermented dairy drink)	Randomized, placebo-controlled, and double-blind trial in adults	Significant decrease in major symptoms of influenza-like illness. IFN-α elicited by A/H1N1 on peripheral blood mononuclear cells prepared from volunteers tended to be higher, and IFN-stimulated gene 15 was significantly higher	(94)

antiviral factors expression (71), altered vascular permeability (72), and incremented the levels of D-dimers indicating that coagulation and fibrinolysis were triggered. In line with these findings, it was observed that the levels of D-dimers in TLR3$^{-/-}$ mice were significantly lower than in wild-type animals after poly(I:C) administration (63). In addition, by using siRNA technology it was demonstrated that TLR3 is a key receptor in the induction of the procoagulant state in ECs (63). Challenge of those cells with the TLR3 agonist poly(I:C) induced a decrease of TM and an enhancement of TF expression in a time- and dose-dependent manner. The results obtained in our own *in vivo* experiments were in line with these preceding reports (41, 42). We observed that three daily doses of nasally administered poly(I:C) to BALB/c mice induced a marked enhancement of inflammatory cells

(neutrophils and macrophages) and proinflammatory mediators (IL-1β, TNF-α, IL-8, and IL-6) in the respiratory tract. Moreover, TLR3 activation also induced an increase in TF expression and thrombin–antithrombin complex (TATc) levels in the lung while it reduced TM expression. These inflammatory–coagulative modifications were accompanied by respiratory tissue alterations and impairment of lung function (41, 42).

Of interest, we demonstrated that orally (41) or nasally (42) administered immunobiotics before the challenge with poly(I:C) differentially modulated the inflammatory-coagulative response. *L. rhamnosus* CRL1505 was able to reduce and increase the expression of TF and TM, respectively, after the respiratory activation of TLR3. Thus, the CRL1505 strain significantly diminished coagulation activation in blood and in the respiratory tract after the nasal stimulation with poly(I:C).

We also evaluated pulmonary coagulation during IFV infection (41, 42). The respiratory virus induced activation of coagulation in the lungs of infected mice as demonstrated by the increased levels of respiratory TATc. These procoagulant changes were related to alterations in the expression of TM and TF in lungs. Our findings are in line with previous studies in humans and animal models of influenza infection demonstrating increased lung fibrin deposition and enhanced numbers of intravascular thrombi in the respiratory tract (59, 73, 74). We demonstrated that immunobiotic treatment is able to significantly diminish the activation of coagulation in IFV-challenged mice. In fact, lower levels of respiratory TATc and a reduced expression of TF was observed in *L. rhamnosus* CRL1505-treated mice infected with IFV when compared to controls (41, 42).

As mentioned before, IFV promote a procoagulant state directly through its capacity to infect ECs and monocytes stimulating the expression of TF (75, 76). In addition, IFV induce activation of coagulation indirectly by the enhancement of proinflammatory factors such as IL-6 (75, 76). Therefore, the ability of immunobiotics to modulate the IFV-triggered immune-coagulative response could be explained by their direct influence on viral replication related to the enhancement of the antiviral state in the respiratory mucosa, and indirectly through the modulation of the inflammatory response. Considering this last point, we performed experiments using anti-IL-10R blocking antibodies in order to evaluate the role of the regulation of the inflammatory response in the reduction of coagulation activation. Results showed that IL-10 is important for the regulation of coagulation induced by the immunobiotic *L. rhamnosus* CRL1505 (41). Blocking of IL-10R abolished the capacity of the CRL1505 strain to change the expression of TM and TF in the lungs. This was in line with our previous studies evaluating the ability of *L. rhamnosus* CRL1505 to confer protection against inflammatory damage induced by TLR3 activation or RSV infection, which showed that IL-10 is a key factor for the reduction of lung injury (67). Additionally, it was demonstrated that lethal disease caused by IFV infection is prevented by IL-10 administration through the reduction of lung immunopathology (77). Moreover, TF expression and procoagulant activity of macrophages and ECs are reduced by IL-10 (78, 79).

Therefore, we demonstrated that immunobiotic administration induce an early increase in the levels of TNF and IL-6 in the respiratory tract after poly(I:C), RSV, or IFV challenge, while the levels of those proinflammatory factors are significantly reduced later during infection (41, 42, 67). The early increase of proinflammatory mediators and the augmented levels of IFN-γ explain the ability of *L. rhamnosus* CRL1505 to diminish viral replication while the improved production of IL-10 would lead to a beneficial modulation of the immune-coagulative response which results in a reduced severity of lung damage. It has been suggested that respiratory viral infections increase the risk of venous thromboembolism and ischemic heart disease through ECs perturbation, coagulation activation, reduction of anticoagulant factors, and inhibition of fibrinolysis (80–82). Then, our studies suggest that immunobiotics could be an interesting alternative not only to reduce the incidence and/or severity of respiratory viral infections, but in addition to reduce the risk of atherothrombotic alterations associated to respiratory viral infections.

CONCLUSION

Research from the last decade has clearly demonstrated that beneficial microorganisms are able to modulate respiratory tract immunity and promote the resolution and lessen the severity of respiratory infections caused by pathogens such as IFV. Studies in animal models have demonstrated that orally or nasally administered immunobiotics are able to improve protection against IFV by three main mechanisms. First, immunobiotics increase the respiratory antiviral state by their capacity to improve levels of type I IFNs, the number and activity of antigen presenting cells, NK cells, $CD4^+IFN\text{-}\gamma^+$ T, and IgA^+ B lymphocytes, as well as the levels of systemic and mucosal specific antibodies. Second, immunobiotics beneficially modulate the IFV-triggered respiratory inflammatory response by inducing changes in the levels and kinetics of proinflammatory factors and immunoregulatory cytokines such as IL-10 that allow the clearance of virus with a minimal inflammatory lung tissue damage. Finally, as demonstrated by our recent research works, immunobiotics modulate lung immune-coagulative response triggered by TLR3 activation or IFV infection, mainly by down-regulating lung TF and restoring TM levels. Studies in animal models suggest that immunobiotics would influence principally the innate immune response, modulating in that way the early antiviral inflammatory response and the subsequent cellular and humoral immune responses. Therefore, immunobiotics would have mainly an adjuvant effect. However, the exact molecular mechanisms by which immunobiotics differentially modulate the innate antiviral immune response against IFV remain to be elucidated.

Additionally, a growing number of studies in humans have examined the effect of immunobiotics on the incidence and severity of IFV infection. Considering the impact of immunobiotics in the innate immune response clinical studies have evaluated principally their potential adjuvant effects on IFV vaccination (**Table 2**). Although mechanistic studies have not been addressed in depth, there is promising evidence for beneficial effects of immunobiotics on human respiratory health and resistance against IFV. These observations might be helpful to propose new

preventive approaches to improve IFV control using immunobiotics by developing functional foods, pharmabiotics, or vaccine adjuvants.

AUTHOR CONTRIBUTIONS

HZ, SA, HK, and JV have designed, written, and revised the review article.

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MINI REVIEW
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The Role of Microbiota and Immunobiotics in Granulopoiesis of Immunocompromised Hosts

Susana Salva[1] and Susana Alvarez[1,2]**

[1] Immunobiotechnology Laboratory, CERELA-CONICET, Tucuman, Argentina, [2] Applied Biochemistry Institute, Universidad Nacional de Tucumán, Tucuman, Argentina

The number of granulocytes is maintained by a regulated balance between granulopoiesis in the bone marrow and clearance and destruction in peripheral tissues. Granulopoiesis plays a fundamental role in the innate immune response. Therefore, factors affecting the normal granulopoiesis lead to alterations in innate defenses and reduce the resistance against infections. In this study, we give a description on recent advances regarding the molecular and cellular events that regulate steady-state and emergency granulopoiesis, which are crucial processes for the generation of protective innate immune responses. Particular attention will be given to emergency granulopoiesis alterations in immunosuppression states caused by malnutrition and chemotherapy. The role of microbiota in maintaining a steady-state granulopoiesis and the immunological mechanisms involved are also discussed. Moreover, we describe the findings of our laboratory demonstrating that the dietary supplementation with immunobiotics is an interesting alternative to improve steady-state and emergency granulopoiesis, the respiratory innate immune response, and the resistance against respiratory pathogens in immunocompromised hosts.

Keywords: immunobiotics, granulopoiesis, immunocompromised hosts, respiratory infections, *Lactobacillus rhamnosus* CRL1505

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****Correspondence:***
Susana Salva
ssalva@cerela.org.ar;
Susana Alvarez
salvarez@cerela.org.ar

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INTRODUCTION

The microbiota is a complex community of bacteria, fungi, archaea, and viruses that colonize the mucosal surfaces and skin of the human body (1). The gut microbiota is typically integrated by bacteria and specifically by members of the divisions *Bacteroidetes* and *Firmicutes* (2). However, each individual's microbiota is unique, and its composition depends on multiple factors, such as, diet, lifestyle, host genetic, use of antibiotics, and environment (3–5). The gut microbiota plays a fundamental role in the health maintenance of its host. In particular, we highlight that microbiota is able to control immunity in distant tissues through its capacity to regulate hematopoiesis at primary immune sites as the bone marrow (BM) (6). On the other hand, the immunomodulatory probiotic lactic acid bacteria (immunobiotics) are capable to improve the recovery of myeloid cells production affected by malnutrition or chemotherapy, and to increase the immune response against bacterial pathogens (7, 8). The mechanisms for systemic immunomodulation by the microbiota provide a probable mechanism for immunobiotics activity, demonstrating that translocated microbial products benefit the host by enhancing systemic innate immune function.

In this mini review, we examine the interaction between microbiota and immune system, and how this crosstalk affects the maintenance of a steady-state granulopoiesis that is crucial for the generation of protective innate immune responses. We also revise the alterations of emergency granulopoiesis in immunosuppression states caused by malnutrition and chemotherapy. In addition, we discuss

TABLE 1 | Regulation of granulopoiesis by microbiota and immunobiotics.

Experiemental models	Outcome
Microbiota-granulopoiesis crosstalk	
Polymyxin treated and germ-free (GF) mice (18)	Decreased levels of splenic and bone marrow (BM) progenitor cells forming GM-CFU colonies
Kanamycin-treated mice (17)	Decreased numbers of granulocytes in the BM and blood
GF mice Antibiotic-treated mice Nod1-deficient mice (23)	Translocation of peptidoglycan from the gut to neutrophils in the BM Peptidoglycan concentrations in sera correlate with neutrophil function Antimicrobial capacity of neutrophils depends on recognition of microbiota-derived peptidoglycan from Gram-negative bacteria *via* the pattern-recognition receptor Nod1 but not Nod2 orTLR4 Consistent with impaired innate priming Nod1-deficient mice were unable to control *Streptococcus pneumoniae* early sepsis
GF mice *TLR4*$^{-/-}$ mice *TRIF*$^{-/-}$ mice *MyD88*$^{-/-}$ mice (14)	Total lack of microbial colonization is associated with decreased neutrophil numbers and decreased G-CSF levels in the steady state *In vivo* feedback is impaired in *TLR4*$^{-/-}$ and *TRIF*$^{-/-}$, but not *MyD88*$^{-/-}$ animals Steady-state neutrophil homeostasis is G-CSF-dependent and regulated through pattern-recognition receptors, thereby directly linking toll-like receptor (TLR)-triggering to granulopoiesis
MyD88-deficient mice (20)	Loss of MyD88 reduce numbers and proliferation rate of hematopoietic stem cells in the BM
GF mice (27)	Reduction of neutrophil recruitment to the peritoneal cavity in response to diverse stimuli including microbial components and sterile ligands Microbiota-derived signals *via* a MyD88-dependent pathway are required to precondition the neutrophil inflammatory response
Antibiotic-treated neonatal mice GF neonatal mice (16)	Decreased numbers of circulating and BM neutrophils and lower granulocyte-macrophages in the BM Decreased number of interleukin (IL)-17-producing cells in the intestine and production of G-CSF Increased susceptibility to *Escherichia coli* K1 and *Klebsiella pneumoniae* sepsis, which could be partially reversed by administration of G-CSF. Microbiota-derived components, such as LPS *via* TLR4/MyD88 signaling, induced IL-17 production mainly by group 3 innate lymphoid cells in the intestine and increased plasma levels of G-CSF leading to granulocytosis
GF mice (19)	Damage of differentiation of specific myeloid cell progenitors of both yolk sac and BM origin Innate immune defects lead to impaired early responses to pathogens
GF mice (21)	Complexity of the intestinal microbiota correlates with the number of BM myeloid cells Transfer of sterile filtered, boiled serum from colonized mice was sufficient to expand the BM myeloid cell pool of GF mice in a MyD88/Toll-IL-1 receptor-containing adaptor molecule-dependent manner, indicating that heat-stable, circulating, commensal-derived products may be responsible for maintaining steady-state myelopoiesis levels
Antibiotic-treated mice (28)	Impairment of resistance to lung infection with *K. pneumoniae* and reduction of ROS-mediated bacterial killing by alveolar macrophages and required Nod1- and Nod2- but not TLR-dependent signaling
GF mice Antibiotic-treated mice (29)	Reduction steady-state numbers of tissue-resident and BM-derived phagocytes rendered GF and antibiotic-treated mice susceptible to acute systemic *Lysteria monocytogenes* infection
Neonatal non-obese diabetic mice (22)	The gut microbiota enriched in *Staphylococcus* in neonatal non-obese diabetic mice, correlated with increased immature granulocytes in the liver and spleen
Immunobiotics-granulopoiesis crosstalk	
Protein-malnourished mice (33)	Protein malnutrition altered B cell development in BM. The treatment of malnourished mice with *Lactobacillus rhamnosus* CRL1505 was able to induce a recovery of B cells that would explain its ability to increase immunity against infections
Protein-malnourished mice (35)	Repletion of malnourished mice with supplemental *Lactobacillus rhamnosus* improved neutrophils recruitment, phagocytic activity, and resistance against pneumococcal infection
Protein-malnourished mice (7)	Protein-malnutrition impaired the emergency myelopoiesis induced by the generation of the innate immune response against pneumococcal infection Repletion of malnourished mice with *Lactobacillus rhamnosus* CRL1505 was able to accelerate the recovery of granulopoiesis and improve innate immunity
Chemotherapy-treated mice (8)	*Lactobacillus casei* CRL431 and *Lactobacillus rhamnosus* CRL1506 accelerated the recovery of Cy-caused myelosuppression and the recovery of the immune response against the opportunistic pathogen *Candida albicans*
Chemotherapy-treated mice (53)	*Bifidobacterium longum* BB536 increased resistance to sepsis caused by an intestinal infection of *Pseudomonas aeruginosa* in mice treated with Cy
Chemotherapy-treated mice (42, 54)	*Lactobacillus plantarum* HY7712 as *Lactobacillus casei* HY7213 accelerated the recovery of Cy-induced immunosuppression by immunopotentiating NK cells and cytotoxic T lymphocytes derived from BM and spleen, in addition to restoring the phagocytic capacity of peritoneal macrophages
Chemotherapy-treated mice (55)	*Lactobacillus plantarum* stimulated the proliferation of splenocytes from mice immunocompromised by Cy treatment in response to LPS

the research of our laboratory demonstrating that dietary supplementation with immunobiotics is an interesting alternative to improve steady-state and emergency granulopoiesis, respiratory innate immune response, and resistance against respiratory pathogens in immunocompromised hosts.

GRANULOPOIESIS AND ITS REGULATION BY THE GUT MICROBIOTA

Granulocytes are key players of the innate immune response. They are short-lived cells, and their number is kept by a balance

between BM granulopoiesis and peripheral tissues' clearance and destruction (9). These cells are continuously generated in steady-state conditions from long-lived self-renewing hematopoietic stem cells (HSCs) that give rise to short-lived HSCs and multipotent progenitors (MPPs) in BM. MPPs differentiate into common lymphoid and myeloid progenitors (CMP). CMPs give rise to granulocyte-macrophage (GMP), megakaryocyte-erythrocytes, and dendritic cell progenitors. Neutrophils, monocytes, as well as other granulocyte populations derive from GMPs (10). In front of an infectious challenge, neutrophils are recruited in large numbers to the infected tissues, the hematopoietic system must rapidly respond to the demand of these cells by turning from the steady-state to an emergency granulopoiesis (11).

The tissue macrophages activate the LRX family transcription factors during the ingestion of apoptotic neutrophils in steady-state granulopoiesis. This, in turn, suppresses the pro-inflammatory cytokines' production (12). Those macrophages decrease their production of interleukin (IL)-23 and thereby reduce the stimulus for IL-17 production by innate lymphoid cells, natural killer T cells, γδ-T cells, or Th17 cells. The reduced IL-17 levels account for low G-CSF expression (13). Involvement of IL-23/IL-17/G-CSF axis in regulation of granulopoiesis was confirmed in several independent murine models (14) and human studies (15). Moreover, steady-state neutrophil homeostasis is G-CSF-dependent and regulated through pattern-recognition receptors (PRRs), thereby directly linking Toll-like receptor (TLR)-triggering to granulopoiesis (14). Microbiota-derived components, such as LPS *via* TLR4/MyD88 signaling, induced intestinal IL-17 production and increased plasma levels of G-CSF leading to granulocytosis (16). Studies using antibiotics-treated and germ-free (GF) mice showed a decrease in GMPs in BM, and a lower number of neutrophils in the periphery (16–20). Furthermore, it was reported that a live complex flora is needed to restore granulopoiesis completely (21, 22). By using MyD88-deficient mice, it was shown that MyD88-dependent TLR signaling induced by microbiota can impact on the early hematopoietic development and terminal myeloid differentiation (20, 21) (**Table 1**).

During emergency granulopoiesis, pathogen-associated molecule patterns (PAMPs) are detected by PRRs of innate immune system. In addition, bacteria and bacteria-derived products (e.g., LPS) are sensed by TLR-expressing endothelial cells. Consequently, the granulopoiesis and the neutrophils' release into the circulation are produced by the increased amounts of G-CSF and GM-CSF (11). A large body of evidence suggests that circulating microbiota-derived products or pathogens may reach the BM or extramedullar sites, where they can be directly sensed by HSPCs and committed myeloid progenitors (23, 24). On the other hand, circulating HSCs can encounter bacteria or their products in the periphery before re-entering the BM (10). LPS-sensing by hematopoietic cells is dispensable for the induction of emergency granulopoiesis. TLR4 and MyD88 expression of non-hematopoietic cell type is absolutely required for this process (25, 26). Thus, levels of growth factors determine the rate at which neutrophils are induced the proliferation and differentiation of neutrophil precursors by JAK–STAT pathways. In this context, transcription factor C/EBP-α regulates steady-state granulopoiesis, whereas C/EBP-β is critical for triggering emergency granulopoiesis in response to GM-CSF (11). On the other hand, recent studies demonstrate that microbiota priming is required for neutrophil extravasation to injured tissues after inflammatory stimuli (27). In addition, antimicrobial capacity of neutrophils was shown to be dependent on the recognition of microbiota-derived peptidoglycan from Gram-negative bacteria *via* Nod1 but not Nod2 or TLR4 (23). An example of the systemic effect of gut microbiota on granulopoiesis during infection has been provided by a research work demonstrating that early innate resistance to *Klebsiella pneumoniae* lung infection was impaired in microbiota-depleted mice, and that the peptidoglycan translocated from the gut was able to modulate the systemic innate immunity (28). Therefore, factors affecting the normal granulopoiesis lead to alterations in innate defenses and reduce the resistance against pathogens (23, 28, 29) (**Table 1**).

GRANULOPOIESIS AND MALNOURISHED HOSTS

Granulopoietic homeostasis requires an important cellular renewal, because of the cells' generation and death. Approximately $0.5–1.0 \times 10^{11}$ granulocytes are generated each day during steady-state conditions in adults (11). In contrast to local infection that can be contained by the innate immune response, in severe infections, the emergency granulopoiesis is triggered and neutrophilia occurs. Therefore, the hematopoietic system is capable of rapid adaptation when augmenting cellular output several-fold levels to respond to the higher demand for neutrophils (10). Steady-state growth and development, physical activity, and response to serious illness are affected by nutritional status (30). In agreement to several research works, we have established that malnutrition affects the hematopoietic tissue that has a high turnover rate and cell proliferation, inducing a damage of blood cells production and causing hypoplasia and histological alterations of BM (31–33). This is characterized by a reduction of hematopoietic space, which is occupied by components of extracellular matrix (31, 33). These histological alterations may be responsible for the damage of the hematopoietic niches, which may influence the crosstalk between hematopoietic cells and the growth factors that regulate the granulopoiesis. In line with Borelli et al. (34), a reduction of GMPs was observed in BM of malnourished mice that could explain the reduction of myeloid cells of BM and blood (7, 35). Thus, nutritional deficiencies affect hematopoiesis, leading to an immunocompromised condition (35).

The relationship between malnutrition and infection can be viewed under two aspects: malnutrition compromising host defense, or infections either aggravating a previously existing deficient nutritional status or triggering malnutrition through disease pathogenesis. It was described that malnutrition alters both innate and adaptive immune responses as consequence of multiple abnormalities induced in the immune system (36). Herrera et al. (7) demonstrated that protein-malnutrition significantly reduces the capacity to recruit neutrophils into infected lungs and that this effect could be related to impairment in granulopoiesis. Several factors could be involved in the impairment of emergency granulopoiesis in malnourished mice, in addition

to those mentioned above. CXCL12 expression in response to pneumococcal infection in BM requires special attention. The HSCs homing into BM is regulated by CXCL12 and their receptor CXCR4 (9). There is no change in the expression of CXCL12 during an infection in malnourished mice, which could be a mechanism for the preservation of HSCs in the BM. Malnutrition also impairs the expression of both GM-CSF and IL-1 in BM and contributes to the altered emergency granulopoietic response (7). It is known that the steady-state and emergency granulopoiesis are directed by GM-CSF while the BM stromal cells function is to support hematopoiesis mainly regulated by IL-1 (37). Hence, the neutrophilia induced by infection or inflammation is assisted by both GM-CSF and IL-1, which in turn accelerates granulopoiesis by expanding MPP and CMP compartments (38). Thus, the reduced capacity of malnourished hosts to develop adequate levels of GM-CSF may explain BM's defective response against an infectious challenge.

On the other hand, there is a great increase in the number of patients with secondary immunodeficiencies related to chemotherapeutic treatments. Cyclophosphamide (Cy) is a drug widely used as an antineoplastic alkylating agent because of its significant therapeutic range and broad spectrum of activity to treat different types of cancer (39). For the World Health Organization, Cy is one of the essential medicines needed in a health system (40). However, this drug induces serious side effects, such as apoptosis and necrosis in BM cells (41), alterations of basal and emergency hematopoiesis, immunosuppression (8, 42), increased susceptibility to infections (8, 43), and even change of intestinal microbiota composition (44). Because of the increased susceptibility to infections, chemotherapy is commonly used in combination with antibiotics in cancer therapy (45). Paradoxically, the consequent propagation of antibiotic resistance among pathogens and depletion of intestinal microbiota lead to increased vulnerability of these patients. For these reasons, it is vital to support treatments aimed at recovering the hematopoietic capacity to increase the efficiency of the immune response triggered in infectious hematopoietic alternative resources processes.

CAN ORAL ADMINISTRATION OF IMMUNOBIOTICS REGULATE GRANULOPOIESIS?

In the last years, a number of research project were meant to find alternative treatments to favor hematopoiesis, improve immunity, booster anticancer effects, and clear anticancer drugs (46). A long list of health benefits has been described for immunobiotics, are likely to modulate and enhance immunity functions in malnourished mice (8, 47–49) (**Table 1**).

