frontiers in Immunology

ORIGINAL RESEARCH
published: 09 February 2017
doi: 10.3389/fimmu.2017.00060

Dexamethasone Inhibits *S. aureus*-Induced Neutrophil Extracellular Pathogen-Killing Mechanism, Possibly through Toll-Like Receptor Regulation

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Neutrophils release neutrophil extracellular traps (NETs) in a pathogen-killing process called NETosis. Excessive NETs formation, however, is implicated in disease pathogenesis. Therefore, to understand how NETosis is regulated, we examined the effect of dexamethasone (DXM), an anti-inflammatory drug, on this process and the role of toll-like receptors (TLRs). We stimulated human neutrophils with phorbol 12-myristate 13-acetate (PMA) or *Staphylococcus aureus* (*S. aureus*) and quantified NETs formation. We also examined the effect of DXM on the bactericidal effect of NETs and the role of reactive oxygen species (ROS) and nuclear factor (NF)-κB in DXM-regulated NETosis. DXM significantly inhibited *S. aureus*-induced NETosis and extracellular bacterial killing. ROS production and NF-κB activation were not involved in DXM-regulated NETosis. TLR2 and TLR4, but not TLR5 or TLR6, modified *S. aureus*-induced NETs formation. Neither DXM nor TLRs were involved in PMA-induced NETosis. Furthermore, TLR2 and TLR4 agonists rescued DXM-inhibited NETosis, and neither TLR2 nor TLR4 antagonists could further inhibit NETosis reduction induced by DXM, indicating that DXM may inhibit NETosis by regulating TLR2 and TLR4. In conclusion, the mechanisms of *S. aureus*- and PMA-induced NETosis are different. DXM decreases NETs formation independently of oxidant production and NF-κB phosphorylation and possibly *via* a TLR-dependent mechanism.

Keywords: neutrophil extracellular traps, dexamethasone, TLRs, *S. aureus*, PMA

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *25 June 2016*
Accepted: *16 January 2017*
Published: *09 February 2017*

Citation:
Wan T, Zhao Y, Fan F, Hu R and Jin X (2017) Dexamethasone Inhibits S. aureus-Induced Neutrophil Extracellular Pathogen-Killing Mechanism, Possibly through Toll-Like Receptor Regulation. Front. Immunol. 8:60. doi: 10.3389/fimmu.2017.00060

INTRODUCTION

Neutrophils are the most abundant leukocytes in human blood and play an essential role in innate immunity since they are the first cells recruited to sites of infection and inflammation (1). They engulf microorganisms or opsonized particles and degrade them intracellularly as well as releasing microbicidal proteins and reactive oxygen species (ROS) (2). Recently, these cells have been shown to release structures called neutrophil extracellular traps (NETs), which consist of chromatin along with histones and many granular antimicrobial proteins—including elastase, myeloperoxidase, and calprotectin; this is a novel extracellular pathogen-killing mechanism described as NETosis (3–5).

Although NETosis contributes to pathogen control, it is essential for the balance between the formation and removal of NETs to be regulated to ensure tissue homeostasis, because large amounts of NETs may contribute to collateral damage within inflamed tissues. Excessive amounts of NETs are associated with the pathogenesis of inflammatory and autoimmune diseases, including preeclampsia (6), cystic fibrosis (7), and lupus (8). Moreover, NETs have been observed to act as a scaffold for thrombus formation (9, 10), which is increasingly being recognized as a critical phenomenon linking inflammation with venous thrombosis. Therefore, NETosis is a double-edged sword: while it is an effective first-line antimicrobial mechanism, it might also lead to organ failure and death if it is unregulated. Hence, it is important to understand the mechanism of NETs regulation, but little information is available about this topic thus far.

Since an inflammatory microenvironment is essential for NETs formation, we believed that using glucocorticoids, which are potent anti-inflammatory drugs, can help elucidate how NETs formation is regulated. They are commonly used to resolve inflammation and are closely related to neutrophil function. They have been shown to inhibit neutrophil apoptosis and cytokine release during inflammation (11) and are also associated with many neutrophil functions, including chemotaxis, migration, and phagocytosis (12). Therefore, we examined the effect of a commonly used glucocorticoid drug, dexamethasone (DXM), on NETs formation. On the other hand, toll-like receptors (TLRs), which are essential pattern-recognizing receptors (PRRs) that mediate the recognition of microbial structures, have been reported to activate neutrophil extracellular traps to ensnare bacteria in septic blood (13). Moreover, most of the TLRs were reported to be expressed in neutrophils and were involved in neutrophils activation (14). So, we also investigated the role of different TLRs in NETs formation.

We found that DXM significantly inhibited NETs formation induced by *Staphylococcus aureus* (*S. aureus*) but not that induced by phorbol 12-myristate 13-acetate (PMA), which suggested that DXM can serve as a potential drug to regulate NETosis. In addition, the modulation of TLR-2 and TLR4 had an effect on NETs production, thus indicating the involvement of TLRs in this process.

MATERIALS AND METHODS

Reagents

Phorbol 12-myristate 13-acetate, DXM, DNase I, cytochalasin D, and dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Percoll, from GE Healthcare (Little Chalfont, UK); and TLR agonists and TLR antagonists, from InvivoGen (San Diego, CA, USA). Anti-histone H2B and neutrophil elastase antibodies, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, anti-phosphorylated nuclear factor κB (anti-p-NF-κB, p65) antibody, secondary antibodies coupled to AF488 or AF555, and horseradish peroxidase (HRP) secondary antibody were purchased from Santa Cruz Biotechnology (CA, USA). SYTOX Green, Luria broth, Quant-iT PicoGreen double-stranded deoxyribonucleic acid (dsDNA) assay kit and micro-plates were purchased from ThermoFisher Scientific (Basingstoke, UK).

Isolation of Human Neutrophils

Neutrophils were isolated from the peripheral blood of fasting healthy donors by Percoll gradient centrifugation, as previously described (7). For those donors, comprehensive history and physical examination were performed, basic laboratory tests were used to exclude occult disease. This study was conducted according to the principles expressed in the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of Affiliated Second Hospital, School