Therefore, when the diet induced *Lactobacillus casei* CRL431 or *Lactobacillus rhamnosus* CRL1505, the recovery of the respiratory immunity in immunocompromised hosts was reduced from 21 to 7 days (32, 50, 51). Furthermore, the supplementation of repletion diet with immunobiotics induced recovery of mielopoyesis and normalization of emergency granulopoiesis in response to pneumococcal infection (7, 32). We demonstrated that the treatments with immunobiotics were efficient to recover the architecture of BM tissue, subendosteal epithelium, and BM cellularity altered by malnutrition (7). Moreover, the administration of *L. rhamnosus* CRL1505 induced the growth of mitotic pool cells, mature myeloid cells, and neutrophils in BM. Although the mechanisms involved are not completely elucidated, it is known that cell wall components reach the gut mucosa and from there to circulation during colonization of gut mucosa by commensal bacteria or probiotics. Indeed, the detection of peptidoglycan in the neutrophil fraction shows that it can accumulate in the BM (23). Considering these findings, a probable mechanism for the immunobiotic activity of the microbiota was observed, demonstrating that microbial products favor the systemic innate immune function of the host.

On the other hand, it was described that some immunobiotics can influence ILs levels in blood, which agree with our findings demonstrating the capacity of immunobiotics to normalize the levels of TNF-α, IL-1β, IL-4, IL-6, and IL-10 in malnourished mice (52). It is probably that the changes in ILs levels induced by immunobiotics could influence on the normalization of granulopoiesis. Moreover, we demonstrated for the first time that dietary supplementation with immunobiotics can modulate the production of GM-CSF in infected lungs and its expression in the BM. Moreover, immunobiotics modulate the CXCR4/CXCL12 signaling axis, which is associated with the recovery of hematopoiesis induced by *L. rhamnosus* CRL1505 (7). The detailed study of the mechanisms that explain the influence of immunobiotics on the regulation of granulopoiesis in BM is an interesting topic for future investigations.

Some works have described beneficial effects of immunobiotics on myelosuppression and immunosuppression in Cy-treated mice, although no deep mechanistic studies were performed (8, 42, 53–55) (**Table 1**). Taking into consideration the capacity of *L. casei* CRL431 and *L. rhamnosus* CRL1506 to modulate hematopoiesis in malnourished mice, we also aimed to evaluate the ability of these immunobiotic strains in Cy-treated mice. We showed that preventive treatment with immunobiotics is capable to increase GMPs in BM ($CD34^+$ and $CD34^+Gr\text{-}1^+$ cells), which enables a prompt recovery of peripheral blood neutrophils after Cy-administration (8). These immunobiotic treatments were also able to improve recruitment of phagocytic cells to the site of infection and increase resistance against *Candida albicans* (8). Further studies to evaluate the mechanisms involved in these activities are needed. However, these results support the idea that immunobiotic strains can improve the recovery from Cy-immunosuppression, enhancing myeloid population in BM. Therefore, immunobiotics can serve as alternatives to reduce the immunosuppression in patients treated with chemotherapy drugs.

CONCLUDING REMARKS

This review exposes wide evidence that the gut microbiota regulates granulocyte homeostasis, and therefore influences the host's innate immune response. Additionally, research from the last years demonstrated that the oral administration of immunobiotics improves the recovery of steady-state granulopoiesis and stimulate the emergency granulopoiesis

in malnourished and Cy-immunocompromised host. Future research is needed in order to elucidate the mechanisms by which specific immunobiotic strains enhance the recovery of granulopoiesis in immunocompromised hosts. Although the use of colony-stimulating factors can reduce the increased risk of infections induced by chemotherapy treatments, they are also the cause of several important side effects including bone pain, low-grade fever, and fatigue. Interestingly, the results expressed provide the basis for new applications of immunobiotics in order to stimulate the production of neutrophils and other types of leukocytes in the BM which would strength the ability of the host to fight against infections, without the side effects observed for stimulating factors. Certainly, this immunobiotic effect would improve the quality of life of patients receiving chemotherapy.

AUTHOR CONTRIBUTIONS

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Peptidoglycan from Immunobiotic *Lactobacillus rhamnosus* Improves Resistance of Infant Mice to Respiratory Syncytial Viral Infection and Secondary Pneumococcal Pneumonia

Patricia Clua[1,2†], Paulraj Kanmani[3,4†], Hortensia Zelaya[1,5], Asuka Tada[3], A. K. M. Humayun Kober[3,4], Susana Salva[1,2], Susana Alvarez[1,2,5], Haruki Kitazawa[3,4] and Julio Villena[1,2,3]**

[1] Immunobiotics Research Group, Tucuman, Argentina, [2] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina, [3] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [4] Livestock Immunology Unit, International Education and Research Center for Food and Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [5] Institute of Applied Biochemistry, National University of Tucumán, Tucuman, Argentina

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Edited by:
Hao Shen,
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Mariela Segura,
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University of Pennsylvania,
United States

***Correspondence:**
Haruki Kitazawa
haruki.kitazawa.c7@tohoku.ac.jp;
Julio Villena
jcvillena@cerela.org.ar

†These authors have contributed equally to this work.

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Several research works have demonstrated that beneficial microbes with the capacity to modulate the mucosal immune system (immunobiotics) are an interesting alternative to improve the outcome of bacterial and viral respiratory infections. Among the immunobiotic strains with the capacity to beneficially modulate respiratory immunity, *Lactobacillus rhamnosus* CRL1505 has outstanding properties. Although we have significantly advanced in demonstrating the capacity of *L. rhamnosus* CRL1505 to improve resistance against respiratory infections as well as in the cellular and molecular mechanisms involved in its beneficial activities, the potential protective ability of this strain or its immunomodulatory cellular fractions in the context of a secondary bacterial pneumonia has not been addressed before. In this work, we demonstrated that the nasal priming with non-viable *L. rhamnosus* CRL1505 or its purified peptidoglycan differentially modulated the respiratory innate antiviral immune response triggered by toll-like receptor 3 activation in infant mice, improving the resistance to primary respiratory syncytial virus (RSV) infection, and secondary pneumococcal pneumonia. In association with the protection against RSV-pneumococcal superinfection, we found that peptidoglycan from *L. rhamnosus* CRL1505 significantly improved lung $CD3^+CD4^+IFN\text{-}\gamma^+$, and $CD3^+CD4^+IL\text{-}10^+$ T cells as well as $CD11c^+SiglecF^+IFN\text{-}\beta^+$ alveolar macrophages with the consequent increases of IFN-γ, IL-10, and IFN-β in the respiratory tract. Our results also showed that the increase of these three cytokines is necessary to achieve protection against respiratory superinfection since each of them are involved in different aspect of the secondary pneumococcal pneumonia that have to be controlled in order to reduce the severity of the infectious disease: lung pneumococcal colonization, bacteremia, and inflammatory-mediated lung tissue injury.

Keywords: immunobiotics, peptidoglycan, toll-like receptor 3, viral immunity, *Streptococcus pneumoniae*, respiratory syncytial virus

INTRODUCTION

Respiratory viral attack often result in mild to moderate infection; however, life-threatening disease can occur in high-risk populations such as infants, elderly, and immunocompromised hosts. Moreover, secondary bacterial pneumonia is an important complication responsible for high morbidity and mortality during epidemic and pandemic viral respiratory infections in infants and children (1–3). It was demonstrated that secondary bacterial respiratory infections are caused primarily by *Streptococcus pneumoniae*. The majority of the clinical observations and experiments in animal models have focused in post-influenza pneumococcal pneumonia (4). In fact, numerous studies have investigated how primary influenza virus (IFV) infection enhances the susceptibility to secondary pneumococcal disease, by increasing bacterial attachment and colonization, disrupting epithelial barriers, and altering the innate immune response in the respiratory tract (3). Although IFV and *S. pneumoniae* interaction has been extensively studied because of its great impact in the severity of respiratory infections, other viruses like the Respiratory Syncytial Virus (RSV) have been associated to an increased susceptibility to secondary pneumococcal pneumonia.

Clinical and epidemiologic data suggest that RSV is linked to increases in the frequency (5) and severity (6) of pneumococcal disease. It was also demonstrated that mice infected with RSV before pneumococcal challenge as well as mice infected with both respiratory pathogens simultaneously showed enhanced lung alterations and elevated levels of bacteremia (7, 8). Mechanisms underlying pneumococcal superinfection include RSV-induced local destruction of the epithelium and respiratory ciliary dyskinesia that impairs mucociliary clearance in the airways (8). Elevated pneumococcal adherence to the respiratory tract epithelium is also considered one of the mechanisms facilitating *S. pneumoniae* infection. It was reported that intercellular adhesion molecule 1 (ICAM-1), carcinoembryonic adhesion molecule 1 (CEACAM1), and platelet activating factor receptor (PAF) are upregulated by RSV infection in respiratory epithelial cells, which are molecules used by pneumococci for colonization (9). Moreover, *in vitro* experiments with HEp-2 cells (human nasopharyngeal), A549 cells (pneumocyte type II), or human airway epithelial cell primary cultures showed that RSV virions enhance pneumococcal adherence through the expression of the viral G protein in epithelial surfaces that serve as an adhesion molecule for pneumococci (7, 8, 10). Surprisingly, transcriptomic analysis performed by Smith et al. (8) showed that the direct interaction between RSV and *S. pneumoniae* alters bacterial gene expression. The work demonstrated that the pneumococcal penicillin-binding protein 1a binds RSV G protein and that this interaction alters *S. pneumoniae* transcriptome increasing the expression of the virulence factors pneumolysin and neuraminidase A/B. These results indicate that complex interactions exist between RSV, *S. pneumoniae*, and host, which must be fully characterized in order to reduce the severity and mortality of respiratory superinfections caused by these pathogens.

During the last years, several research works have demonstrated that beneficial microbes with the capacity to modulate the mucosal immune system (immunobiotics) are a potential alternative to improve the outcome of bacterial (11) and viral (12) respiratory infections. Among the immunobiotic strains with the capacity of beneficially modulate respiratory immunity, our research group has demonstrated that *Lactobacillus rhamnosus* CRL1505 has outstanding properties. Nasal priming with *L. rhamnosus* CRL1505 is able to significantly increase the resistance against the respiratory pathogens *S. pneumoniae*, IFV, or RSV (13–15). We have also reported that immunobiotic *L. rhamnosus* CRL1505 is able to differentially regulate the levels and kinetics of respiratory inflammatory cells and cytokines in mice after activation of Toll-like receptor 3 (TLR3) by the nasal administration of poly(I:C), or after the challenge with RSV or IFV (13, 14). This beneficial regulation of virus-triggered inflammatory response in the respiratory tract by the CRL1505 strain correlated with a significant reduction in lung damage and improved survival of infected mice (13, 14). Of interest, we have demonstrated that viability of the immunobiotic strain is not necessary to achieve the protective effect. In fact, protection against RSV or *S. pneumoniae* infections can be improved by nasal administration of non-viable *L. rhamnosus* CRL1505 (13) or its peptidoglycan (15), respectively. Although we have significantly advanced in demonstrating the capacity of *L. rhamnosus* CRL1505 to improve resistance against respiratory infections as well as in the cellular and molecular mechanisms involved in its beneficial activities (12), the potential protective ability of this strain or its immunomodulatory cellular fractions in the context of a secondary bacterial pneumonia has not been addressed before.

We hypothesized that the effect of immunobiotics or their immunomodulatory cellular fractions in the respiratory antiviral innate immune response could beneficially influence the resistance to secondary bacterial infections. Therefore, in the present study, we investigated how the exposure of infant mice to the nasal priming with non-viable *L. rhamnosus* CRL1505 or its peptidoglycan influences the respiratory innate immune response triggered by TLR3 activation, the susceptibility to primary RSV infection, and the resistance to secondary pneumococcal pneumonia. We demonstrated that peptidoglycan from immunobiotic *L. rhamnosus* CRL1505 improves respiratory antiviral innate immune response, reduces bacterial transmigration across the lung, and limits pulmonary inflammatory damage caused by *S. pneumoniae* after the challenge with poly(I:C) or the infection with RSV.

MATERIALS AND METHODS

Microorganisms and Peptidoglycan

Lactobacillus rhamnosus CRL1505 was obtained from the CERELA culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). The culture was kept freeze-dried. For experiments the culture was rehydrated using a medium containing 15 g of peptone, 10 g tryptone, and 5 g of meat extract in 1 l of distilled water, pH 7. Then, lactobacilli were cultured for 12 h at 37°C (final log phase) in Man–Rogosa–Sharpe broth (MRS, Oxoid). The bacteria were harvested by centrifugation at 3,000 × *g* for 10 min, washed three times with sterile 0.01 mol/l

phosphate buffer saline (PBS, pH 7.2), and resuspended in sterile PBS. Non-viable *L. rhamnosus* CRL1505 (HK1505) was obtained as described previously (13). Bacteria were killed by tyndallization in a water bath at 80°C for 30 min, and the lack of bacterial growth was confirmed using MRS agar plates. Peptidoglycan from *L. rhamnosus* CRL1505 (PG1505) was obtained as described previously (15). Briefly, the bacterium was grown in MRS broth for 18 h at 37°C, washed three times with sterile PBS, and lyophilized. Lactobacilli were resuspended in sterile water (0.1 g/ml) and lysed by sonication in an Ultrasonic Homogenizer (Cole Parmer) with cycles of 2.5 min and amplitude of 70%. The cell wall obtained was delipidated by successive refluxing with methanol, methanol–chloroform (1:1), and chloroform. The delipidated preparation was resuspended in Tris–HCl buffer 50 μM (pH 7.5) and treated with bovine pancreatic DNAse I (Sigma) (50 μg/ml) and ribonuclease A (Sigma) (100 μg/ml) at 37°C, 4 h. Finally, cell wall was treated with 50% hydrogen chloride at 4°C for 20 h. The PG1505 obtained was washed with sterile water, adjusted to pH 7.2, and lyophilized until use.

Animals and Feeding Procedures

Infant (3-week-old) BALB/c mice were obtained from the closed colony kept at CERELA (San Miguel de Tucumán, Argentina). They were housed in plastic cages at room temperature. Mice were housed individually during the experiments and the assays for each parameter studied were performed in 5–6 mice per group for each time point. HK1505 was nasally administered to infant mice for 2 consecutive days at a dose of 10^8 cells/mouse/day in 50 μl of PBS (13). PG1505 was nasally administered to infant mice for 2 consecutive days at a dose of 8 μg/ml, in 50 μl of PBS (15). Anesthesia was not necessary for HK1505 or PG1505 administration since mice did not show any sing of discomfort. The treated groups and the untreated control group were fed a conventional balanced diet *ad libitum*. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of CERELA and all efforts were made to minimize suffering.

Intranasal Administration of Poly(I:C)

Administration of the viral pathogen molecular pattern poly(I:C) was performed on day 3, after the 2-day treatments with HK1505 or PG1505 as shown in **Figure 1A**. Mice were lightly anesthetized and 100 μl of PBS, containing 250 μg poly(I:C) (equivalent to 10 mg/kg body weight), was administered dropwise, *via* the nares (13, 16, 17). Control animals received 100 μl of PBS. Mice received three doses of poly(I:C) or PBS with 24 h rest period between each administration.

RSV Infection

Human RSV strain A2 was grown in Vero cells as described previously (13, 16). Briefly, Vero cells were infected with RSV at

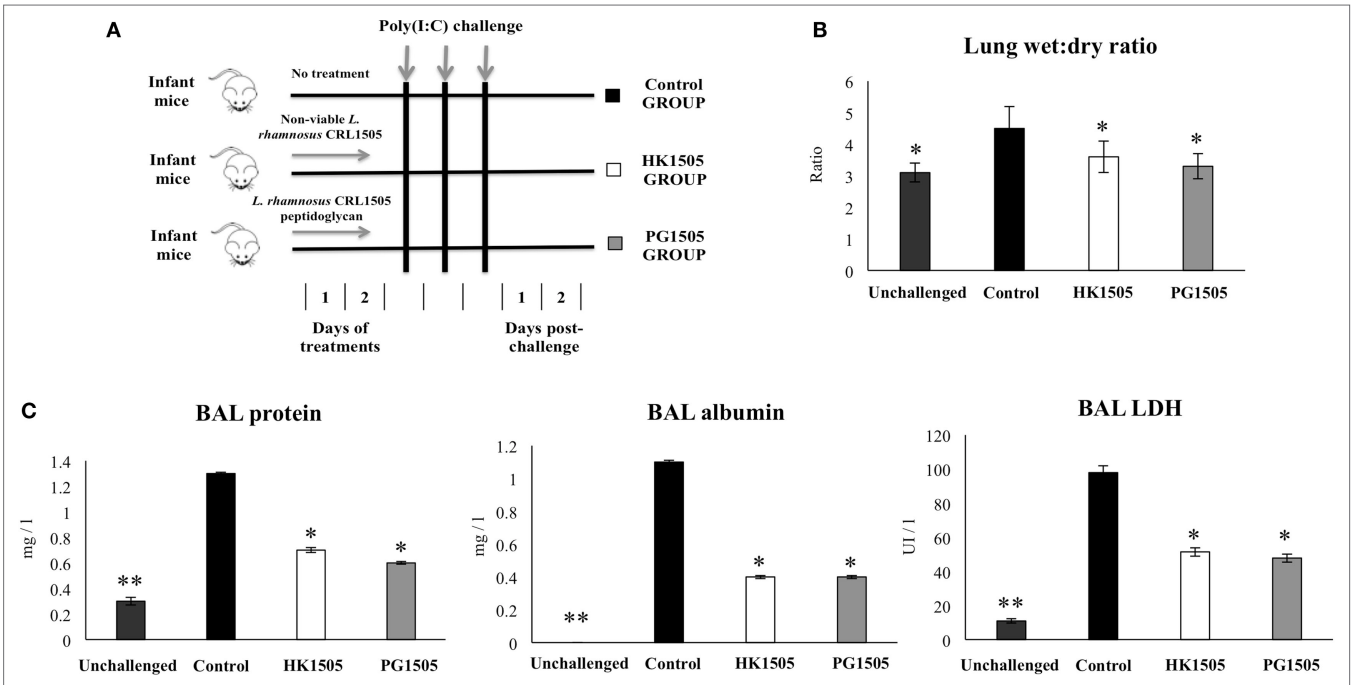

FIGURE 1 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on lung tissue damage induced by the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days and then challenged with three once-daily doses of poly(I:C) **(A)**. Non-treated infant mice challenged with poly(I:C) were used as controls. 2 days after the last poly(I:C) administration lung wet:dry weight ratio **(B)**, lactate dehydrogenase (LDH) activity and, albumin and protein concentrations in bronchoalveolar lavages (BAL) **(C)** were determined. The results represent data from three independent experiments. Significant differences between treated and control groups, $^*P < 0.05$, $^{**}P < 0.05$.

a multiplicity of infection of 1 in 5 ml of Dulbecco's modified Eagle's medium (DMEM). Cells were infected for 3 h at 37°C, 5% CO_2. After infection, 7 ml of DMEM with 10% fetal bovine serum (Sigma, Tokyo, Japan), 0.1% penicillin–streptomycin (Pen/Strep) (Sigma, Tokyo, Japan), and 0.001% ciprofloxacin (Bayer) was added to the flask, and cells were incubated until extensive syncytium formation was detected. Then, Vero cells were scraped and sonicated three times, 5 s per time, at 25 W on ice. Cell debris was removed by centrifugation at 700 g for 10 min at 4°C. Virus supernatant was sucrose density gradient purified and stored in 30% sucrose at −80°C. For *in vivo* infection, mice were lightly anesthetized with isoflurane and intranasally challenged with 3.1×10^6 PFU of RSV (13, 16). Viral challenge was performed on day 3, after the 2-day treatments with HK1505 or PG1505. Lung RSV titers and tissue damage were evaluated during 5 days after viral infection.

For the evaluation of viral infection, the RSV immunoplaque assay was performed as described previously (13, 16). In brief, lung tissue was removed from infant mice and stored in 30% sucrose for plaque assay. Lungs were homogenized using a pellet pestle and centrifuged at 2,600 × *g* for 10 min at 4°C to clarify supernatant. Serial dilutions of lung tissue-clarified supernatants were added into fresh Vero cells monolayers, and incubated at 37°C, 5% CO_2 for 3 h. All samples were run in triplicate. After incubation and removal of supernatant, 1 ml of fresh DMEM medium containing 10% FBS, 0.1% Pen/Strep, and 0.001% ciprofloxacin was added to monolayers. When extensive syncytia developed, monolayers were fixed with 1 ml of ice-cold acetone:methanol (60:40). Then, well were treated with primary RSV anti-F (clones 131-2A; Chemicon) and anti-G (Mouse monoclonal [8C5 (9B6)] to RSV glycoprotein, Abcam) antibodies for 2 h, followed by secondary horseradish peroxidase anti-mouse immunoglobulin antibody (Anti-mouse IgG, HRP-linked Antibody #7076, Cell signaling Technology) for 1 h. Plates washed twice with PBS containing 0.5% Tween 20 (Sigma) after each antibody incubation step. Individual plaques were developed using a DAB substrate kit (ab64238, Abcam) following manufacture's specifications. Results were expressed as log10 PFU/g of lung.

S. pneumoniae Secondary Infection

Streptococcus pneumoniae serotype 6B (ANLIS, Argentina) was obtained from the respiratory tract of a patient from the Children's Hospital, Tucuman, Argentina. Pneumococci were grown on blood agar for 18 h. Colonies were suspended in Todd Hewitt broth (Oxoid), incubated overnight at 37°C, harvested, and washed with sterile PBS. Cell density was adjusted to 4×10^7 CFU/ml.

Challenge with pneumococci was performed 1, 3, and 5 days after the last administration of poly(I:C) (data not shown), and a higher susceptibility to bacterial infection was found when the challenge was performed 5 days after TLR3 activation. In addition, several published articles performed bacterial infection 5–7 days after respiratory viral challenge considering that viral load and cytokine environment are different in the earlier and later stages of viral infection (4, 18). Then, challenge with pneumococci was also performed 5 days after the infection with RSV. HK1505- and PG1505-treated as well as control infant mice were challenged intranasally with the pathogen by dripping 25 µl of inoculums containing 10^3, 10^4, 10^6, or 10^7 CFU (log phase) in PBS into each nostril.

Treated and control mice were sacrificed 2 days after *S. pneumoniae* infection. Lungs were excised, weighed, and homogenized in sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 h at 37°C. *S. pneumoniae* was identified by standard techniques and the results were expressed as log of CFU/g of lung. Bacteremia was monitored by blood samples obtained by cardiac puncture which were plated on blood agar. Results were reported as negative or positive hemocultures.

Blocking Experiments

In order to evaluate the role of IFN-β, IFN-γ, and IL-10 in the immunoprotective effect of HK1505 and PG1505, anti-IFN-β, anti-IFN-γ, and anti-IL-10 receptor (IL-10R) blocking antibodies were used (16). Different groups of mice were nasally primed with HK1505 or PG1505 for 2 days and then challenged with poly(I:C) for 3 days as described above. On days 2 and 4 after poly(I:C) challenge, mice were nasally treated with 50 µg of purified IFN-β (LEAF™ Purified anti-mouse IFN-β antibody, #519202 BioLegend), purified anti-IFN-γ (LEAF™ Purified anti-mouse IFN-γ antibody, #505706 BioLegend), or anti-IL-10R (LEAF™ Purified anti-mouse IL-10R antibody, #112708, BioLegend, Tokyo, Japan) antibodies or 250 µg isotype control antibodies (LEAF™ Purified Rat IgG1, Isotype Ctrl, BioLegend). Twelve hours later mice were challenged with *S. pneumoniae* (Figure S1 in Supplementary Material). The efficiency of blocking antibodies was determined by evaluating serum and respiratory concentration of IFN-β, IFN-γ, or IL-10 12 h after the last administration.

Lung Injury Parameters

Bronchoalveolar lavages (BAL) samples were obtained as described previously (13, 16). Briefly, the trachea was exposed and intubated with a catheter, and two sequential lavages were performed in each mouse by injecting sterile PBS. The recovered fluid was centrifuged for 10 min at 900 × *g*; and frozen at −70°C for subsequent analyses.

Protein and albumin content, a measure to quantitate increased permeability of the bronchoalveolar–capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were determined in the acellular BAL fluid. Protein content was measured by the bicinchoninic (BCA) protein assay (Pierce Biotechnology Inc., Rockford, IL, USA). Albumin content was determined colorimetrically based on albumin binding to bromcresol green using an albumin diagnostic kit (Wiener Lab, Buenos Aires, Argentina). LDH activity, expressed as units per liter of BAL fluid, was determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide using the Wiener reagents and procedures (Wiener Lab). Lung wet:dry weight ratio was measured as previously described (13, 17). Wet:dry weight ratio was calculated as an index of intrapulmonary fluid accumulation, without correction for blood content.

Histopathological examination was also performed in order to further evaluate tissue damage. Lungs were aseptically removed, fixed in 4% formalin, and embedded in histowax (Leica Microsystems). Histopathological assessment was performed on

5-μm tissue sections stained with hematoxylin–eosin. At least four tissue sections from various areas of the lung of each mouse in all experimental groups were examined.

Cytokine Concentrations in Serum and BAL

Tumor necrosis factor (TNF)-α (Mouse TNF-alpha Quantikine enzyme-linked immunosorbent assay (ELISA) Kit, sensitivity: 7.2 pg/ml), interferon (IFN)-α (Mouse IFN-alpha ELISA Kit, sensitivity: 12.5 pg/ml), IFN-β (Mouse IFN-beta ELISA Kit, sensitivity: 15.5 pg/ml), IFN-γ (Mouse IFN-gamma Quantikine ELISA Kit, sensitivity: 2 pg/ml), interleukin (IL)-6 (Mouse IL-6 Quantikine ELISA Kit, sensitivity: 1.8 pg/ml), IL-8 (Mouse IL-8 Quantikine ELISA Kit, sensitivity: 2 pg/ml), IL-10 (Mouse IL-10 Quantikine ELISA Kit, sensitivity: 5.2 pg/ml), and monocyte chemoattractant protein (MCP)-1 (Mouse/Rat CCL2/JE/MCP-1 Quantikine ELISA Kit, sensitivity: 2 pg/ml) concentrations in serum and BAL were measured with commercially available ELISA technique kits following the manufacturer's recommendations (R&D Systems, MN, USA).

Lung Cells Preparation and Flow Cytometry Studies

Single lung cells from mice were prepared as previously described (13, 17). Lungs were removed, finely minced, and incubated for 90 min with 300 U of collagenase (Yakult Honsha Co., Tokyo, Japan) in 15 ml of RPMI 1640 medium (Sigma, Tokyo, Japan). To dissociate the tissue into single cells, collagenase-treated minced lungs were gently tapped into a plastic dish. After removal of debris, erythrocytes were depleted by hypotonic lysis. The cells were washed with RPMI medium supplemented with 100 U/ml of penicillin and 100 mg/ml of streptomycin and then resuspended in a medium supplemented with 10% heat-inactivated fetal calf serum. Cells were counted using Trypan Blue exclusion and then resuspended at an appropriate concentration of 5×10^6 cells/ml.

Lung cell suspensions were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 15 min at 4°C. Cells were incubated in the antibody mixes for 30 min at 4°C and washed with FACS buffer. Then, cells were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD8, CD11c, CD11b, CD103, MHC-II, IFN-γ, IL-10, sialic acid-binding immunoglobulin-like lectin F (SiglecF) (BD Bioscience), IFN-β, and CD45 (eBioscience). Cells were then acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue (13, 17).

Statistical Analysis

Experiments were performed in triplicate and results were expressed as mean ± SD. After verification of the normal distribution of data, 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $P < 0.05$.

RESULTS

Nasally Administered Peptidoglycan from *L. rhamnosus* CRL1505 Reduces Poly(I:C)-Induced Lung Injuries

We have previously demonstrated that nasal administration of non-viable *L. rhamnosus* CRL1505 (HK1505) reduced lung injuries triggered by TLR3 activation (13). Here, we aimed to evaluate the effect of the nasal priming with the peptidoglycan from *L. rhamnosus* CRL1505 (PG1505) on the immune response triggered by the viral pathogen-associated molecular pattern poly(I:C) and compare it with the effect induced by HK1505. For this purpose, infant mice were treated with PG1505 and then challenged with poly(I:C) as shown in the experimental protocol of **Figure 1A**. HK1505 treatment was used as a positive control. Lung injury was studied on day 2 post-challenge as we described previously (13, 17). An altered lung wet:dry weight ratio (**Figure 1B**), as well as increased levels of LDH activity and protein and albumin concentrations (**Figure 1C**) were found in BAL samples of poly(I:C)-treated infant mice, indicating local cellular damage and impairment of the alveolar-capillary barrier. Nasally administered HK1505 or PG1505 did not induce significant changes in the BAL biochemical parameters evaluated before poly(I:C) administration (data not shown). Both, HK1505 or PG1505 significantly diminished wet:dry weight ratio and the three biochemical parameters evaluated in BAL after poly(I:C) challenge (**Figure 1**). HK1505 and PG1505 were equally effective to reduce the lung alterations induced by TLR3 activation.

Nasally Administered Peptidoglycan from *L. rhamnosus* CRL1505 Beneficially Modulates Immune Response Triggered by Poly(I:C) Challenge

In order to evaluate the effect of HK1505 and PG1505 in the respiratory immune system of infant mice, we first determined the levels of different cytokines in BAL before poly(I:C) administration (**Figure 2**). Both, HL1505 and PG1505 treatments significantly increased the levels of BAL IFN-α, IFN-β, IFN-γ, IL-10, and IL-6. BAL concentrations of IFN-α in HK1505 mice and IL-6 in PG1505 mice were higher than the other experimental groups. No significant differences were found when the levels of TNF-α, MCP-1, and IL-8 of control mice were compared with mice receiving HK1505 or PG1505 (**Figure 2**). The treatments were also able to increase serum IFN-α, IFN-β, IFN-γ, IL-10, and IL-6, being both HK1505 and PG1505 equally effective to achieve this effect (Figure S2 in Supplementary Material). No differences were found in the levels of serum TNF-α, MCP-1, or IL-8 when comparing HK1505 and PG1505 with controls. We also evaluated the changes induced by nasally administered HK1505 and PG1505 in lung immune cells using flow cytometry. Nasal priming with HK1505 and PG1505 enhanced the number of $CD3^+CD4^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+IL\text{-}10^+$ T cells in lungs

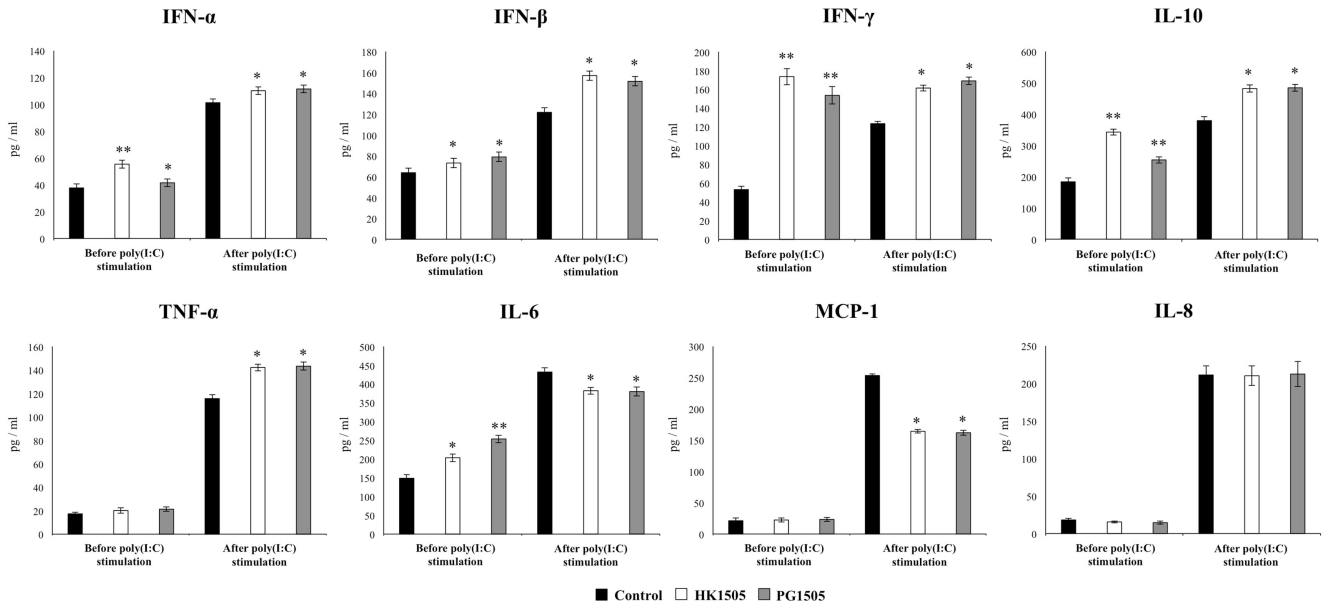

FIGURE 2 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on respiratory cytokines before and after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days and then challenged with three once-daily doses of poly(I:C). Non-treated infant mice challenged with poly(I:C) were used as controls. Before and 2 days after poly(I:C) administration, the levels of tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-β, IFN-γ, interleukin (IL)-6, IL-8, IL-10, and monocyte chemoattractant protein-1 (MCP-1) were determined in bronchoalveolar lavages. The results represent data from three independent experiments. Significant differences between treated and control groups, $^*P < 0.05$.

while no effect was observed for $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells when compared to controls (**Figure 3**). In addition, three population of antigen presenting cells were studied in lungs: myeloid DCs ($MHC\text{-}II^+CD11c^+CD11b^{low}CD103^+$ and $MHC\text{-}II^+CD11c^+CD11b^{high}CD103^-$ cells) and alveolar macrophages ($CD45^+MHC\text{-}II^-CD11c^+SiglecF^+$). HK1505 and PG1505 significantly increased the number of both lung $CD11c^+CD11b^{low}CD103^+$ and $CD11c^+CD11b^{high}CD103^-$ DCs, while no quantitative changes were detected in $CD45^+CD11c^-SiglecF^+$ macrophages (**Figure 3**) or $CD45^+Gr1^+$neutrophils (data not shown).

The respiratory immune response triggered by poly(I:C) in infant mice and the effect of the nasal priming with HK1505 and PG1505 in that response were next studied. As we described previously (13, 17), the nasal administration of poly(I:C) significantly increased respiratory levels of type I IFNs (IFN-α, IFN-β), IFN-γ, and pro-inflammatory cytokines and chemokines (TNF-α, IL-6, MCP-1, IL-8) in BAL of infant mice (**Figure 2**). Both HK1505 and PG1505 treatments significantly increased the levels of BAL IFN-α, IFN-β, IFN-γ, and TNF-α while they diminished the concentration of IL-6 and MCP-1 (**Figure 2**). In addition, IL-8 was not modified in HK1505 or PG1505 groups when compared to control mice (**Figure 2**). Poly(I:C) stimulation also induced an increase in the respiratory levels of IL-10 that were significantly higher in HK1505- or PG1505-treated mice when compared to controls (**Figure 2**). Lung immune cells populations were also evaluated in poly(I:C)-challenged mice (**Figure 3**). Poly(I:C) administration increased the numbers of $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^+CD4^+IL\text{-}10^+$, $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells, and myeloid DCs ($MHC\text{-}II^+CD11c^+CD11b^{low}CD103^+$ and $MHC\text{-}II^+CD11c^+CD11b^{high}CD103^-$ cells) in lungs as we described previously (13, 17). In addition, we also observed an increase in alveolar macrophages ($CD45^+CD11c^+SiglecF^+$) and neutrophils ($CD45^+Gr1^+$) after the challenge with poly(I:C) when compared to basal levels (**Figure 3**). Nasal priming with HK1505 or PG1505 increased the numbers of lung $CD3^+CD4^+IFN\text{-}\gamma^+$, and $CD3^+CD4^+IL\text{-}10^+$ T cells as well as $MHC\text{-}II^+CD11c^+CD103^+$ and $MHC\text{-}II^+CD11c^+CD11b^{high}$ DCs (**Figure 3**). No significant modification of the numbers of lung $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells or $CD45^+CD11c^+SiglecF^+$ macrophages (**Figure 3**) were observed after HK1505 or PG1505 treatments when compared to controls. Significantly reduced numbers of neutrophils in BAL and lung were found in HK1505- or PG1505-treated mice when compared to controls (**Figure 3**).

Nasally Administered Peptidoglycan from *L. rhamnosus* CRL1505 Improves Resistance against RSV Infection

We next evaluated whether the changes induced by PG1505 in the respiratory immune system affected the outcome of RSV infection in infant mice as described previously for HK1505 (13). Therefore, infant mice were treated with HK1505 or PG1505 by the nasal route and then challenged with 10^6 PFU of RSV (13, 16). Evaluation of viral loads of infected mice showed that RSV was present in lungs of all the experimental groups during the 5 days studied (**Figure 4A**). However, significantly lower viral titers were found in mice treated with HK1505 or PG1505 when compared to controls, being both treatments equally effective to reduce

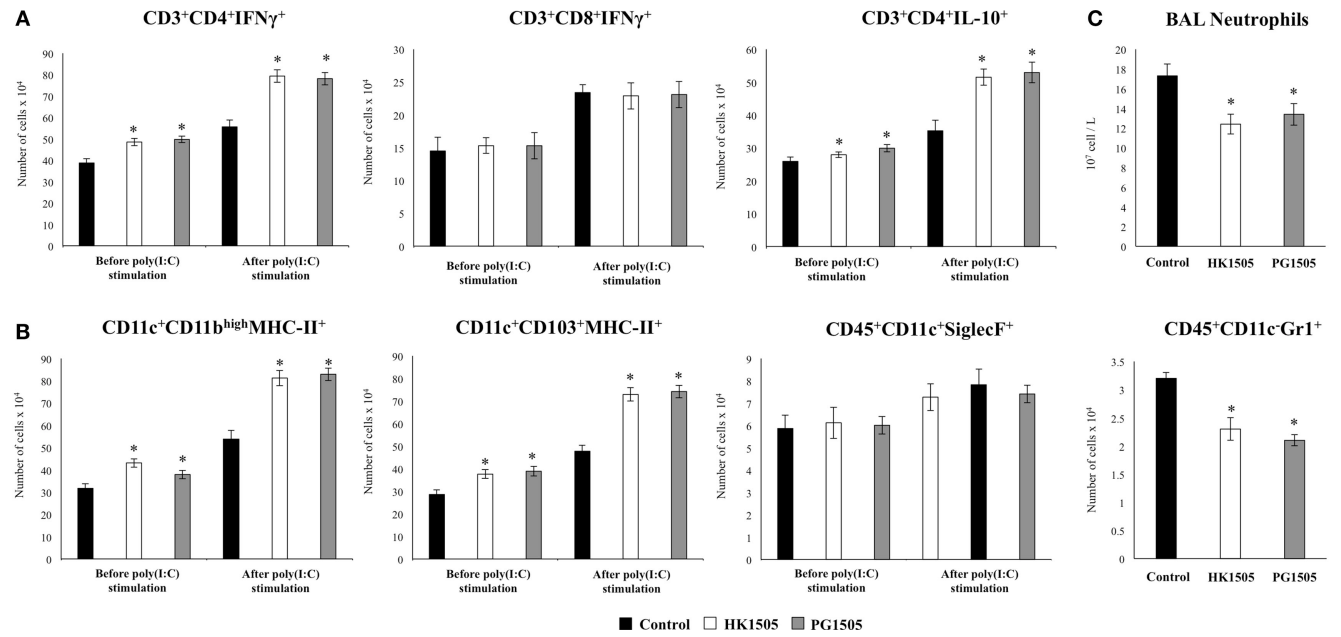

FIGURE 3 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on respiratory immune cell populations before and after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days and then challenged with three once-daily doses of poly(I:C). Non-treated infant mice challenged with poly(I:C) were used as controls. Before and 2 days after poly(I:C) administration, the numbers of lung T cells **(A)** including $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^+CD4^+IL\text{-}10^+$, and $CD3^+CD8^+IFN\text{-}\gamma^+$ T lymphocytes, as well as antigen presenting cells **(B)** including $MHC\text{-}II^+CD11c^+CD11b^{low}CD103^+$ and $MHC\text{-}II^+CD11c^+CD11b^{high}CD103^-$ dendritic cells, and $CD45^+CD11c^+SiglecF^+$ alveolar macrophages were determined by flow cytometry. In addition, bronchoalveolar lavages (BAL) neutrophils and lung $CD45^+CD11c^-Gr1^+$ cells **(C)** were determined. The results represent data from three independent experiments. Significant differences between treated and control groups, $^*P < 0.05$.

RSV replication in the respiratory tract. Moreover, HK1505 and PG1505 significantly improved the body weight gain during RSV infection when compared to controls (**Figure 4B**). The markers of lung tissue damage in RSV-infected mice showed that the viral infection induced a significant cellular damage and alveolar–capillary barrier alterations (**Figure 4C**). Both, BAL LDH and albumin concentrations were significantly lower in infant mice previously treated with HK1505 or PG1505 than in RSV-challenged control mice (**Figure 4C**).

Nasally Administered Non-Viable *L. rhamnosus* CRL1505 and Its Peptidoglycan Improve Resistance to Secondary Pneumococcal Pneumonia after Poly(I:C) Treatment

As mentioned before, respiratory viral infections increase the susceptibly of secondary bacterial pneumonia in infants. Taking into consideration the beneficial effects of nasal HK1505 (13) or PG1505 on the respiratory antiviral innate immune response, we next addressed whether these treatments where able to increase the resistance of infant mice to secondary pneumococcal pneumonia. For this purpose, we first performed comparative studies of secondary pneumococcal infection in Swiss albino and BALB/c mice that are naturally susceptible and resistant to pneumococci, respectively. We found that BALB/c mice, which are highly susceptible to poly(I:C) and RSV, were a better animal model for studying poly(I:C)-pneumococcal or RSV-pneumococcal infection than Swiss albino mice (data not shown). In line with our findings, some works have established that BALB/c mice are suitable for studying poly(I:C)-induced respiratory damage and RSV-bacterial infections. Stark et al. (19) demonstrated that exposure of BALB/c mice to RSV significantly decreased *S. pneumoniae*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa* clearance. In addition, the work showed that the effect of RSV infection on bacterial clearance was dependent on the mouse genetic background by performing experiments with C57BL/6J and FVBN/J mice, which are relatively resistant and susceptible to RSV infection respectively. C57BL/6J mice showed a modest change in pneumococcal clearance following RSV challenge, whereas FVBN/J mice showed a decrease in pneumococcal clearance following RSV.

As shown in the experimental protocol illustrated in **Figure 5A**, mice were nasally primed with HK1505 or PG1505, stimulated with poly(I:C), and 5 days after the last poly(I:C) administration challenged with the respiratory bacterial pathogen *S. pneumoniae*. Pneumococcal colonization and bacteremia as well as lung tissue damage were evaluated on day 2 post-pneumococcal challenge. Two doses (10^3 and 10^4 cells) of *S. pneumoniae* were evaluated. Pneumococci were detected in lungs (**Figure 5B**) and blood (**Figure 5C**) of control infant mice for the two doses of the respiratory pathogen. HK1505 significantly reduced lung bacterial cell counts and the dissemination of *S. pneumoniae* into the blood in infant mice infected with

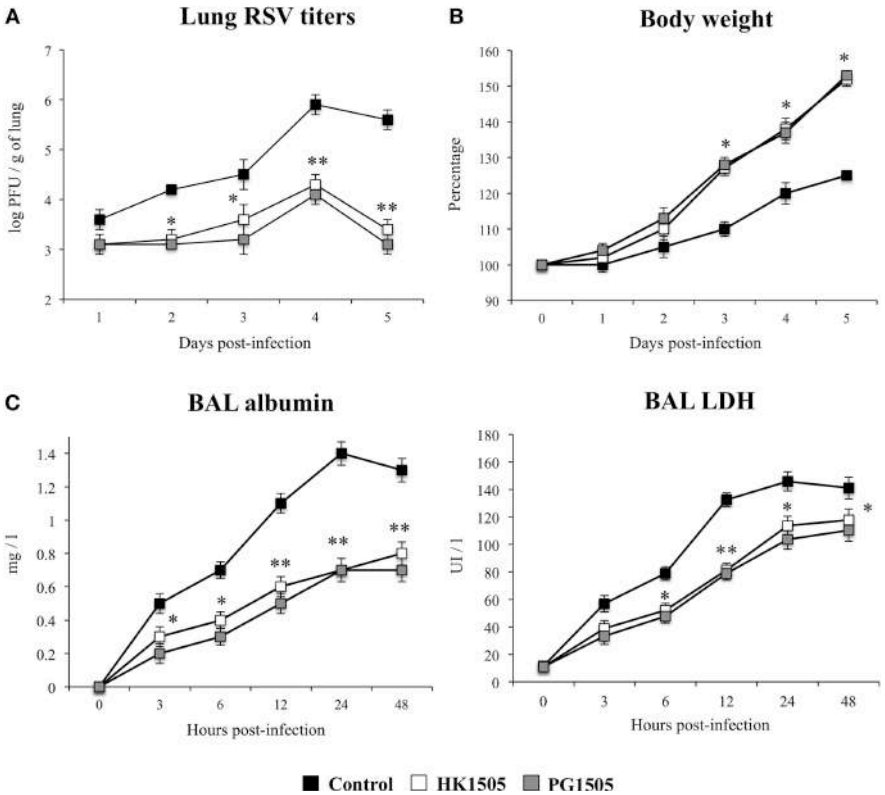

FIGURE 4 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on the resistance to primary Respiratory Syncytial Virus (RSV) infection. Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days and then challenged with RSV. Non-treated infant mice challenged with the viral pathogen were used as controls. Lung RSV titers **(A)**, changes in body weight **(B)**, and lactate dehydrogenase (LDH) activity and albumin concentrations in bronchoalveolar lavages (BAL) **(C)** were evaluated on different time points after the viral challenge. The results represent data from three independent experiments. Significant differences between treated and control groups $^{*}P < 0.05$, $^{**}P < 0.01$.

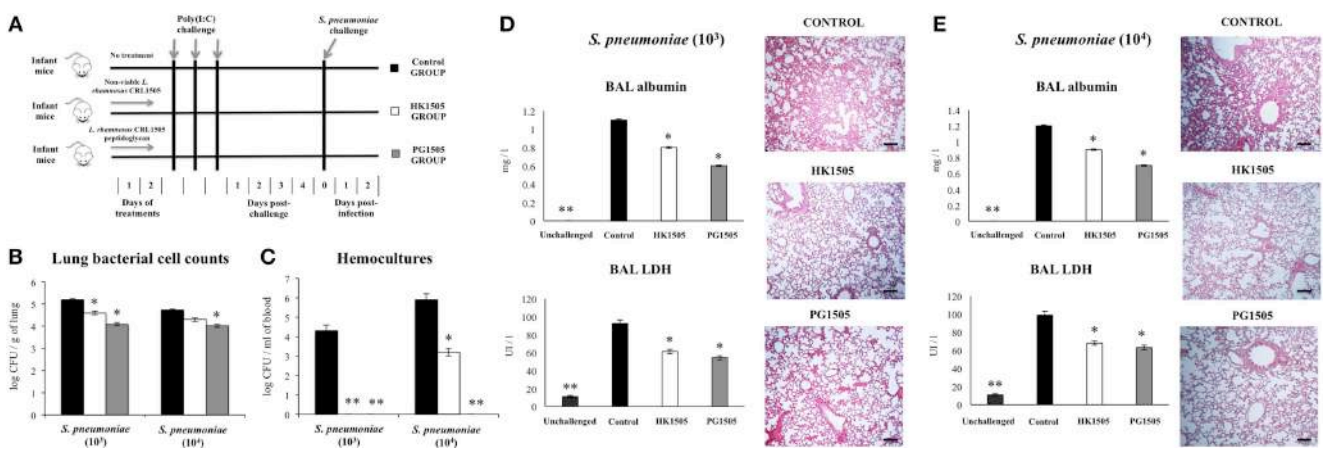

FIGURE 5 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on the resistance to secondary pneumococcal pneumonia after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days, challenged with three once-daily doses of poly(I:C) and, infected with two different doses of *Streptococcus pneumoniae* 5 days after the last poly(I:C) administration **(A)**. Non-treated infant mice stimulated with poly(I:C) and challenged with *S. pneumoniae* were used as controls. Lung bacterial cells counts **(B)**, hemocultures **(C)**, lactate dehydrogenase (LDH) activity and albumin concentrations in bronchoalveolar lavages (BAL) **(D,E)**, and lung histopathological examination **(D,E)** were determined on day 2 post-pneumococcal challenge. Scale bar = 100 μm. The results represent data from three independent experiments. Significant differences between treated and control groups, $^{*}P < 0.05$, $^{**}P < 0.01$.

10^3 pneumococcal cells while no significant differences in these parameters were found in animals infected with 10^4 pneumococcal cells (**Figure 5**). Interestingly, PG1505 significantly reduced lung bacterial cell counts and the dissemination of the respiratory pathogen into the blood in infant mice infected with both 10^3 and 10^4 pneumococcal cells (**Figures 5B,C**). Evaluation of lung tissue injury showed that secondary pneumococcal pneumonia induced a significant cellular damage and alveolar–capillary barrier alterations as demonstrated by the significant higher levels of BAL LDH and albumin when compared to basal levels (**Figures 5D,E**). Lung histological examination revealed inflammatory cell recruitment around alveoli and blood vessels, focal hemorrhage, and a significant reduction of gas exchange spaces (**Figures 5D,E**). Biochemical markers and histology also showed that lung tissue injuries were comparable in mice infected with both 10^3 and 10^4 pneumococcal cells. BAL LDH and albumin concentrations were significantly lower in infant mice treated with HK1505 or PG1505 when compared to controls for both 10^3 and 10^4 pneumococcal cells (**Figures 5D,E**). Moreover, lung histology of HK1505- or PG1505-treated mice showed a significant reduction in the alterations of gas exchange spaces, hemorrhage, and inflammatory cells infiltration.

Nasally Administered Non-Viable *L. rhamnosus* CRL1505 and Its Peptidoglycan Differentially Modulate the Immune Response Triggered by Secondary Pneumococcal Pneumonia

Our previous results indicate that IFN-β, IFN-γ, and IL-10 are involved in the immunoregulatory effects of nasally administered immunobiotics (12). Moreover, as we described in **Figure 2**, the levels of these three cytokines were significantly increased in the respiratory tract of HK1505- or PG1505-treated mice. Therefore, we aimed to investigate the levels of IFN-β, IFN-γ, and IL-10 before (day 0) and after (day 2) the infection with *S. pneumoniae* as indicated in the experimental protocol of **Figure 5A**. The three cytokines were increased in HK1505 and PG1505 groups indicating that their elevated levels persisted up to 5 days of the last poly(I:C) administration (**Figure 6A**). Pneumococcal challenge

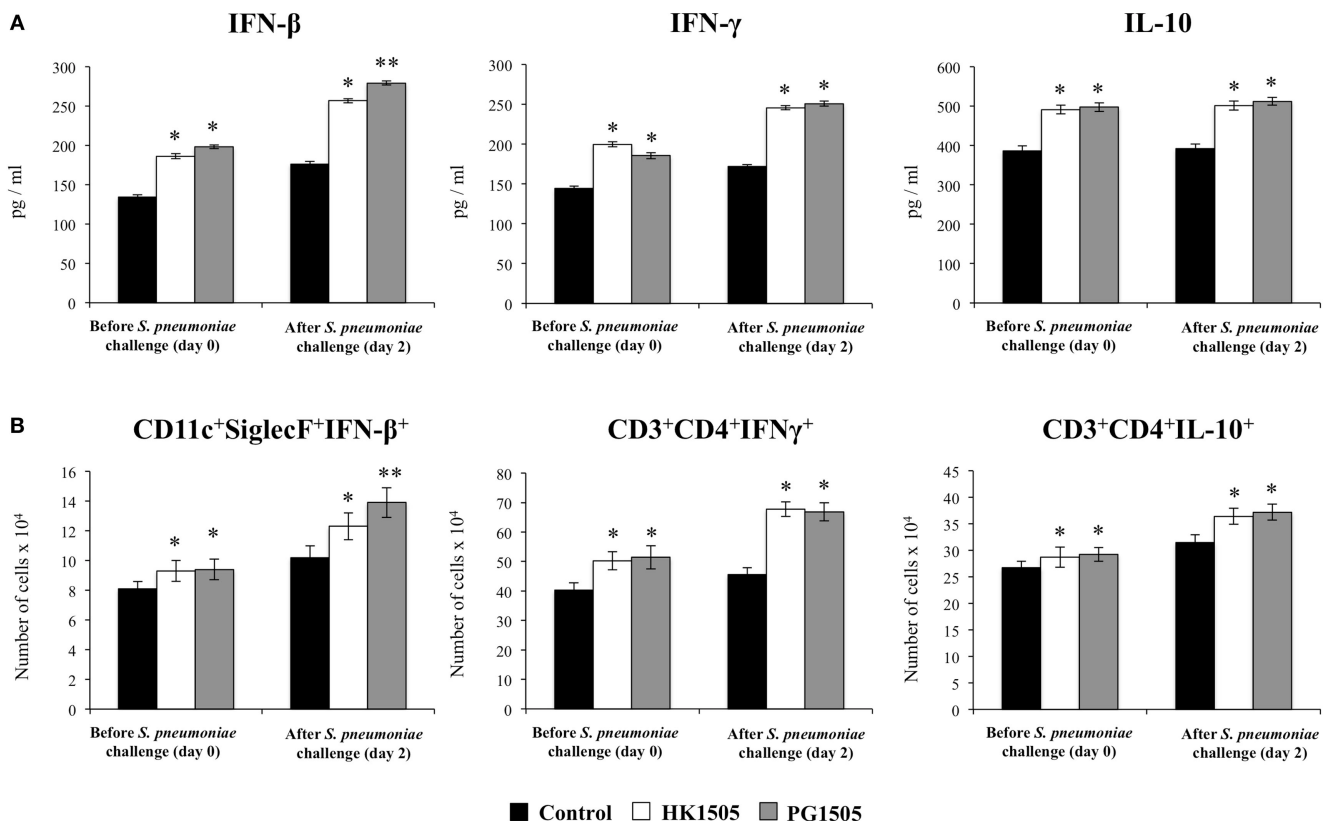

FIGURE 6 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on the respiratory immune response to secondary pneumococcal pneumonia after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days, challenged with three once-daily doses of poly(I:C) and, infected with two different doses of *Streptococcus pneumoniae* 5 days after the last poly(I:C) administration. Non-treated infant mice stimulated with poly(I:C) and challenged with *S. pneumoniae* were used as controls. The levels of interferon (IFN)-β, IFN-γ, and interleukin (IL)-10 in bronchoalveolar lavages **(A)**, as well as the numbers of lung $CD3^+CD4^+IFN\text{-}\gamma^+$, and $CD3^+CD4^+IL\text{-}10^+$ T cells and $CD45^+CD11c^+SiglecF^+$ alveolar macrophages **(B)** were determined before (day 0) and after (day 2) pneumococcal challenge. The results represent data from three independent experiments. Significant differences between treated and control groups, $^*P < 0.05$, $^{**}P < 0.01$.

further increase BAL IFN-β, IFN-γ, and IL-10 that were higher in HK1505- or PG1505-treated mice when compared to controls (**Figure 6A**). We also investigated the potential source of these cytokines within the immune cell populations of lungs. As we described previously, we found that IFN-γ and IL-10 were mainly produced by $CD4^+$ T cells. In this work, we also demonstrated that IFN-β was mainly produced by alveolar macrophages and the $CD45^-$ population of lung (probably respiratory epithelial cells) (data not shown). Similar to the results of cytokines' levels, we found that lung $CD3^+CD4^+$IFN-γ^+, and $CD3^+CD4^+$IL-10^+ T cells as well as $CD11c^+SiglecF^+$IFN-β^+ macrophages were significantly increased before and after the infection with *S. pneumoniae* in HK1505- or PG1505-treated mice when compared to control animals (**Figure 6B**). Of interest, both $CD11c^+SiglecF^+$IFN-β^+ macrophages and BAL IFN-β levels were higher in the PG1505 group than in HK1505 mice (**Figure 6**).

In order to further evaluate the role of IFN-β, IFN-γ, and IL-10 in the immunoregulatory effect of HK1505 and PG1505 during secondary pneumococcal infection we used blocking anti-IFN-β, anti-IFN-γ, and anti-IL-10R antibodies as described in the experimental protocol of Figure S2 in Supplementary Material. Treatment of mice with anti-IFN-β significantly abolished the capacity of HK1505 and PG1505 to avoid pneumococcal dissemination into the blood although it had no effect on lung bacterial cell counts (**Figure 7A**). In addition, the ability of HK1505 and PG1505 to protect against pulmonary damage was lost with anti-IFN-β antibodies administration (**Figure 7D**). Treatment with anti-IFN-γ significantly abolished the capacity of HK1505 and PG1505 to reduce lung bacterial cell counts and had no effect on bacteremia (**Figure 7B**) or in the protection against lung tissue damage (**Figure 7E**). On the other hand, administration of anti-IL-10R to infant mice significantly abolished the capacity of HK1505 and PG1505 to reduce lung tissue injuries (**Figure 7F**), while they did not affect lung pneumococcal colonization or dissemination into the blood (**Figure 7C**).

Nasally Administered Non-Viable *L. rhamnosus* CRL1505 and Its Peptidoglycan Improve Resistance to Secondary Pneumococcal Pneumonia after RSV Infection

Finally, we aimed to evaluate whether HK1505 or PG1505 treatments were able to protect against secondary pneumococcal pneumonia after the infection of infant mice with RSV. For this purpose, mice were nasally primed with HK1505 or PG1505, infected with RSV, and 5 days after the infection they were challenged with 10^3 cells of *S. pneumoniae*. Similar to the experiments performed with poly(I:C), pneumococcal colonization and bacteremia were evaluated on day 2 post-pneumococcal challenge. In addition, RSV titers as well as lung tissue damage were studied before (day 0) and after (day 2) the infection with *S. pneumoniae* (**Figure 8**). RSV was detected in the lungs of infected infant mice before and after pneumococcal infection (**Figure 8A**). In addition, pneumococci were detected in lungs (**Figure 8B**) and blood (**Figure 8C**) of control infant mice. Both, HK1505 and PG1506 significantly reduced RSV titers as well as lung bacterial cell counts and the dissemination of *S. pneumoniae* into the blood (**Figures 8A–C**), which was in line with the improved survival of HK1505- and PG1506-treated mice when compared to controls (**Figure 8D**). Of interest, PG1505 was more effective than HK1505 to diminish lung bacterial cell counts. When lung injury was studied, it was observed that the secondary pneumococcal pneumonia induced a significant increase of the BAL biochemical parameters that evaluate cellular damage and alveolar–capillary barrier alterations (**Figure 8E**). In addition, histological examination of lungs showed a significant reduction of gas exchange spaces, inflammatory cell recruitment, and focal hemorrhage (**Figure 8F**). HK1505 and PG1505 significantly reduced pulmonary damage as demonstrated by the diminished BAL LDH and albumin concentrations and the lower histological alterations when compared to controls (**Figures 8E,F**).

DISCUSSION

It was established that the mortality associated to respiratory viral infections is not due to the viral infection alone but instead, secondary bacterial pneumonia complicates many severe cases in infected hosts (1–3). Therefore, it is crucial to understand how respiratory viral infections alter the host's respiratory microenvironment and the local innate immunity to benefit the establishment of secondary bacterial infections, in order develop preventive or therapeutic strategies aimed to protect against mortality. Considering the elevated incidence of viral infections and the high frequency of associated secondary bacterial infections that contribute to aggravate the health status, several approaches are been investigated for preventing or treating respiratory superinfections, including antibiotics and immunomodulatory drugs (20). To our knowledge, no study has evaluated the potential protective ability of immunobiotics or their immunomodulatory cellular fractions in the context of a secondary bacterial pneumonia. Therefore, we demonstrated here for the first time, that the nasal priming with peptidoglycan from the immunobiotic *L. rhamnosus* CRL1505 is able to improve the resistance of infant mice to primary RSV infection and secondary pneumococcal pneumonia.

The first conclusion that can be obtained from the data presented in this work is that peptidoglycan from immunobiotic *L. rhamnosus* CRL1505 preserves the immunomodulatory properties of the viable strain. We have demonstrated previously that the nasal priming with viable *L. rhamnosus* CRL1505 beneficially modulates the respiratory immune response triggered by TLR3 activation and increases the resistance to RSV infection (13). Similar to viable *L. rhamnosus* CRL1505, its purified peptidoglycan administered before the nasal challenge with three once-daily doses of poly(I:C) significantly increased the levels of BAL type I IFNs, especially IFN-β. Thus, PG1505 would enhance the expression of hundreds of IFNs-induced genes that counteract viral replication. This is in line with the improved resistance of PG1505-treated infant mice to the infectious challenge with RSV. Moreover, PG1505 differentially regulated the production of pro- and anti-inflammatory cytokines in response to TLR3 activation which are also important for the generation of an effective respiratory antiviral response. PG1505 improved the

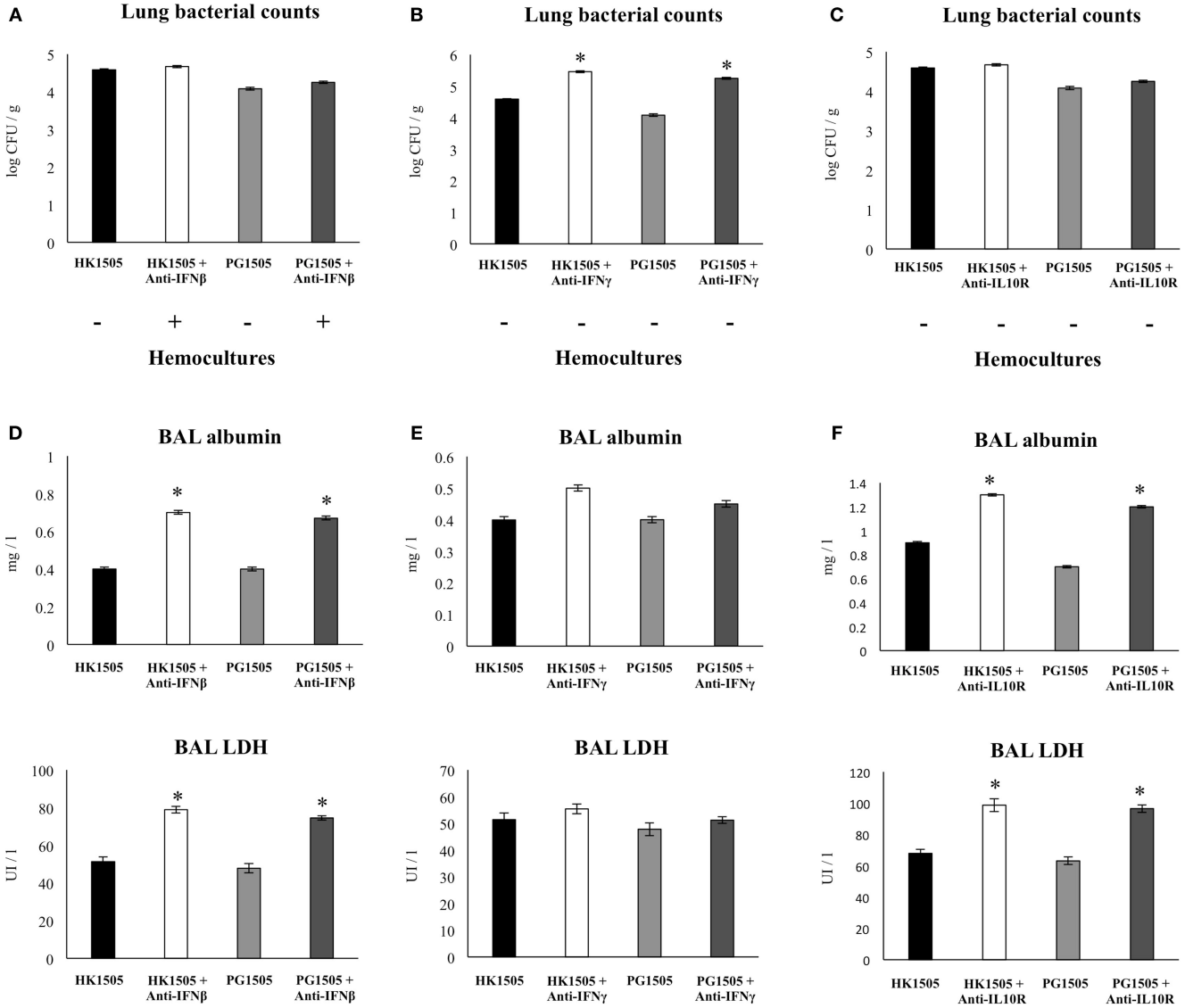

FIGURE 7 | Role of interferon (IFN)-β, IFN-γ, and interleukin (IL)-10 in the immunomodulatory effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on the resistance to secondary pneumococcal pneumonia after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days, and then challenged with three once-daily doses of poly(I:C). On days 2 and 4 after poly(I:C) challenge, mice were nasally treated anti-IFN-β, anti-IFN-γ, or anti-IL-10 receptor (IL-10R) blocking antibodies. Control HK1505 and PG1505 received isotype control antibodies. Twelve hours later mice were challenged with *Streptococcus pneumoniae*. Lung bacterial cells counts and hemocultures **(A–C)**, as well as lactate dehydrogenase (LDH) activity and albumin concentrations in bronchoalveolar lavages (BAL) **(D–F)** were determined on day 2 post-pneumococcal challenge. The results represent data from three independent experiments. Significant differences between treated and control groups, $^*P < 0.05$.

production of some cytokines (TNF-α, IL-10) while it reduced the levels of other pro-inflammatory factors (IL-6, MIP-1) indicating that the inflammatory response was differentially modulated. In fact, PG1505-treated mice showed an improved capacity to control RSV replication in the respiratory tract and a reduced inflammatory damage in the lung tissue. Resembling the effect of viable *L. rhamnosus* CRL1505 (13), PG1505 improved the numbers of $CD3^+CD4^+IFN\text{-}\gamma^+$, $MHC\text{-}II^+CD11b^{low}CD103^+$, and $MHC\text{-}II^+CD11b^{high}CD103^-$ DCs as well as the respiratory levels of IFN-γ indicating the generation of a Th1 response, that is also involved in the immune protection against respiratory viral attack. These results indicate that peptidoglycan is a key bacterial component involved in the immunomodulatory and antiviral capacity of the immunobiotic strain *L. rhamnosus* CRL1505.

An important finding of this work is that both HK1505 and PG1505 were able to differentially modulate the response of infant mice to the secondary pneumococcal pneumonia produced after the challenge with poly(I:C) or RSV. Slight but still significant reduction of pneumococcal cell counts were found in the lungs of HK1505- or PG1505-treated mice when compared

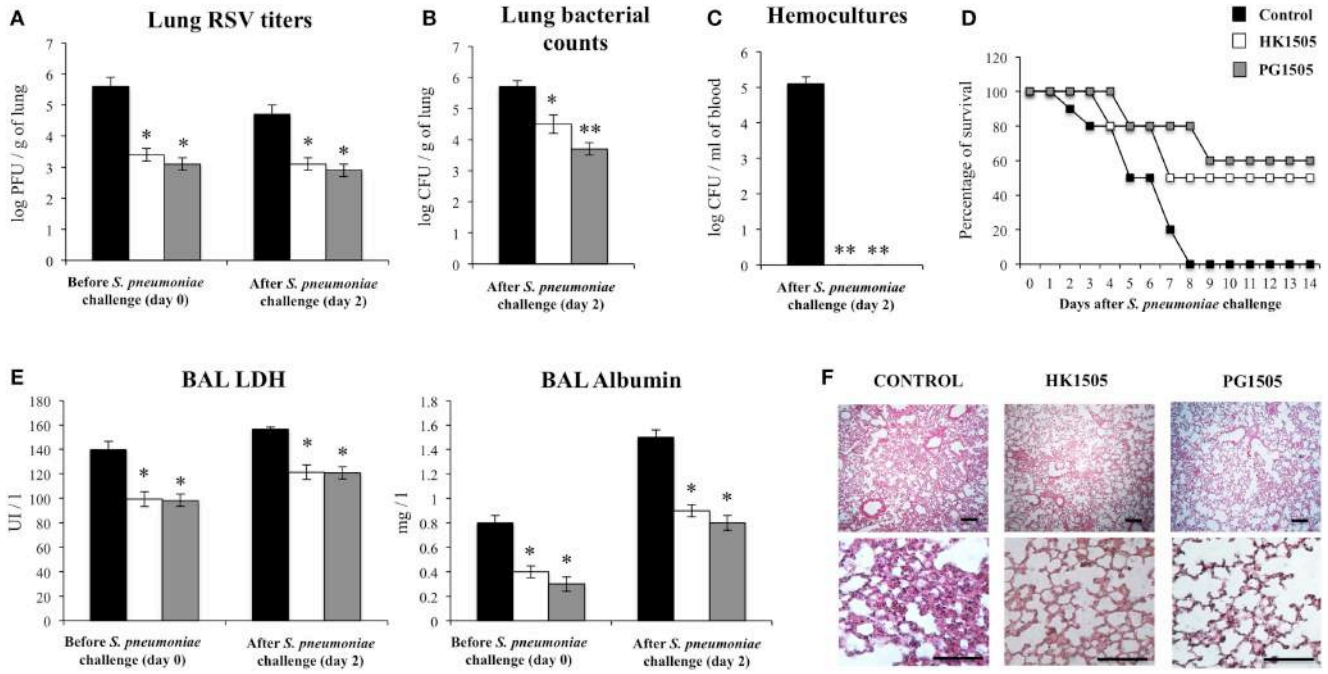

FIGURE 8 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on the resistance to secondary pneumococcal pneumonia after the primary infection with Respiratory Syncytial Virus (RSV). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days, challenged with RSV and, infected with *Streptococcus pneumoniae* 5 days after the viral infection. Non-treated infant mice infected with RSV and challenged with *S. pneumoniae* were used as controls. Lung RSV titers **(A)**, lung bacterial cells counts **(B)**, hemocultures **(C)**, survival **(D)**, lactate dehydrogenase (LDH) activity, and albumin concentrations in bronchoalveolar lavages (BAL) **(E)**, and lung histopathological examination **(F)** were determined on day 2 post-pneumococcal challenge. Scale bar = 100 μm. The results represent data from three independent experiments. Significant differences between treated and control groups, $^{**}P < 0.05$, $^{**}P < 0.01$.

to controls. The effect of HK1505 and PG1505 in reducing lung pneumococcal cell counts was modest compared with our own previous studies. We had reported that the nasal administration of viable *Lactococcus lactis* NZ9000 to adult and infant mice reduced in more than 2 log *S. pneumoniae* cell counts in lungs (21). In addition, non-viable *L. casei* CRL431 (22), and non-viable *L. rhamnosus* CRL1505 or its peptidoglycan (15) allowed to immunocopromised malnourished mice to completely eliminate *S. pneumoniae* from lungs. Moreover, HK1505 and PG1505 evaluated in an infant mice model of primary pneumococcal infection also reduced in more than 2 log *S. pneumoniae* cell counts in lungs when compared to untreated controls (data not shown). Then, immunobiotics and their cellular fractions would be more efficient to improve resistance to primary than secondary pneumococcal pneumonia.

Despite the modest results obtained by measuring the burden of the pathogen in the respiratory tract, PG1505 treatment was able to significantly reduce lung tissue damage and bacterial dissemination into the blood stream. These findings are of importance because studies in clinical trials (5, 6) and animal models of RSV-*S. pneumoniae* superinfection (7, 8) showed that enhanced lung injuries and elevated levels of bacteremia are critical factors that determine the severity of infection and the rate of mortality. In fact, PG1505-treated infant mice showed a significant improvement of survival after superinfection with RSV and *S. pneumoniae*. As mentioned before, several modifications induced by respiratory viruses are involved in facilitating *S. pneumoniae* infection, including the destruction of the respiratory epithelium, ciliary dyskinesia, enhancement of adhesion factors, and alterations of the immune response (7–10). In relation to immunopathology, it was reported that sequential infection with RSV and *S. pneumoniae* induced a significantly greater inflammation with high levels of infiltrated neutrophils and elevated levels of pro-inflammatory factors in the lung compared with mice that were inoculated with each pathogen separately (19). It is possible to speculate that the protective effect of PG1505 would be exerted at different levels. (a) The improvement of antiviral response and the consequent reduction of RSV titers in the lung, would contribute to the reduction of respiratory epithelium damage. (b) A reduction *S. pneumoniae* adhesion by diminishing the expression of RSV G protein and adhesion molecules in respiratory epithelial cells. In relation to this point, although we have not evaluated the impact of PG1505 in the expression of adhesion molecules in the respiratory tract, our recent transcriptomic studies evaluating the effect of *L. rhamnosus* CRL1505 in poly(I:C) challenged intestinal epithelial cells showed that the immunobiotic strain is able to differentially modulate the expression of several adhesion molecules including selectins E and L as well as ICAM-1, and EPCAM (23). Therefore, evaluating the effect of PG1505 on the expression of

relevant adhesion molecules in respiratory epithelial cells is an interesting topic for future investigations. (c) The differential modulation of the respiratory innate immune response that allow a reduction of RSV and *S. pneumoniae* replication with minimal inflammatory damage of lung tissue.

Our results suggest that pneumococcal growth in lungs, dissemination into blood, and inflammatory tissue damage are events which are not strictly coupled with each other. Moreover, they are regulated by different factors during the development of secondary pneumococcal pneumonia as became evident when experiment with blocking anti-IFN-β, anti-IFN-γ, and anti-IL-10R antibodies were performed. In line with our results, Damjanovic et al. (24) demonstrated in a mice model of IFV-*S. pneumoniae* superinfection that uncontrolled bacterial outgrowth and excessive inflammation are not strictly coupled events, and that both are contributors to deleterious lung immunopathology and death. In fact, the work reported that the use of a bacteriostatic antibiotic effectively improved clinical outcome by controlling pneumococcal replication but it failed to significantly diminish pulmonary immunopathology. On the other hand, the use of dexamethasone slightly reduced immunopathology while it had no impact on bacterial clearance. Interestingly, administration of dexamethasone in combination the bacteriostatic antibiotic significantly improved pulmonary immunopathology, bacterial elimination, and most importantly the survival of infected mice (24). All these results together suggest that effective intervention strategies for respiratory superinfections need to involve an efficient control of pathogens growth and aberrant host inflammatory responses.

We found that at least three cytokines are involved in the immunomodulatory protective effect of HK1505 and PG1505 in the respiratory superinfection: IL-10, IFN-β, and IFN-γ. Our results indicated that IL-10 was involved in the protection against inflammatory damage while IFN-γ participated in the reduction of pneumococcal growth in the lungs. This is in line with our previous findings demonstrating that the protection of infant mice against poly(I:C) or RSV challenge induced by viable *L. rhamnosus* CRL1505 was dependent of both IL-10 and IFN-γ (16). Moreover, as observed for viable *L. rhamnosus* CRL1505 (16), both HK1505 and PG1505 improved the numbers of $CD3^+CD4^+IFN\text{-}\gamma^+$, and $CD3^+CD4^+IL\text{-}10^+$ T cells in the lungs of infant mice. These immune cell populations remained significantly elevated when compared to controls after pneumococcal challenge, indicating their participation in the protection against secondary bacterial pneumonia.

In addition, we showed that IFN-β was involved in the protection against lung tissue injury as well in the control of pneumococcal dissemination into the blood. The lung epithelial–endothelial layer is an important barrier in pneumococcal pathogenesis, since its alteration results in serious complications such as bacteremia and meningitis that are associated with high mortality. Some works have demonstrated an important role of IFN-β in the control of pneumococcal infection and invasiveness. Studies in IFNAR1 or IFN-β knockout mice showed that the abolishment of the IFN-β-IFNAR1 pathway increased nasopharyngeal carriage and enhanced mortality upon pneumococcal infection (25, 26). It was also demonstrated by LeMessurier et al. (27) that IFN-β increase the expression of tight junction proteins and reduce PAF receptor expression, which correlates with diminished pneumococcal invasion and transmigration of pulmonary epithelial and endothelial cells. In addition, intranasal administration of IFN-β was found to protect mice against the development of pneumococcal systemic disease (27). The role of IFN-β in secondary pneumococcal pneumonia has been also studied. No significant differences in *S. pneumoniae* counts in the lungs of $IFNAR1^{-/-}$ and $IFNAR1^{+/+}$ mice were observed after pneumococcal challenge. However, pneumococci were observed earlier and at higher numbers in blood samples of $IFNAR1^{-/-}$ mice compared to wild-type animals (27). We demonstrated here that the improvement of IFN-β by PG1505 treatment would be related to the enhancement of $CD11c^+SiglecF^+IFN\text{-}\beta^+$ alveolar macrophages. This is in line with studies demonstrating that alveolar macrophages are the main producers of type I IFNs during pulmonary viral infections (28, 29). However, it should be noted that the primary type I IFN response is mediated not only by alveolar macrophages but also by epithelial cells of the respiratory tract (25, 27). Therefore, respiratory epithelial cells may represent promising cellular target for future mechanistic studies in order to explain the protective effect of PG1505.

Our results demonstrated that the modulation of the three cytokines (IFN-β, IFN-γ, and IL-10) are necessary to achieve protection against secondary pneumococcal pneumonia. Therefore, an appropriate balance between pro- and anti-inflammatory factors would be necessary in order to obtain full protection without affecting lung structure and function. In line with this statement, some works have demonstrated that the excessive production of one particular cytokine have negative consequences in the outcome of respiratory infections. It was showed that excessive production of IFN-β during viral infection induce impaired neutrophils responses due to inadequate production of neutrophil chemoattractants (30). Although neutrophils infiltration has been linked to lung tissue damage, the phagocytic and bactericidal activities of these cells are necessary for the initial control of pneumococci. In addition, it was observed that neutralization of IFN-γ in IFV-infected mice considerably diminished bacterial susceptibility and improved survival after secondary pneumococcal pneumonia (31). This effect was explained by the inhibition of bacterial phagocytosis by the excessive production of IFN-γ. Data obtained from mice infected with IFV and *S. pneumoniae* demonstrated that the exaggerated production of IL-10 was associated with increased pneumococcal colonization and enhanced mortality (32). The work showed that the treatment of mice with blocking anti-IL-10 antibodies before secondary pneumococcal infection resulted in reduced bacterial cell counts in lungs and prolonged survival (32). Moreover, it can be anticipated that other cytokines are involved in susceptibility to secondary bacterial respiratory infections. In this regard, recent findings of Chen et al. (33) demonstrated that the anti-inflammatory cytokine IL-35 contributes to the decreased resistance to secondary pneumococcal pneumonia. The work reported that IFV infection induced a high expression of IL-35 in the respiratory tract and that secondary pneumococcal infection leaded to a synergistic production

of this cytokine. Lung enhancement of IL-35 inhibited the early immune response against *S. pneumoniae*.

In summary, we demonstrated that the nasal priming with peptidoglycan from *L. rhamnosus* CRL1505 differentially modulates the respiratory innate antiviral immune response triggered by TLR3 activation in infant mice, improving the resistance to primary RSV infection, and secondary pneumococcal pneumonia. In association with the protection against RSV-pneumococcal superinfection, we found that peptidoglycan from *L. rhamnosus* CRL1505 significantly improved lung $CD3^+CD4^+IFN\text{-}\gamma^+$, and $CD3^+CD4^+IL\text{-}10^+$ T cells as well as $CD11c^+SiglecF^+IFN\text{-}\beta^+$ alveolar macrophages with the consequent increases of IFN-γ, IL-10, and IFN-β in the respiratory tract. Our results also demonstrated that the increase of the three cytokines is necessary to reduce the severity of the respiratory superinfection since each of them are involved in different aspect of the secondary pneumococcal pneumonia that have to be controlled in order to reduce the severity of the infectious disease: lung pneumococcal colonization, bacteremia, and inflammatory-mediated lung tissue injury. The findings of this work will lead us in new directions to explore the molecular mechanisms *via* which peptidoglycan from *L. rhamnosus* CRL1505 interacts with immune and non-immune cells of the respiratory tract and to investigate whether its immunomodulatory properties are unique or common to peptidoglycans of other immunobiotic strains with antiviral capabilities.

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of CERELA and all efforts were made to minimize suffering.

AUTHOR CONTRIBUTIONS

SA, HK, and JV designed the study and wrote the manuscript. PC, PK, HZ, AT, AK, and SS did the laboratory work. PC, PK, SS, and JV performed statistical analysis. SS, SA, HK, and JV contributed to data analysis and interpretation. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00948/full#supplementary-material.

FIGURE S1 | Role of interferon (IFN)-β, IFN-γ, and interleukin (IL)-10 in the immunomodulatory effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on the resistance to secondary pneumococcal pneumonia after the nasal administration of the viral pathogen-associated molecular pattern poly(I:C). Infant mice were nasally primed with HK1505 or PG1505 during two consecutive days, and then challenged with three once-daily doses of poly(I:C). On days 2 and 4 after poly(I:C) challenge, mice were nasally treated anti-IFN-β, anti-IFN-γ, or anti-IL-10 receptor blocking antibodies. Control HK1505 and PG1505 received isotype control antibodies. Twelve hours later mice were challenged with *Streptococcus pneumoniae*.

FIGURE S2 | Effect of non-viable *Lactobacillus rhamnosus* CRL1505 (HK1505) and its peptidoglycan (PG1505) on blood cytokines. HK1505 or PG1505 were nasally administered to infant mice during two consecutive days. Non-treated infant mice were used as controls. Levels of tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-β, IFN-γ, interleukin (IL)-6, IL-8, IL-10, and monocyte chemoattractant protein-1 (MCP-1) were determined in bronchoalveolar lavages (BAL) **(A)** and serum **(B)**. The results represent data from three independent experiments. Significant differences between treated and control groups, $^*P < 0.05$, $^{**}P < 0.01$.

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Effects of Microbial Aerosol in Poultry House on Meat Ducks' Immune Function

Guanliu Yu[1], Yao Wang[1], Shouguo Wang[2], Changmin Duan[2], Liangmeng Wei[1], Jing Gao[3], Tongjie Chai[1] and Yumei Cai[1]**

[1] College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Sino-German Cooperative Research Centre for Zoonosis of Animal Origin Shandong Province, Tai'an, China, [2] Deizhou People's Hospital, Deizhou, China, [3] The Central Hospital of Tai'an, Tai'an, China

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***Correspondence:**
Tongjie Chai
chaitj117@163.com
Yumei Cai
caiyum@163.com

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The aim of this study was to evaluate effects of microbial aerosols on immune function of ducks and shed light on the establishment of microbial aerosol concentration standards for poultry. A total of 1800 1-d-old cherry valley ducks were randomly divided into five groups (A, B, C, D, and E) with 360 ducks in each. To obtain objective data, each group had three replications. Concentrations of airborne bacteria, fungi, endotoxin in different groups were created by controlling ventilation and bedding cleaning frequency. Group A was the control group and hygienic conditions deteriorated progressively from group B to E. A 6-stage Andersen impactor was used to detect the aerosol concentration of aerobes, gram-negative bacteria, fungi, and AGI-30 microbial air sampler detect the endotoxin, and Composite Gas Detector detect the noxious gas. In order to assess the immune function of meat ducks, immune indicators including H5 AIV antibody titer, IgG, IL-2, T-lymphocyte transformation rate, lysozyme and immune organ indexes were evaluated. Correlation coefficients were also calculated to evaluate the relationships among airborne bacteria, fungi, endotoxin, and immune indicators. The results showed that the concentration of airborne aerobe, gram-negative bacteria, fungi, endotoxin have a strong correlation to H5 AIV antibody titer, IgG, IL-2, T-lymphocyte transformation rate, lysozyme, and immune organ indexes, respectively. In addition, when the concentration of microbial aerosol reach the level of group D, serum IgG (6–8 weeks), lysozyme (4 week) were significantly higher than in group A ($P < 0.05$); serum IL-2 (7 and 8 weeks), T-lymphocyte transformation rate, lysozyme (7 and 8 weeks), spleen index (6 and 8 weeks), and bursa index (8 week) were significantly lower than in group A ($P < 0.05$ or $P < 0.01$). The results indicated that a high level of microbial aerosol adversely affected the immune level of meat ducks. The microbial aerosol values in group D provide a basis for recommending upper limit concentrations of microbial aerosols for healthy meat ducks.

Keywords: microbial aerosol, immune indicators, stress, cherry valley ducks, poultry houses

INTRODUCTION

The air in poultry houses is usually heavily contaminated by large quantities of airborne microorganisms, endotoxins and toxic gases (NH_3, H_2S), etc. (Nimmermark et al., 2009; Cambra-López et al., 2010; Lawniczek-Walczyk et al., 2013). In airborne microorganisms, there is a high concentration of non-pathogenic microorganisms leading to animal immunosuppression (Douwes et al., 2003; Fiegel et al., 2006). The high level of airborne aerobe could reduce animal immunity and growth rate (Wolinsky, 2006). Many studies have documented that exposure to fungal aerosol may be associated with asthma, acute toxic and allergic, and it may threaten caretakers and external ambient in animal houses as well (Bush and Portnoy, 2001; Pavan and Manjunath, 2014). The percentage of airborne gram-negative bacteria in the bacterial aerosol is small, but it contains a lot of pathogenic bacteria (Zucker et al., 2000). Endotoxins are ubiquitous in the environment. They are a biologically active lipopolysaccharide that is a component of the outer membrane of gram-negative bacteria (Balasubramanian et al., 2012). According to Pirie, inhaled endotoxin contributes significantly to the induction of airway inflammation and dysfunction (Pirie et al., 2003). Many occupational studies have shown positive associations between endotoxin exposure and respiratory disorders including asthma-like syndrome, chronic airway obstruction, organic duct toxic syndrome, byssinosis, bronchitis, etc. (Madsen, 2006). Zucker et al. have used it as an important symbol of organic dust in the air of poultry house (Zucker et al., 2000). Endotoxin also affects human humoral and cellular immunity (Burrell, 1990). Furthermore, in terms of toxic gases in animal house, ammonia and hydrogen sulfide are two well-known toxic components (Yao and Li, 2010). They can cause respiratory, eye diseases and even poisoning death (Teye et al., 2008; Yao and Li, 2010; Barrasa et al., 2012).

To date, numerous correlation studies have focused on microbial aerosol composition, concentration and mechanisms of spread to the surrounding ambient (Zucker et al., 2000; Madsen, 2006; Duan et al., 2007; Masclaux et al., 2013; Matković et al., 2013). However, studies of microbial aerosol on immune function have not been found. Therefore, the aim of this study was to clarity the effect of microbial aerosol on the immune function of ducks, which was based on comparing the significance between control group and the treatment groups of ducks' specific immune indexes (e.g., IgG, H5 AIV antibody titer, IL-2, etc.) and the non-specific immune factors (e.g., lysozyme), as well as the relationship between major microbial aerosol concentration and immune indicators. Moreover, this study also could enlighten future studies on the establishment of microbial aerosol concentration standards for poultry breeding.

MATERIALS AND METHODS

Experimental Design

This study was conducted at the Animal Husbandry & Veterinary Station of Shandong Agricultural University, China during January–March, 2014. Five groups were set up, with a control group A and 4 treatment groups (B, C, D, and E, with hygienic conditions deteriorating progressively from group B to E). Each group had three replications with each in a separate poly-tunnel. The poly-tunnel is covered by a double layer of clear plastic with 2 cm insulation in between and with steel or wood arch frames and bedding on the ground. It is naturally ventilated and the duck feces are cleaned manually. All 15 poly-tunnels were identical, equipped with similar exhaust fan, radiator and incandescent light bulb (80 W). Air warmed by the heat from the sun in the day and the bulb at night was retained in the building by the roof and walls. Temperature of each group was maintained between 20 and 24°C using radiators and exhaust fan. A regime of 16 h light (between 05:00 and 21:00) and 8 h darkness was used, with a 25 min twilight phase at the end of each day, and light intensity was about 60 lx at bird-eye height. The size of poly-tunnel was 4.0 × 4.0 × 3.0 m, with a window (2.0 × 1.5 m) facing the sun. A glass door (0.8 × 1.8 m) was used to observe the behavior of ducks in each poly-tunnel. A total of 1800 1-day old cherry valley ducks were placed in the ducks houses, with 360 ducks in each poly-tunnel. The ducks were reared on the floor with thick bedding (wood-shavings), and food and water were automatically refilled. Phosphoric acid (H_3PO_4), calcium superphosphate [$Ca(H_2PO_4)_2$], ferrous sulfate ($FeSO_4{\cdot}7H_2O$), caustic lime (CaO), acticarbon and alum [$Al_2(SO_4)_3{\cdot}18H_2O$] were used to absorb noxious gases (such as NH_3; Moore et al., 1996; Do et al., 2005; Yao and Li, 2010). Before the trial began, environmental management measures under rearing conditions in poly-tunnels in North China were investigated, while health management measures in different groups were examined. Based on these findings, the health management measures of all treatment groups in this experiment are listed (**Table 1**; Yu et al., 2016). All animal experiments were performed according to the guidelines of the Committee on the Ethics of Animals of Shandong and the appropriate biosecurity guidelines, and the protocol was approved by Shandong Agricultural University Animal Care and Use Committee (No. SDAUA-2014-066).

Sample Collection and Analysis

Determination of Airborne Aerobe, Fungi, and Gram-Negative Bacteria

A 6-stage Andersen impactor (airflow 28.3 L/min), at a height of about 0.2 m (duck's breathing zone) above the ground in the central part of each poly-tunnel, was used to sample airborne aerobe, fungi and gram-negative bacteria weekly at 7:00, 14:00,

TABLE 1 | Management regimes in different groups.

Groups	Ventilation method	Ventilation time (h)	Frequency of troughs cleaning, sterilization, and bedding replacement
A (Control)	Natural and mechanical	24	Once/day
B (Treatment)	Mechanical	24	Once/2 days
C (Treatment)	Mechanical	18	Once/3 days
D (Treatment)	Mechanical	12	Once/4 days
E (Treatment)	Mechanical	10	Once/5 days

and 20:00 h, respectively. The samples were selected three times for 1–2 min a time in every poly-tunnel. Soy agar medium with 5% defibrinated sheep blood, Sabouraud's medium (HB0253-8, Hope Bio-Technology Co., Ltd, Qingdao, China) and a gram-negative bacteria selective medium (HB8643, Hope Bio-Technology Co., Ltd, Qingdao, China) were used as sampling media for aerobes, fungi, and gram-negatives, respectively. For Sabouraud's medium, after high temperature steam sterilization, add Chloramphenicol (dose is 0.2 g/L) into it. The samples were taken to the microbe laboratory and cultured in incubators (aerobic condition)–the aerobes at 37°C for 1 day, fungi at 25°C for 4 days and gram-negative bacteria at 37°C for 3 days. After incubation, the numbers of colonies on plates were determined with a Colony Star counter and concentrations were expressed as colony forming units per m^3 (CFU/m^3; Andersen, 1958).

Determination of Airborne Endotoxin

Air samples for endotoxin were collected by the AGI-30 Sampler (airflow 12.5 L/min) weekly at the height of 0.2 m (Duck's breathing zone) for 20 min, with 50 mL pyrogen-free water as media (Brachmann et al., 1964). Sampling sites were set in the central part of each poly-tunnel. Endotoxin content was determined by Limulus amebocyte lysate (LAL) assay (QLC2100 Bio Whittaker, Walkersville, MD, USA). A standard curve obtained from an *Escherichia* was used to express concentrations as endotoxin units (EU) which were presented as EU/m^3.

Determination of Noxious Gas

Noxious gas was detected by Composite Gas Detector (GC310, Chicheng Electric Co., Ltd, Henan, China) in all groups in real time. The instrument was hung 0.2 m above the ground on the wall. The concentration of noxious gas was presented as mg/Kg.

Determination of Immune Indicators

At the age of 10 days, ducks were immunized with H5 AIV vaccine (Reassortant Avian Influenza Virus H5 Subtype Vaccine, Inactivated Strain Re-6+Strain Re-4, Qingdao Yebio Biological Engineering Co., Ltd., Qingdao, China) by neck subcutaneous, 1.5 mL of each one.

Five mL of blood sample was collected in EDTA vacuum tubes through vena digitalis from each duck of 4-, 5-, 6-, 7- and 8-week old (60 ducks in each group). After centrifugation for 10 min at 800 g, serum samples were stored at −20°C until analysis. Duck IgG detection kit, duck IL-2 detection kit (both of them were purchased from Nanjing SenBenJia Biological Technology Co., Ltd. Nanjing, China), lysozyme detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and hemagglutination-inhibition (HI) test were used to detect the serum IgG, IL-2, lysozyme and H5 AIV antibody titer, respectively.

MTT (Methy Thiazolyl Tetrazolium) colorimetric assay was used to detect T lymphocytes transformation rate (Lazar et al., 2010; Hsiao and Huang, 2011; Yin et al., 2015). The procedure as follows:

One mL of blood sample was collected in EDTA vacuum tubes through vena digitalis from each duck of 4-, 5-, 6-, 7-, and 8-week old (60 ducks in each group).

One mL whole blood dilution (Shanghai Yanjin Biotechnology Co.Ltd. Shanghai, China) was added to the above blood sample (1 mL), then mixed. The mixture was added on 4 mL lymphocyte separation fluid (Beijing Dingguo Chengsheng Biotechnology Co., Ltd. Beijing, China) for 15 min centrfugation at 800 g. The white coat that under the plasma layer was sucked out and washed 2 times with 3–5 times volume RPMI 1640 culture liquid (Sigma, USA) without calf serum, each time with centrfugation for 10 min at 800 g.

Counting with Trypan Blue (Sigma, USA), the living cells was more than 95%. Single cell suspensions (final concentration was 3×10^6 /mL) were prepared by RPMI 1640 complete culture liquid containing 10% calf serum. The single cell suspension was cultured in a cell incubator at 37°C, 6.5% CO_2 for 14 h. Peripheral blood lymphocyte were obtain and then prepared for lymphocyte suspension (final concentration was 2×10^6 /mL).

Cells are grown in microtiter plates (tissue culture grade, 96 wells, flat bottom). 100 μL of the lymphocyte suspension and 100 μL of the PHA (phytohaemagglutinin; Beijing Baiaosentai Biotechnology Co., Ltd. Beijing, China; final concentration was 25 μg / mL) was added (final concentration was 2×10^6 /mL) into each test well. 100 μL of the lymphocyte suspension and 100 μL of the RPMI 1640 was added into each control well. The replications is five. After the incubation in a cell incubator at 37°C, 6.5% CO_2 for 44 h, 20 μL of MTT (5 mg/mL) was added into each well and then continued to incubate for 4 h. The supernatant of each well was discarded carefully.

After that, 150 μL of dimethyl sulfoxide was added into each well and then oscillated for 10 min on microoscillator. The value of OD 570 nm was measured by Microplate Reader (Antai AY-858, Shanghai, China).

$$\text{T-lymphocyte transformation rate} = \frac{\text{Mean value of test } OD_{570}}{\text{Mean value of control } OD_{570}} \times 100\%$$

Determination of Immune Organ Indexes

The ducks of 4-, 6-, 8-week were weighed and recorded (60 ducks in each group). After that, thymus, spleen and bursa were collected from those ducks, respectively, and then weighed and recorded. At last, the immune organ indexes were calculated according to the follow formula.

$$\text{Immune organ indexes} = \frac{\text{Immune organ (g)}}{\text{Body weight (Kg)}} \times 100\%$$

Statistical Analysis

Data collection ran from week 4 to week 8. Data for each group were expressed as the mean of three replications. The maximum, minimum and median value were used for the air ambient parameter (Duan et al., 2007). All statistical analyses were performed using SAS 9.1 Software (SAS Institute, Inc., Cary, NC, USA). One-way ANOVA analysis with multiple-range test was used to evaluate the difference among groups (Duncan, 1955). Results are expressed as mean ± standard deviation (SD). Correlation between major microbial concentrations and

immune indicators were analyzed by Pearson's. $P < 0.05$ was considered statistically significant.

RESULTS

The Concentrations of Microbial Aerosol and Noxious Gas under Different Hygienic Conditions

Over time, the concentrations of airborne aerobe, airborne fungi, airborne gram-negative, airborne endotoxin, and NH_3 showed an overall trend of increase with the deteriorating of hygienic conditions, however, concentration of NH_3 in each group was lower than the poultry harmless criterion (10 ppm, GB/T 18407.3–2001), and H_2S was not found in all groups (**Table 2**; Yu et al., 2016).

The Effect of Microbial Aerosol on Specific Immunity of Meat Ducks

H5 AIV Antibody Titer

Under the condition without booster immunization, the H5 AIV antibody titer in serum of meat ducks of groups A and B reached a peak at week 5 (6.00 ± 1.00 and 6.33 ± 1.53, respectively), however, groups C, D, and E reached the peak at weeks 6, 7, and 8 (5.00 ± 1.00, 4.33 ± 1.53, and 3.00 ± 2.00, respectively; **Figure 1**). At the same week age, with the microbial aerosol concentrations increasing, the concentration of H5 AIV antibody titer in the serum of meat ducks generally showed a tendency of decline.

Serum H5 AIV antibody titers were lower in groups E than in group A ($P < 0.01$) at weeks 4 and 7; groups E were lower than group A ($P < 0.05$) at weeks 5 and 6.

IgG

At the same week age, with the increase of the microbial aerosol concentrations, the concentration of IgG in serum of meat ducks generally showed a tendency of increase (**Figure 2**).

Concentration of serum IgG were higher in groups E than in groups A ($P < 0.05$) at week 4 and 5; groups D were higher than

TABLE 2 | Airborne aerobe, airborne fungi, airborne gram-negative bacteria, airborne endotoxin, and noxious gas concentrations under different hygienic conditions.

Parameter	Value	Groups				
		A	B	C	D	E
Aerobe (×10E5 CFU/mE3)	Minimum	0.46	0.69	0.68	0.59	0.71
	Maximum	2.30	5.10	5.76	5.96	8.96
	Mean	1.05	2.45	2.94	**2.96**	4.31
Fungi (×10E4 CFU/mE3)	Minimum	0.11	0.21	0.19	0.85	0.78
	Maximum	3.49	3.54	3.95	5.73	8.05
	Mean	1.02	1.32	1.44	**2.63**	3.07
Gram-negative bacteria (×10E4 CFU/mE3)	Minimum	0.20	0.32	0.36	0.89	0.98
	Maximum	2.04	1.95	3.62	8.87	5.03
	Mean	0.93	1.24	1.68	**3.09**	2.64
Endotoxin (×10E3 EU/mE3)	Minimum	0.20	0.40	0.28	0.13	0.56
	Maximum	25.6	72.4	102.4	144.8	144.8
	Mean	6.49	10.48	23.03	**41.78**	47.79
NH_3 (mg/Kg)	Minimum	0	0	2	4	4
	Maximum	4	12	10	15	14
	Mean	2.56	2.42	5.67	**9.48**	8.97
H_2S (mg/Kg)	Minimum	–[a]	–	–	–	–
	Maximum	–	–	–	–	–
	Mean	–	–	–	–	–

All value for total experimental period.

[a]*Below the limit of detection.*

The bold values could be used as a basis for recommending upper limit concentrations of microbial aerosols for healthy meat ducks.

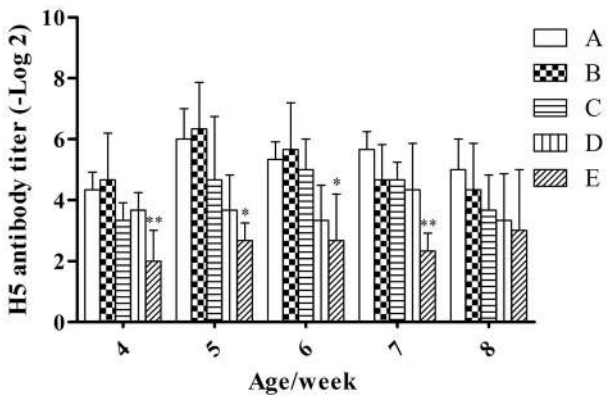

FIGURE 1 | H5 AIV antibody titer under different hygienic conditions ($n = 60$). Note: The comparison was between treatment groups (B–E) and control group (A) at the same age/week, *$P < 0.05$ and **$P < 0.01$. The same as below. *Means that the difference between treatment groups (B–E) and control group (A) was significant.

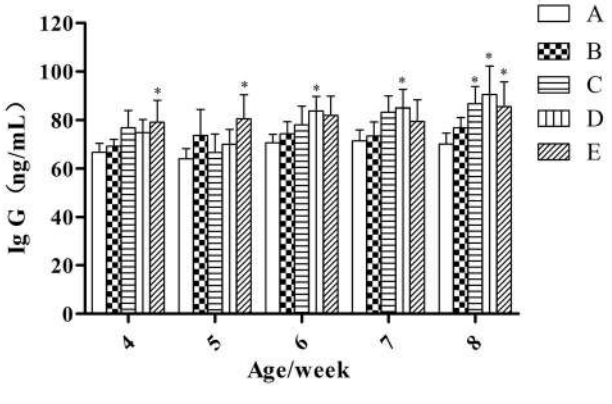

FIGURE 2 | IgG concentration under different hygienic conditions ($n = 60$). *Means that the difference between treatment groups (B–E) and control group (A) was significant.

group A ($P < 0.05$) at week 6 and 7; groups C, D, and E were higher than group A ($P < 0.05$) at week 8.

IL-2

At the same week age, with the increase of the microbial aerosol concentrations, the concentration of IL-2 in serum of meat ducks generally showed a tendency of decline (**Figure 3**).

Serum IL-2 in groups E were lower than in group A ($P < 0.05$) at week 4 and 6; groups D and E were lower than group A ($P < 0.01$) at week 7; groups D and E were lower than group A ($P < 0.05$ and $P < 0.01$, respectively) at week 8.

T-Lymphocyte Transformation Rate

At the same week age, with the increase of the microbial aerosol concentrations, T-lymphocyte transformation rate of meat ducks generally showed a tendency of decline (**Figure 4**), and the decline range was obvious.

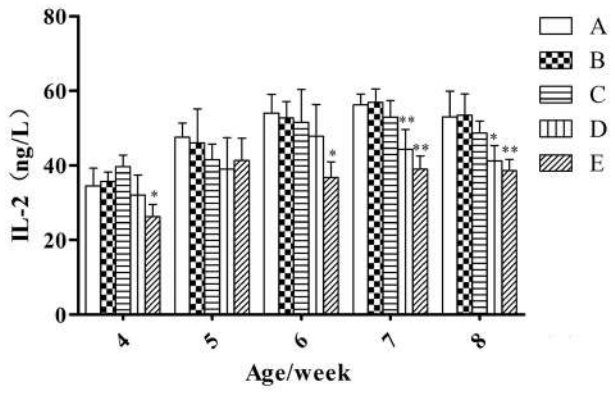

FIGURE 3 | IL-2 concentration under different hygienic conditions ($n = 60$). *Means that the difference between treatment groups (B–E) and control group (A) was significant. **Means that the difference between treatment groups (B–E) and control group (A) was extremely significant.

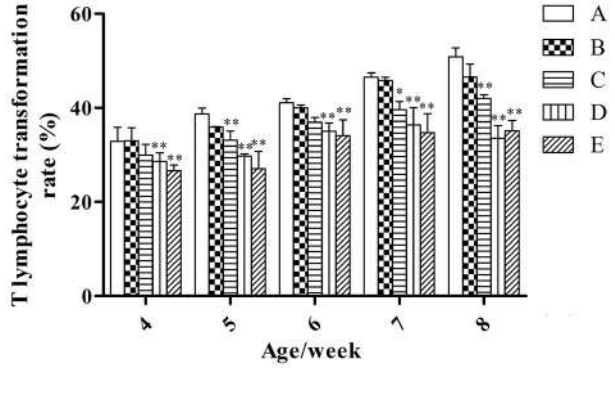

FIGURE 4 | T-lymphocyte transformation rate under different hygienic conditions ($n = 60$). *Means that the difference between treatment groups (B–E) and control group (A) was significant. **Means that the difference between treatment groups (B–E) and control group (A) was extremely significant.

T-lymphocyte transformation rates of groups D and E were lower than that of group A ($P < 0.01$) at weeks 4 and 6; groups C, D, and E were lower than that of group A ($P < 0.01$) at weeks 5 and 8; groups C, D, and E were lower than that of group A ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) at week 7.

The Effect of Microbial Aerosol on Non-specific Immunity of Meat Ducks

Lysozyme

At the same week age, as microbial aerosol concentrations increasing, the concentration of lysozyme in serum of meat ducks generally showed a tendency of increase at first (at weeks 4 and 5) and then showed a tendency of decline (at weeks 6, 7, and 8; **Table 3**).

Serum lysozyme in groups D and E were higher than group A ($P < 0.05$) at week 4; groups B, C, D and E were lower than group A ($P < 0.01$) at week 7; groups D and E were lower than group A ($P < 0.01$), but the group B was higher than group A ($P < 0.01$) at week 8.

Immune Organ Indexes

At the same week age, with the increase of the microbial aerosol concentrations, the index of thymus, spleen and bursa of meat ducks generally showed a tendency of decline (**Table 4**).

For thymus index, there was no significant effect ($P > 0.05$). As for spleen index, groups D and E were lower than group A ($P < 0.05$) at week 6; groups D and E were lower than group A ($P < 0.05$ and $P < 0.01$, respectively) at week 8. For bursa index, groups C, D and E were lower than group A ($P < 0.05$) at week 8.

Relationships between Microbial Aerosol Constituents and Immune Indicators

The correlation between microbial aerosol and immune indicators is shown in **Table 5**.

The concentration of aerobe showed a strong correlation to IgG, H5 AIV antibody titer, T-lymphocyte transformation rate, Thymus Index, Spleen Index and Bursa Index ($r = 0.91$ at $P < 0.05$, $r = -0.90$ at $P < 0.05$, $r = -0.89$ at $P < 0.05$, $r = -0.92$ at $P < 0.05$, $r = -0.94$ at $P < 0.05$, $r = -0.88$ at $P < 0.05$, respectively).

As for fungi, a significant negative correlation was recorded between fungi and H5 AIV antibody titer, IL-2, T-lymphocyte transformation rate, Lysozyme, Thymus Index, Spleen Index and Bursa Index ($r = 0.95$ at $P < 0.05$, $r = -0.99$ at $P < 0.01$, $r = -0.95$ at $P < 0.05$, $r = -0.97$ at $P < 0.01$, $r = -0.96$ at $P < 0.01$, $r = -0.96$ at $P < 0.01$, $r = -0.93$ at $P < 0.05$, respectively).

The concentration of endotoxin revealed the same dependency on H5 AIV antibody titer, IL-2, T-lymphocyte transformation rate, Lysozyme, Thymus Index, Spleen Index, and Bursa Index ($r = 0.98$ at $P < 0.01$, $r = -0.99$ at $P < 0.01$, $r = -0.95$ at $P < 0.05$, $r = -0.97$ at $P < 0.01$, $r = -0.98$ at $P < 0.01$, $r = -0.95$ at $P < 0.05$, $r = -0.89$ at $P < 0.05$, respectively).

However, the gram-negative bacteria correlated negatively with IgG, IL-2, T-lymphocyte Transformation Rate, Lysozyme,

TABLE 3 | Lysozyme concentration (U/mL) under different hygienic conditions ($n = 60$).

Weeks	Groups				
	A	B	C	D	E
4	120.14 ± 4.22	126.10 ± 4.99	124.60 ± 9.73	130.62 ± 7.99*	133.62 ± 6.14*
5	148.32 ± 5.21	150.85 ± 5.79	149.22 ± 6.68	152.30 ± 7.76	156.83 ± 9.35
6	153.72 ± 6.77	152.80 ± 11.29	158.16 ± 8.43	145.65 ± 7.03	148.98 ± 9.43
7	176.42 ± 6.43	158.47 ± 6.92**	162.38 ± 9.68**	151.90 ± 7.56**	141.37 ± 5.98**
8	166.31 ± 4.46	180.53 ± 6.65**	156.90 ± 8.21	144.82 ± 8.60**	139.43 ± 7.34**

*The comparison was between treatment groups (B–E) and control group (A) at the same age/week, * $P < 0.05$ and ** $P < 0.01$. The same as below. *Means that the difference between treatment groups (B–E) and control group (A) was significant. **Means that the difference between treatment groups (B–E) and control group (A) was extremely significant.*

TABLE 4 | Immune organ indexes under different hygienic conditions ($n = 60$).

Items	Weeks	Groups				
		A	B	C	D	E
Thymus index	4	2.81 ± 0.17	2.60 ± 0.11	2.49 ± 0.63	2.16 ± 0.33	2.09 ± 0.51
	6	2.44 ± 0.47	2.16 ± 0.38	1.95 ± 0.13	1.98 ± 0.48	1.80 ± 0.34
	8	2.21 ± 0.45	2.19 ± 0.28	1.96 ± 0.26	1.56 ± 0.33	1.57 ± 0.41
Spleen index	4	1.32 ± 0.12	1.24 ± 0.26	1.25 ± 0.33	1.03 ± 0.38	0.96 ± 0.08
	6	1.26 ± 0.22	1.06 ± 0.14	0.90 ± 0.14	0.79 ± 0.28*	0.69 ± 0.20*
	8	1.19 ± 0.21	0.96 ± 0.08	0.98 ± 0.18	0.80 ± 0.20*	0.72 ± 0.12**
Bursa index	4	1.84 ± 0.39	1.74 ± 0.56	1.86 ± 0.17	1.49 ± 0.47	1.54 ± 0.33
	6	1.44 ± 0.08	1.27 ± 0.16	1.31 ± 0.25	1.16 ± 0.20	1.19 ± 0.19
	8	1.46 ± 0.23	1.16 ± 0.16	1.03 ± 0.20*	0.97 ± 0.23*	0.89 ± 0.23*

**Means that the difference between treatment groups (B–E) and control group (A) was significant. **Means that the difference between treatment groups (B–E) and control group (A) was extremely significant.*

TABLE 5 | Correlation between concentrations of major microbial aerosol components and values of immune indicators.

Immune index	Aerobe	Fungi	Gram-negative bacteria	Endotoxin
IgG	***r* = 0.91***	$r = 0.86$	***r* = −0.90***	$r = 0.86$
H5 AIV antibody titer	***r* = −0.90***	***r* = −0.95***	$r = -0.87$	***r* = −0.98****
IL-2	$r = -0.84$	***r* = −0.99****	***r* = −0.88***	***r* = −0.99****
T-lymphocyte trans-formation rate	***r* = −0.89***	***r* = −0.95***	***r* = −0.95***	***r* = −0.95***
Lysozyme	$r = -0.79$	***r* = −0.97****	***r* = −0.95***	***r* = −0.97****
Thymus index	***r* = −0.92***	***r* = −0.96****	$r = -0.83$	***r* = −0.98****
Spleen index	***r* = −0.94***	***r* = −0.96****	***r* = −0.92***	***r* = −0.95***
Bursa index	***r* = −0.88***	***r* = −0.93***	***r* = −0.94***	***r* = −0.89***

Significant relationships ($P < 0.05$, **$P < 0.01$) expressed as Pearson correlation coefficients (**r**) in bold.*

Spleen Index, and Bursa Index ($r = -0.90$ at $P < 0.05$, $r = -0.88$ at $P < 0.05$, $r = -0.95$ at $P < 0.05$, $r = -0.95$ at $P < 0.05$, $r = -0.92$ at $P < 0.05$, $r = -0.94$ at $P < 0.05$, respectively).

The prediction models are as follows:

$Y = 75.49 - 2.78 \times 10E\text{-}6\ X_1 + 2.13 \times 10E\text{-}5\ X_2$, $R^2 = 0.3414$, $p = 0.5086 > 0.05$

Y: IgG (ng/mL); X_1: airborne aerobe (CFU/mE3); X_2: airborne gram-negative bacteria (CFU/mE3)

$Y = 4.76 + 2.41 \times 10E\text{-}6\ X_1 + 1.00 \times 10E\text{-}4\ X_2 - 1.03 \times 10E\text{-}4\ X_3$, $R^2 = 0.9760$, $p = 0.0230$

Y: H5 AIV antibody titer (−Log 2); X_1: airborne aerobe (CFU/mE3); X_2: airborne fungi (CFU/mE3); X_3: airborne endotoxin (EU/mE3)

$Y = 55.61 - 5.01 \times 10E\text{-}4\ X_1 + 6.01 \times 10E\text{-}6\ X_2 - 1.03 \times 10E\text{-}4\ X_3$, $R^2 = 0.8795$, $p = 0.0141$

Y: IL-2 (ng/mL); X_1: airborne fungi (CFU/mE3); X_2: airborne gram-negative bacteria (CFU/mE3); X_3: airborne endotoxin (EU/mE3)

$Y = 43.77 - 5.53 \times 10E\text{-}6\ X_1 + 1.01 \times 10E\text{-}4\ X_2 + 3.69 \times 10E\text{-}6\ X_3 - 3.03 \times 10E\text{-}4\ X_4$, $R^2 = 0.8417$, $p = 0.0392$

Y: T-lymphocyte transformation rate (%); X_1: airborne aerobe (CFU/mE3); X_2: airborne fungi (CFU/mE3); X_3: airborne gram-negative bacteria (CFU/mE3); X_4: airborne endotoxin (EU/mE3)

$Y = 155.45 - 8.35 \times 10E\text{-}6\ X_1 - 1.99 \times 10E\text{-}6\ X_2 - 2.00 \times 10E\text{-}4\ X_3$, $R^2 = 0.9097$, $p = 0.0517 > 0.05$

Y: Lysozyme (U/mL); X_1: airborne fungi (CFU/mE3); X_2: airborne gram-negative bacteria (CFU/mE3); X_3: airborne endotoxin (EU/mE3)

From the analysis above, it could be concluded that the concentration of airborne aerobe, fungi, gram-negative bacteria, endotoxin have a strong correction with the value of H5 AIV antibody titer, IgG, IL-2, T-lymphocyte rate, lysozyme, and immune organ indexes, respectively. Thus, it can provide a substantial evidence to confirm the effect of microbial aerosol on immune level.

DISCUSSION

Microbial aerosol originates from feed, manure, litter, as well as microorganisms, their byproducts and fragments in poultry houses (Millner, 2009; Just et al., 2011). Airborne aerobes, fungi, gram-negative bacteria and their bioproducts or biological fragments (such as endotoxins) are major components (Yu et al., 2016). The concentrations and components of it could reflect the condition of ambient sanitation in animal houses (Zucker and Muller, 2000; Kaliste et al., 2002). High concentrations of microbial aerosol and its metabolites (endotoxin, NH_3, H_2S, etc) are important factors affecting the health and productivity of animals (Prazmo et al., 2003; Banhai et al., 2008).

In this study, four treatment groups with gradually deteriorating hygienic conditions and one control group under standard hygienic sanitary management were set up by changing the frequency of trough cleaning, sterilization, bedding replacement, and ventilation (**Table 1**). The concentrations of airborne aerobes, fungi, gram-negative bacteria, and endotoxin in groups B, C, D increased both over time and as hygienic conditions deteriorated (**Table 2**). The results show that routine hygienic management measures, such as ventilation, bedding replacement and sterilization can reduce bioaerosols in duck poly-tunnels, which is important in order to maintain optimal microclimate and hygiene. Phosphoric acid, calcium superphosphate, ferrous sulfate, caustic lime, acticarbon, and alum were effective in absorbing noxious gases.

To the best of our knowledge, this study is the first to evaluate the effects of microbial aerosol on duck immunity. As we all known, immunity of animal can be divided into specific and non-specific immunity, and specific immunity can be divided into humoral and cellular immunity. In order to explore the effects of microbial aerosol on the immune function of duck. We chose IgG, H5 AIV antibody titer as reliable indicators for humoral immunity; took IL-2, T-lymphocyte transformation rate as representativeness indexes for cellular immunity; and took lysozyme, Immune organ indexes as non-specific immune factors.

Immunity to avian influenza is mainly based on humoral immunity, and detection of antibody titer of avian influenza contributes to indicating the condition of specific immune system protection (Ellis et al., 2004; Liu et al., 2006). As for serum IgG, it is the highest level of immunoglobulin in the blood of bodies. The activity of anti-bacteria, anti-virus and anti-toxin of IgG can be embodied in animal blood, and it plays essential roles in "Main Immune" (Borghesi et al., 2014). IL-2, also called T-cell growth factor, is the main cytokine in regulating cellular immune (Bayer et al., 2013). It is mainly produced by activated T-lymphocytes, and also can activate a variety of immune cells, regulate the body's immunity and enhance the body's anti-inflammatory effects, etc. (Song et al., 2005). In the process of the immune response, T-lymphocyte transformation rate is involved in the cellular immune response, therefore, it is often used to assess the functional status of lymphocytes and the status of body's immunity (Toivanen and Toivanen, 1973; Hovi et al., 1978; Kim et al., 1996).

Lysozyme is a kind of hydrolase that has special effects on the microbial cytoderm, which relaxes cytoderm and loses the protective effect on cells, and results in bacteria dissolution eventually (Sung et al., 2011). In the process of anti-bacterial infection, lysozyme often used as an important indicator that reflects strength of non-specific immunity (Fiolka et al., 2012; Zhao et al., 2014). The weight of thymus, spleen and bursa can be used to evaluate the immune status of poultry. It also reflects the strength of immune function intuitively (Rivas and Farbricant, 1985).

Over the experimental period, as microbial aerosol concentration increasing, serum IgG and lysozyme (4 and 5 weeks) increased, whereas H5 AIV antibody titer, IL-2, T-lymphocyte transformation rate, lysozyme (6 and 8 weeks), and immune organ indexes decreased. When the concentration of microbial aerosol reach the level of group D, serum IgG (6–8 weeks), serum lysozyme (4 week) were significantly higher than in group A ($P < 0.05$); serum IL-2 (7 and 8 weeks), T-lymphocyte transformation rate, serum lysozyme (7 and 8 weeks), spleen index (6 and 8 weeks), and bursa index (8 week) were significantly lower than in group A ($P < 0.05$ or $P < 0.01$).

Microbial aerosols at certain concentrations can stimulate the stress response, and stress can have serious adverse effects on welfare (Yu et al., 2016). Under stress, animals have to activate energy to combat the stressor, which can enhance catabolism and weaken the anabolism of protein and fat. Where animals are reared in environments contaminated with microorganisms, the nutrient status of organs may be compromised (Benson et al., 1993). This might be the reason for the decreasing tendency of immune organ indexes.

Moreover, if the stress in a long period of time, and it could lead to chronic stress, which could lead to cellular immune inhibition (Schedlowski, 1993; Bartolomucci et al., 2003), cutting down the production of IL-2 in serum (McEwen et al., 1997). This might be the reason for the tendency of serum IL-2 and T-lymphocyte transformation rates.

As for the tendency of serum IgG and lysozyme, after the initial increase it later decreased (6–8 weeks; **Figure 2** and **Table 3**), this may be due to the appearance of "malignant stress" in the late stages of this study, that is, under the short-term and mild stress, animals could adapt to it by compensatory reaction, but the long-term stress at any intensity will result in harmful effects, such as deterioration of physiological function in animals, etc. (He et al., 2011).

In addition, high level of microbial aerosol also affected the humoral immunity level of meat ducks, and it not only reduced the H5 AIV antibody titer of ducks, but also delayed the emergence of the antibody titer peak (**Figure 1**). This result is analogous to that of Witter (1998). Witter argued that weakened immune function reduces the immune protective effect of the vaccine.

CONCLUSIONS

In conclusion, a high level of airborne aerobe, gram-negative bacteria, fungi, and endotoxin adversely affected the immune level of meat ducks. This study indicates that good ventilation, bedding replacement and sterilization can decrease microbial aerosol concentration effectively. The present findings suggest that the microbial aerosol concentrations of group D provide a basis for recommending upper limit concentrations of microbial aerosols for healthy meat ducks.

AUTHOR CONTRIBUTIONS

GY and YC designed the experiment and completed most of the works. YW, SW, CD, and JG analyzed some test results and collected materials. TC and LW gave experiment instruction. Thank all the authors' contribution to the experiment.

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Serine-Rich Repeat Adhesins Contribute to *Streptococcus gordonii*-Induced Maturation of Human Dendritic Cells

*Eun Byeol Ko[1], Sun Kyung Kim[1], Ho Seong Seo[2], Cheol-Heui Yun[3] and Seung Hyun Han[1]**

[1] Department of Oral Microbiology and Immunology, DRI, and BK21 Plus Program, School of Dentistry, Seoul National University, Seoul, South Korea, [2] Biotechnology Research Division, Korea Atomic Energy Research Institute, Jeongeup, South Korea, [3] Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, South Korea

Dendritic cells (DCs) play a pivotal role in the induction of immunity by recognition, capture, process, and presentation of antigens from infectious microbes. *Streptococcus gordonii* is able to cause life-threatening systemic diseases such as infective endocarditis. Serine-rich repeat (SRR) glycoproteins of *S. gordonii* are sialic acid-binding adhesins mediating the bacterial adherence to the host and the development of infective endocarditis. Thus, the SRR adhesins are potentially involved in the bacterial adherence to DCs and the maturation and activation of DCs required for the induction of immunity to *S. gordonii*. Here, we investigated the phenotypic and functional changes of human monocyte-derived DCs treated with wild-type *S. gordonii* or the SRR adhesin-deficient mutant. The mutant poorly bound to DCs and only weakly increased the expression of CD83, CD86, MHC class II, and PD-L1 on DCs compared with the wild-type. In addition, the mutant induced lower levels of TNF-α, IL-6, and IL-12 than the wild-type in DCs. When DCs sensitized with the mutant were co-cultured with autologous T cells, they induced weaker proliferation and activation of T cells than DCs stimulated with the wild-type. Blockade of SRR adhesin with 3′-sialyllactose markedly reduced *S. gordonii* binding and internalization, causing attenuation of the bacterial immunostimulatory potency in DC maturation. Collectively, our results suggest that SRR adhesins of *S. gordonii* are important for maturation and activation of DCs.

Keywords: *Streptococcus gordonii*, serine-rich repeat adhesins, dendritic cells, maturation, T cell activation

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***Correspondence:**
Seung Hyun Han
shhan-mi@snu.ac.kr

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INTRODUCTION

Streptococcus gordonii is a Gram-positive facultative anaerobic bacterium belonging to the viridans group of oral streptococci (Loo et al., 2000). Although *S. gordonii* is a part of the normal flora in the oral cavity, it is able to cause various infectious diseases such as septic arthritis (Yombi et al., 2012) and life-threatening infective endocarditis with high mortality (Keane et al., 2010) through systemic spread following tooth extraction, brushing, or flossing (Forner et al., 2006). Upon entering the bloodstream, *S. gordonii* preferentially binds to human platelets, causing their aggregation, facilitating bacterial colonization in the endocardium and

heart valves and resulting in endocarditis (Takahashi et al., 2006). Previous studies have shown that oral streptococci associated with endocarditis promote rapid differentiation of monocytes into mature dendritic cells (DCs) (Hahn et al., 2005) and *S. gordonii* induces the secretion of cytokines including TNF-α, IL-6, and IL-12 in DCs (Corinti et al., 1999), implying the importance of DCs in the disease development and immune responses.

Bacterial adherence is an important step for microbial pathogenesis (Moschioni et al., 2010). As one of the initial colonizers of dental biofilms, *S. gordonii* abundantly expresses diverse adhesins that mediate its binding to host tissues. *S. gordonii* utilizes serine-rich repeat (SRR) adhesins, antigen I/II family proteins, cell-surface fibrillar proteins, and amylase-binding proteins to bind to human platelets and monocytes (Takahashi et al., 2004; Urano-Tashiro et al., 2012). Among them, SRR adhesins play an important role in the development of infective endocarditis (Xiong et al., 2008; Jakubovics et al., 2009). *S. gordonii* adheres to sialic acids on platelets or erythrocytes through SRR adhesins in injured heart valves, exacerbating inflammatory responses by promoting deposition of bacterium-platelet-fibrin complexes and recruiting inflammatory immune cells in tissues (Yajima et al., 2008).

Serine-rich repeat adhesins are sialoglycan-binding glycoproteins expressed on the surface of Gram-positive bacteria. They consist of conserved domains including an N-terminal signal peptide, a short SRR region, a ligand-binding basic region (BR) (Jakubovics et al., 2009), a long SRR region, and a C-terminal cell wall-anchoring domain (Bensing et al., 2016). Although the domains are conserved, BRs are highly divergent in amino acid sequence conferring the binding specificity to their cognate ligand (Takamatsu et al., 2005). For instance, Hsa and GspB, which are homologous SRR adhesins expressed on *S. gordonii* CH1 and M99 strains, respectively, have different BR structures with distinct binding ability: Hsa binds to both 3′-sialyllactose and sialyl-T antigen, whereas GspB binds only to sialyl-T antigen (Urano-Tashiro et al., 2016).

Dendritic cells are antigen-presenting cells that link the innate and adaptive immune responses (Steinman, 2006). Under infectious conditions, DCs exert various functions as sentinels; they recognize, phagocytose, and process infecting microbes to present the microbial epitopes to naïve T lymphocytes (Kapsenberg, 2003). Upon sensing antigens, DCs upregulate the expression of MHC proteins and co-stimulatory molecules such as CD40, CD80, and CD86. DCs also produce cytokines such as TNF-α, IL-6, and IL-12p70 that result in activation and differentiation of T lymphocytes. Mature DCs can migrate to draining lymph nodes to present antigens to T lymphocytes and induce antigen-specific adaptive immune responses (Mempel et al., 2004).

Serine-rich repeat adhesins of *S. gordonii* are important for this microbe to bind to host cells. This interaction appears to be critical in bacterial infection and host immunity. In the present study, we investigated the role of *S. gordonii* SRR adhesins, Hsa and GspB, in maturation and activation of human DCs treated with wild-type *S. gordonii* and SRR adhesin-deficient mutant strains.

MATERIALS AND METHODS

Reagents and Chemicals

Ficoll-Paque PLUS was obtained from GE Healthcare (Uppsala, Sweden). Penicillin-streptomycin solution and RPMI-1640 were purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY, USA). Recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 were purchased from R&D Systems (Minneapolis, MN, USA) and CreaGene (Sungnam, Korea), respectively. Anti-human CD14 magnetic particles and anti-human CD3 magnetic particles were purchased from BD Biosciences (San Diego, CA, USA). Dimethyl sulfoxide, Red Blood Cell Lysis Buffer, and cytochalasin D were purchased from Sigma–Aldrich (St. Louis, MO, USA). 5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was obtained from Molecular Probes (Eugene, OR, USA). PE-labeled anti-human CD83, APC-labeled anti-human CD86, APC-labeled anti-human PD-L1, and APC-labeled anti-human CD25 antibodies were purchased from BioLegend (San Diego, CA, USA). FITC-labeled anti-human HLA-DR, DP, and DQ antibodies for MHC class II were obtained from BD Biosciences. All isotype-matched antibodies were obtained from BioLegend or BD Biosciences. Enzyme-linked immunosorbent assay (ELISA) kits for measuring the concentrations of TNF-α, IL-12p70, and IL-6 were purchased from BioLegend. Todd Hewitt broth was obtained from MB Cell (Seoul, South Korea). Bacto^{TM} yeast extract and Bacto^{TM} agar were purchased from BD Biosciences (Sparks, MD, USA). 3′-Sialyllactose (3′SL) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Generation of Human Monocyte-Derived DCs

All experiments using human blood were conducted under approval of the Institutional Review Board at Seoul National University, South Korea. The Korean Red Cross provided blood from healthy human donors after obtaining informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS, as previously described (Kim et al., 2013). PBMCs were then incubated with CD14 magnetic beads for 30 min at room temperature, followed by separation in a magnetic field to isolate $CD14^+$ monocytes. The purified $CD14^+$ monocytes were suspended in RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin solution, 5 ng/ml GM-CSF, and 10 ng/ml IL-4 and were seeded in 60-mm cell culture dishes at a density of 2×10^6 cells/ml. The monocytes were cultured for 5 days to differentiate into immature DCs. Culture media supplemented with GM-CSF and IL-4 was changed every 3 days.

Bacteria and Culture Conditions

Wild-type *S. gordonii* CH1 and M99 strains, the Hsa-deficient mutant strain PS798, and the GspB-deficient mutant strain PS846 were kindly provided by Dr. Paul M. Sullam (University of California at San Francisco). The mutants were generated by double cross-over recombination, as described previously

(Bensing et al., 2004; Xiong et al., 2008). All bacteria were cultured in TH media containing 0.5% yeast extract until mid-log phase at 37°C. The mutant strains grew comparably well *in vitro* (data not shown). Bacterial cells were harvested by centrifugation at 8,000 rpm for 10 min at 37°C and were washed with PBS. To prepare stocks of wild-type *S. gordonii* and mutant strains, the bacterial pellet was suspended in 50% glycerol THY media to 5×10^8 CFU/ml and stored at −80°C in a freezer.

Analysis of Bacterial Adherence and Internalization

To label *S. gordonii* with CFSE, the bacterial pellet was suspended in 1 ml PBS containing 10 μM CFDA-SE and incubated for 15 min at 37°C. The bacterial cells were then washed with PBS. Immature DCs (5×10^4 cells) were incubated with either CFSE-labeled wild-type *S. gordonii* or SRR adhesin-deficient mutant strains at 5×10^5, 5×10^6, or 5×10^7 CFU in 50 μl PBS for 1 h at 4°C or 37°C, respectively. Flow cytometry (FACSCalibur, BD Biosciences) was used to measure bacterial binding at 4°C and internalization at 37°C. All cytometric data were analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

Analysis of DC Phenotypes

Immature DCs (2.5×10^5 cells/ml) were stimulated with either wild-type *S. gordonii* or SRR adhesin-deficient mutant (1×10^6 CFU/ml) in the presence of GM-CSF (2.5 ng/ml) and IL-4 (5 ng/ml). After 1 h, gentamycin (200 μg/ml) was added to the culture to prevent the bacterial growth and the DCs were further incubated for 23 h. The DCs were stained with fluorochrome-conjugated monoclonal antibodies specific to CD83, CD86, MHC class II, and PD-L1 for 30 min on ice and washed with PBS. The mean fluorescence intensity (MFI) of DCs was analyzed by FACSCalibur, and all flow cytometry data were analyzed by FlowJo software.

Quantification of Cytokines

Immature DCs (2.5×10^5 cells/ml) were stimulated with either wild-type *S. gordonii* or an SRR adhesin-deficient mutant (1×10^6 CFU/ml) in the presence of GM-CSF and IL-4. To kill *S. gordonii*, gentamicin was added to the DCs, and cells were further incubated for 23 h. Concentrations of TNF-α, IL-12p70, and IL-6 in the culture supernatants were measured by ELISA kits, as described previously (Lee et al., 2015).

Co-culture of DCs with Autologous T Lymphocytes

Immature DCs (2.5×10^5 cells/ml) were stimulated with either wild-type *S. gordonii* or an SRR adhesin-deficient mutant (1×10^6 CFU/ml) in the presence of GM-CSF and IL-4. After 1 h, DCs were treated with gentamycin to kill *S. gordonii* and were further incubated for another 15 h. To isolate $CD3^+$ T lymphocytes, PBMCs were incubated with anti-human CD3 magnetic particles for 30 min at room temperature. $CD3^+$ cells were isolated by positive selection according to the manufacturer's instruction. To label isolated $CD3^+$ T lymphocytes with CFSE, the cells were incubated in RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin solution, and 10 μM CFDA-SE for 15 min at 37°C and then washed with PBS. The CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) were mixed with *S. gordonii*-stimulated DCs (5×10^4 cells) for 4 days, and the cells were stained with anti-human CD25 antibody. The proliferative activity and activation marker expression of cells were subsequently analyzed by flow cytometry.

Statistical Analysis

The statistical difference between experimental groups and the control group was analyzed by Student's *t*-test. *P*-values less than 0.05 were considered statistically significant. Results are indicated as mean of triplicated measurements ± standard error of the mean (SEM).

RESULTS

SRR Adhesin-Deficient *S. gordonii* Exhibits Attenuated Binding and Internalization to DCs Compared to the Wild-Type Strain

Bacterial binding and internalization are important processes for DCs to initiate immune responses (Kapsenberg, 2003). The role of SRR adhesins of *S. gordonii* was examined, with focus on Hsa for the CH1 strain and GspB for the M99 strain. Bacterial adherence to DCs was studied at 4°C, while internalization was examined at 37°C. Hsa-deficient *S. gordonii* exhibited attenuated binding and internalizing abilities compared to the wild-type (**Figures 1A,C**). Likewise, GspB-deficient *S. gordonii* more weakly bound and internalized to the DCs than did the wild-type (**Figures 1B,D**). The results indicate that SRR adhesins Hsa and GspB are crucial for the adherence and internalization of *S. gordonii* to DCs.

SRR Adhesin-Deficient *S. gordonii* More Weakly Increases the Expression of Phenotypic Maturation Markers on DCs than Does the Wild-Type Strain

Upon sensing microbial antigens, DCs upregulate a number of molecules such as CD83, CD86, MHC proteins, PD-L1, and PD-L2 to induce an antigen-specific adaptive immune response (Kapsenberg, 2003). To examine the roles of *S. gordonii* Hsa and GspB in inducing phenotypic maturation of DCs, the expression of CD83, CD86, MHC class II, and PD-L1 on DCs upon stimulation with either wild-type *S. gordonii* or an SRR adhesin-deficient mutant was compared. Both wild-type *S. gordonii* CH1 and M99 markedly induced the expression of CD83 (**Figures 2A,E** and Supplementary Figures 1A,E, 2A,E), CD86 (**Figures 2B,F** and Supplementary Figures 1B,F, 2B,F), MHC class II (**Figures 2C,G** and Supplementary Figures 1C,G, 2C,G), and PD-L1 (**Figures 2D,H** and Supplementary Figures 1D,H, 2D,H). However, stimulation with the SRR adhesin-deficient mutant showed lower potency

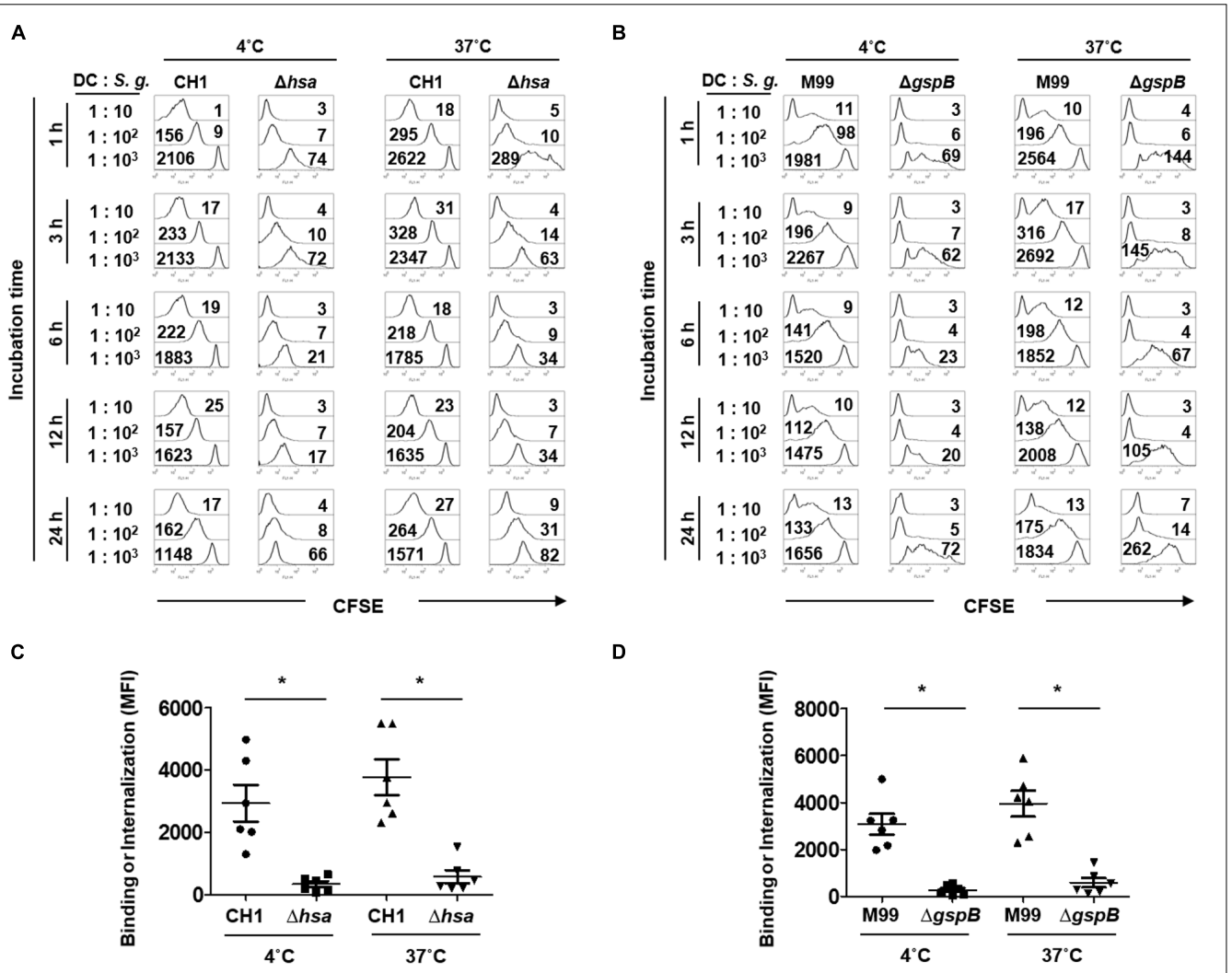

FIGURE 1 | Serine-rich repeat (SRR) adhesin-deficient *Streptococcus gordonii* exhibits attenuated binding and internalization to dendritic cells (DCs). Immature DCs (5×10^4 cells) were co-cultured with CFSE-labeled *S. gordonii* CH1 and M99 strains or their SRR adhesin-deficient mutants (Δ*hsa* for CH1 strain and Δ*gspB* for M99 strain) at 1–10, 10^2, or 10^3 at 4°C (for binding) and 37°C (for internalization). At 1 h after the treatment, gentamycin (200 μg/ml) was added to prevent the bacterial growth followed by incubation for additional 2, 5, 11, or 23 h. Then, the binding and internalization of **(A)** *S. gordonii* CH1 strain and **(B)** M99 strain to DCs were analyzed by flow cytometry. Immature DCs (5×10^4 cells) were co-cultured with CFSE-labeled *S. gordonii* CH1 and M99 strains or their SRR adhesin-deficient mutants (Δ*hsa* for CH1 strain and Δ*gspB* for M99 strain) at 1–10^2 at 4°C (for binding) or 37°C (for internalization) for 1 h. The numbers on the histograms indicate the mean fluorescence intensity (MFI). Binding and internalization of **(C)** *S. gordonii* CH1 strain and **(D)** M99 strain to DCs were analyzed by flow cytometry. Graphs of dot plot represent the mean MFI ± SEM from six independent experiments. Asterisk (*) indicates statistical significance ($P < 0.05$).

in inducing those molecules (**Figures 2A–H** and Supplementary Figures 1A–H, 2A–H). These results indicate that Hsa and GspB of *S. gordonii* contribute to the expression of activation markers on DCs.

SRR Adhesin-Deficient *S. gordonii* Induces Cytokine Production Less Potently than the Wild-Type Bacteria

When DCs are activated, they express cytokines such as IL-12, IL-10, and TNF-α to mediate inflammatory responses and the activation and differentiation of other immune cells including T lymphocytes (Kapsenberg, 2003). Thus, we next examined cytokine production of DCs induced by stimulation with wild-type *S. gordonii* or SRR adhesin-deficient mutants. DCs stimulated with Hsa-deficient mutant (**Figures 3A–C**) or GspB-deficient mutant (**Figures 3D–F**) resulted in significantly lower production of IL-12p70, TNF-α, and IL-6 in the Hsa-deficient CH1 strain as compared to wild-type. Similarly, a substantially lower secretion of these cytokines was also seen in the GspB-deficient mutant as compared to the M99 WT strain. Neither *S. gordonii* CH1 nor M99 strains induced IL-10 production in DCs (data not shown). These results suggest that SRR adhesins play an important role in *S. gordonii*-induced inflammatory cytokine production by DCs.

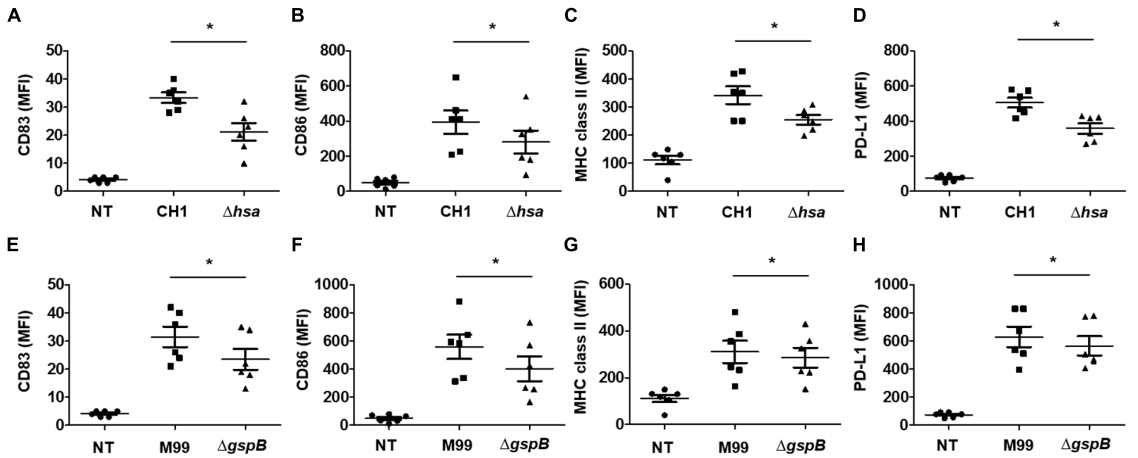

FIGURE 2 | Serine-rich repeat adhesin-deficient *S. gordonii* weakly induces phenotypic maturation of DCs. Immature DCs (2.5×10^5 cells/ml) were stimulated with various concentrations of *S. gordonii* CH1 and M99 strains or their SRR adhesin-deficient strains at 1×10^6 CFU/ml for 24 h. Expression of **(A,E)** CD83, **(B,F)** CD86, **(C,G)** MHC class II, and **(D,H)** PD-L1 on DCs was analyzed by flow cytometry. Graphs of dot plot represent the mean MFI ± SEM from six independent experiments. Asterisk (*) indicates statistical significance ($P < 0.05$). NT, non-treatment.

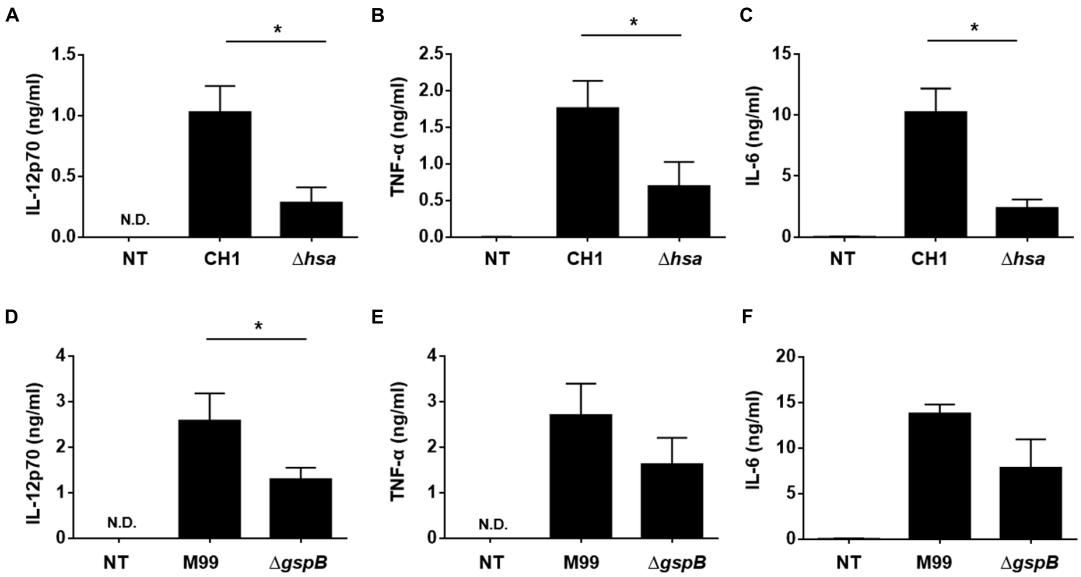

FIGURE 3 | Serine-rich repeat adhesin-deficient *S. gordonii* more weakly induces DC cytokine production than wild-type *S. gordonii*. Immature DCs (2.5×10^5 cells/ml) were stimulated with *S. gordonii* CH1 and M99 strains or their SRR adhesin-deficient strains at 1×10^6 CFU/ml for 24 h. The levels of **(A,D)** IL-12p70, **(B,E)** TNF-α, and **(C,F)** IL-6 in the culture media were measured by ELISA. Concentrations of cytokines are indicated as mean value ± SEM from three independent experiments. Statistical difference between experimental groups was analyzed by Student's *t*-test. *P*-values less than 0.05 were considered statistically significant and are indicated by asterisks (*). N.D., not detected; NT, non-treatment.

DCs Stimulated with SRR Adhesin-Deficient *S. gordonii* More Weakly Induce Proliferation and Activation of Autologous T Cells than DCs Stimulated with Wild-Type *S. gordonii*

Functionally mature DCs exhibit increased expression of MHC proteins, co-stimulatory molecules, and cytokines required for adequate activation of T lymphocytes (Kapsenberg, 2003). To examine the effect of *S. gordonii* SRR adhesins on the T cell-activating capacity of DCs, the proliferative activity and activation marker expression of T lymphocytes were examined through DCs sensitized with wild-type *S. gordonii* or SRR adhesin-deficient mutants. The results showed that Hsa-deficient mutant-sensitized DCs induced proliferation and CD25 expression of T lymphocytes less potently than DCs sensitized with wild-type *S. gordonii* (**Figure 4A**). Likewise, T lymphocytes

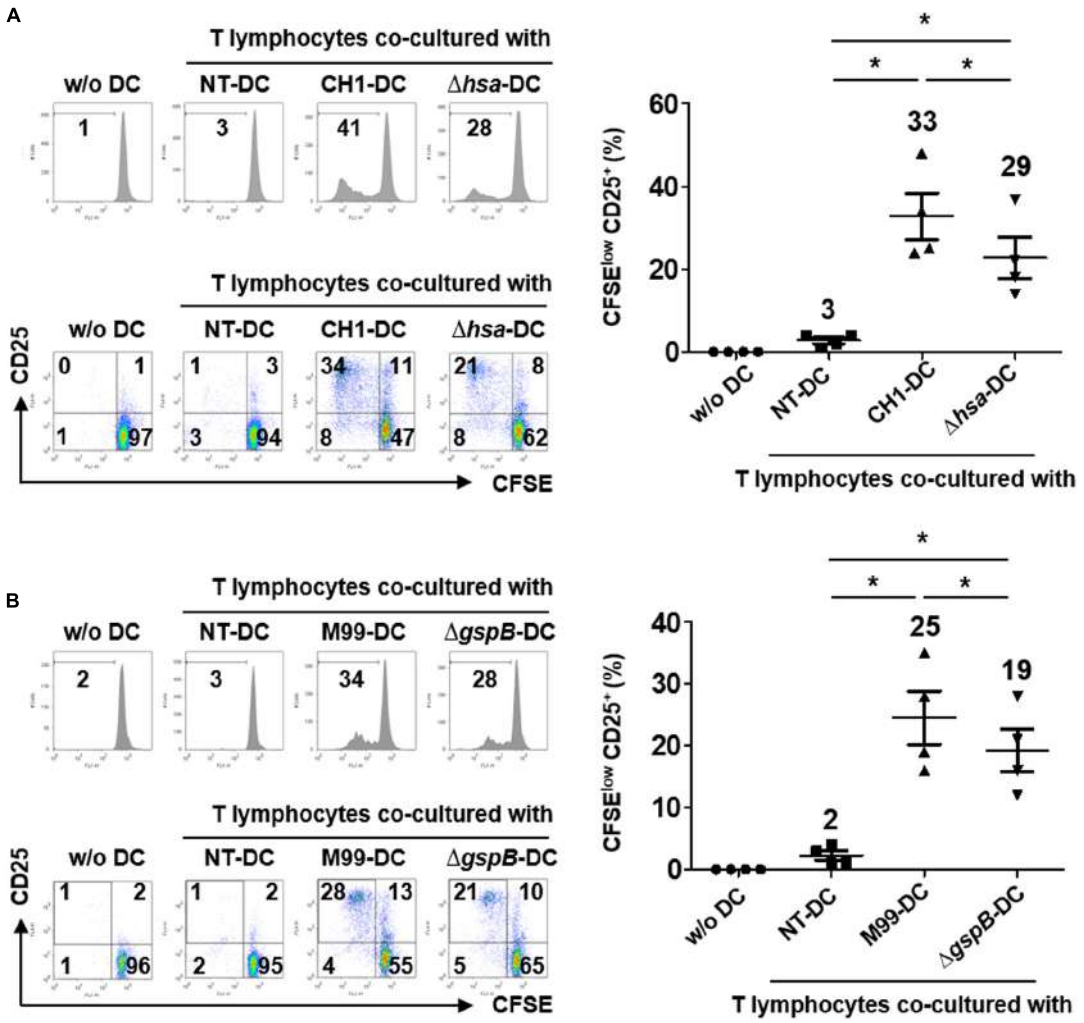

FIGURE 4 | Dendritic cells stimulated with SRR adhesin-deficient *S. gordonii* more weakly induce proliferation and activation of autologous T cells than DCs stimulated with wild-type *S. gordonii*. Immature DCs (2.5×10^5 cells/ml) were stimulated with *S. gordonii* CH1 and M99 strains or their SRR adhesin-deficient strains at 1×10^6 CFU/ml for 16 h. DCs were co-cultured with CFSE-labeled autologous T lymphocytes (2.5×10^5 cells/ml) for 5 days. **(A,B)** Proliferation and CD25 expression of T lymphocytes induced by **(A)** DCs sensitized with *S. gordonii* CH1 strain or its mutant and **(B)** DCs sensitized with *S. gordonii* M99 strain or its mutant were examined by flow cytometry. The numbers in each histogram and quadrant indicate the percentage. Histograms represent the proliferation level of T lymphocytes determined by reduced CFSE fluorescence intensity. Graphs of dot plot indicate the mean values of $CFSE^{low}$ $CD25^+ \pm$ SEM from four independent experiments and the actual mean values are on top of each group. Asterisk (*) indicates statistical significance ($P < 0.05$). NT, non-treatment.

exhibited weakened proliferative activity and activation marker expression in response to GspB-deficient mutant-sensitized DCs (**Figure 4B**). Taken together, these results indicate that Hsa and GspB contribute to DC-mediated immune activation of T lymphocytes by *S. gordonii*.

Blockade of SRR Adhesins Abolishes *S. gordonii* Binding and Internalization to DCs and Attenuates Immunostimulating Potency

Hsa, the SRR adhesion of *S. gordonii* CH1 strain, specifically bind to 3′SL (Urano-Tashiro et al., 2016). In order to further examine the role of Hsa in the bacterial interaction with DCs, *S. gordonii* CH1 strain pretreated with 3′SL (named CH1-SL) was treated with DCs followed by analysis of the bacterial adherence to DCs and phenotypic and functional maturation of DCs. CH1-SL showed marked attenuation in binding and internalizing DCs (**Figure 5A**). In addition, pretreatment with 3′SL abolished the immunostimulatory potency of *S. gordonii*. DCs stimulated with CH1-SL exhibited lower expression of maturation markers such as CD86, MHC class II, and PD-L1 than DCs stimulated with unpretreated *S. gordonii* (**Figure 5B**). Furthermore, stimulation with CH1-SL diminished the production of IL-12p70, TNF-α, and IL-6 by DCs (**Figure 5C**). When CH1-SL-sensitized DCs were co-cultured with autologous T lymphocytes, proliferation and activation of T lymphocytes were more weakly induced than with DCs sensitized by unpretreated *S. gordonii*

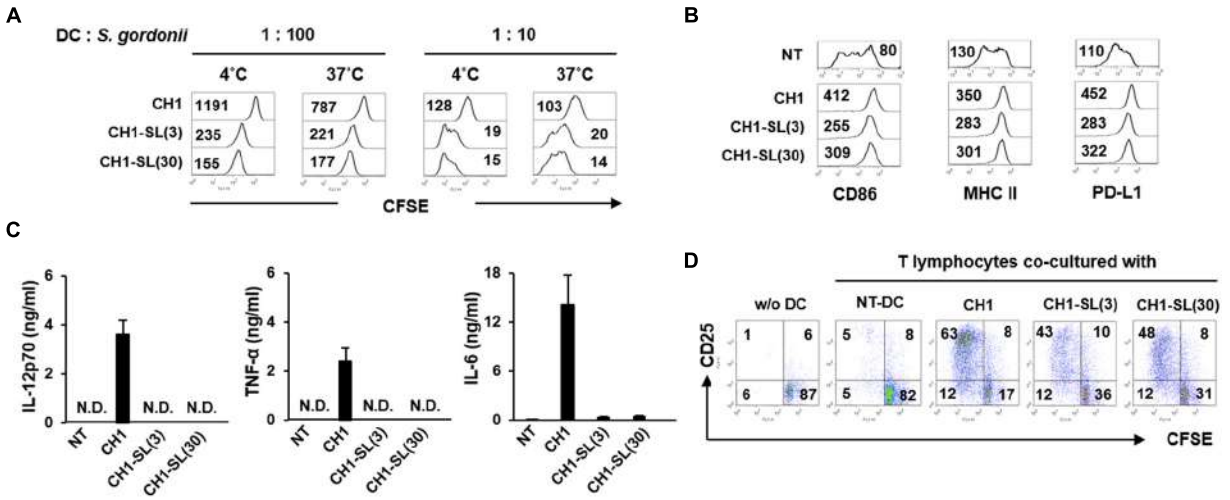

FIGURE 5 | Blockade of SRR adhesins abolishes *S. gordonii* binding and internalization to DCs and attenuates the immunostimulating potency of bacterium in DCs. *S. gordonii* CH1 (6.7×10^6 CFU/ml) was pretreated with 3′-sialyllactose (3 and 30 μM) for 1 h at room temperature to block bacterial Hsa and was washed with PBS. **(A)** Immature DCs (5×10^4 cells) were incubated in the absence or presence of 3′-sialyllactose-pretreated *S. gordonii* (5×10^5 and 5×10^6 CFU) in 50 μl PBS for 1 h at 4 and 37°C, respectively. Bacterial binding (4°C) and internalization (37°C) to DCs were analyzed by flow cytometry. The numbers on the histogram indicate the MFI of DCs. **(B,C)** Immature DCs (2.5×10^5 cells/ml) were either untreated or stimulated with 3′-sialyllactose-pretreated *S. gordonii* (1×10^6 CFU/ml) for 24 h. **(B)** Expression of CD86, MHC II, and PD-L1 on DCs was analyzed by flow cytometry. The numbers on the histogram indicate the MFI of DCs. **(C)** Concentrations of IL-12p70, TNF-α, and IL-6 in DC culture supernatants were measured by ELISA. **(D)** *S. gordonii*-sensitized DCs (5×10^4 cells) were co-cultured with autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. Proliferative activity and CD25 expression of T lymphocytes were analyzed by flow cytometry. The numbers in each quadrant indicate the percentage of T lymphocytes. Results are representative of two similar experiments. N.D., not detected; NT, non-treatment; CH1, *S. gordonii* CH1 unpretreated with 3′-sialyllactose; CH1-SL(3), *S. gordonii* CH1 pretreated with 3 μM 3′-sialyllactose; CH1-SL(30), *S. gordonii* CH1 pretreated with 30 μM 3′-sialyllactose.

(**Figure 5D**). These results suggest that Hsa is a primary target molecule mediating *S. gordonii* binding and internalization to DCs and the immunostimulatory potency of the bacteria in DC maturation.

DISCUSSION

Streptococcus gordonii SRR adhesins, Hsa and GspB, are important not only for bacterial adhesion to host cells, but also for activation of host immune responses. Here, we demonstrated that *S. gordonii* lacking SRR adhesins showed marked reduction in DC maturation, production of inflammatory cytokines, and T cell-activating ability compared to wild-type *S. gordonii*. These results suggest that SRR adhesins Hsa and GspB are major surface molecules that are responsible for *S. gordonii*-induced DC maturation and activation.

Intact *S. gordonii* appears to induce the maturation and activation of human DCs, which is coincident with previous findings that phenotypic maturation and cytokine production take place in murine and human DCs stimulated with *S. gordonii* (Corinti et al., 1999; Mayer et al., 2009). On the other hand, IL-10 was hardly induced in human DCs treated with *S. gordonii* while other cytokines such as TNF-α, IL-6, and IL-12 were substantially increased under the same condition. Interestingly, however, previous reports demonstrated IL-10 induction in human DCs treated with *S. gordonii* (Corinti et al., 1999, 2001). Some possible explanations for this discrepancy can be made. One might be the difference in the intrinsic property of DCs originated from blood of humans with different ethnic backgrounds. Another might be the difference in the experimental conditions used for the preparation of DCs such as the separation method of DC precursors, the concentration of GM-CSF and IL-4, and the culture media composition containing supplementary ingredients. The third one might be the difference in the bacteria-to-DC ratio. The previous report demonstrated no induction of IL-10 at *S. gordonii*-to-DC ratio of 1:1 while a small amount of IL-10 (<100 pg/ml) at 10:1 (Corinti et al., 1999). Another study showed that *S. gordonii*-to-DC ratio of 50:1 was comparable to *S. typhi*-to-DC ratio of 1:1 for the induction of IL-10 at the similar extent, implying the low potentcy of *S. gordonii* to induce IL-10 in DCs (Corinti et al., 2001). Therefore, our results showing no IL-10 induction could be due to the difference in the intrinsic property of DCs derived from different ethnic background, the experimental method, and/or the use of low *S. gordonii*-to-DC ratio at 4:1.

We used SRR adhesin-deficient mutant strains to demonstrate that SRR adhesins Hsa and GspB contribute to *S. gordonii*-induced DC maturation and activation. This is in line with previous findings that *S. gordonii* binding to human monocytes via Hsa promoted their differentiation into DCs (Urano-Tashiro et al., 2012). Interestingly, other surface adhesins of *S. gordonii* are also involved in the activation of innate immune cells. SspA and SspB, famous adhesins of *S. gordonii*, are the best examples for the induction of cytokines in epithelial cells and DCs (Andrian et al., 2012). We suggest

that the SRR adhesins of *S. gordonii* are not only involved in bacterial adherence, but also actively contribute to the induction of innate immunity by maturation and activation of DCs.

Although SRR-deficient *S. gordonii* showed a decreased augmentation on the expression of maturation markers including CD83, CD86, MHC class II, and PD-L1, in comparison with the wild-type strain, the difference between the wild-type and mutant appears to vary in each marker. It is likely due to the difference in the signaling pathways coincident with differential induction and turn-over rate. Furthermore, the more bacteria were treated, the less difference was observed in their expression. It may be because *S. gordonii* also possesses other immunostimulatory molecules such as lipoprotein and LTA in the cell wall that are known to involve the expression of the maturation markers on the host immune cells (Chan et al., 2007; Cho et al., 2013). On the other hand, it is notable that the loss of Hsa in *S. gordonii* CH1 strain was more dramatic than the loss of GspB in *S. gordonii* M99 strain in the phenotypic and functional maturation of DCs. The differential profiles of DC maturation might be due to the difference in the ligand-binding BR structure and glycan specificity between GspB and Hsa (Bensing et al., 2016) as demonstrated by the previous study that Hsa binds to both 3′SL and sialyl-T antigen, whereas GspB binds only to sialyl-T antigen (Urano-Tashiro et al., 2016).

Accumulating reports suggest that *S. gordonii* exhibit similar properties with regard to binding and internalization in various cell types, including monocytes, macrophages, erythrocytes, and platelets (Kerrigan et al., 2007; Urano-Tashiro et al., 2008). For example, *S. gordonii* binds to membrane glycoprotein Ibα on human platelets through bacterial Hsa or GspB, and the lack of GspB decreased platelet binding of the *S. gordonii* M99 strain by approximately 70% (Takamatsu et al., 2005; Xiong et al., 2008). Concordant with previous reports, the current results also showed that *S. gordonii* lacking Hsa or GspB was not efficiently adhered or internalized to DCs, leading to insufficient maturation and activation of DCs. These results support the hypothesis that SRR adhesins Hsa and GspB are important for the interaction of *S. gordonii* and DCs, which stimulate innate immunity mediated through DCs.

Serine-rich repeat adhesins were reported to bind to sialic acids of host cells, contributing to the pathogenesis of *S. gordonii* (Urano-Tashiro et al., 2008). Indeed, *S. gordonii* CH1 exhibited markedly attenuated binding and internalizing ability to DCs in the presence of 3′SL, which might interfere with the interaction of the bacterial Hsa with sialylated motifs on DCs. Moreover, the bacteria pretreated with 3′SL showed weakened induction of maturation, cytokine production, and T cell-activating ability of DCs. Interestingly, the inhibitory effect of 3′SL was dramatic on the induction of cytokine production in comparison with that of phenotypic marker expression. Those differential effects could be due to the distinct intracellular signal transduction pathways required for the expression of cytokines and co-stimulatory receptors. For example, the expression of co-stimulatory receptors including CD80 and CD86 was highly induced by lipopolysaccharide without induction of TNF-α or IL-12 in MyD88-deficient DCs (Kaisho et al., 2001). Therefore, we speculate that the bacterial interaction through SRR with DCs could predominantly participate in the stimulation of signaling pathways for the induction of cytokines rather than phenotypic markers in DCs.

Bacterial binding and internalization are important steps for DC maturation. Many previous studies have reported that a blockade of bacterial adherence and internalization to DCs attenuated the phenotypic and functional activation of DCs. One study showed that clinically isolated Group A Streptococcus did not induce maturation of DCs when binding and/or internalization was perturbed by bacterial hyaluronic acid capsular polysaccharides (Cortes and Wessels, 2009). In addition, encapsulated *Klebsiella pneumoniae* hardly induced DC maturation because a thick capsule layer on the bacterial surface hindered its phagocytosis by Evrard et al. (2010). Consistent with these reports, our findings showed that SRR adhesin-deficient *S. gordonii* mutant strains had weak binding and internalizing abilities. This consequently induced phenotypic and functional activation of DCs to a lesser extent. Furthermore, the inhibition of bacterial internalization with cytochalasin D abrogated *S. gordonii*-induced maturation and activation of DCs (data not shown). Therefore, Hsa- or GspB-mediated binding and internalization of *S. gordonii* to DCs could be the important step for DC maturation and activation.

Although oral streptococci are considered normal flora of the human oral cavity, some have recently been suggested as etiologic agents for systemic diseases including infective endocarditis and osteomyelitis (Li et al., 2000). It is important to understand the exact pathogenic mechanisms of oral bacteria and to characterize their major virulence factors in order to develop preventive and therapeutic agents against *S. gordonii* infection. Because DCs are the primary sentinel cells used to monitor infections and bridge innate and adaptive immunity for host protection, the SRR adhesins of *S. gordonii* might be major immunomodulatory molecules. Further studies are needed to identify the DC receptors that specifically bind to Hsa and GspB and elucidate downstream signal pathways to activate DCs. The results suggest that the SRR adhesins of *S. gordonii* are major virulence factors involved in bacterial adherence to the host and also trigger DC maturation and activation.

AUTHOR CONTRIBUTIONS

SH conceived the idea and contributed to the discussion of the results followed by writing and reviewing the manuscript. SH, EBK, and SK designed the experiments, performed the experiments, and/or interpreted the data. HS and C-HY provided critical comments and contributed to the discussion of the results followed by writing and reviewing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00523/full#supplementary-material

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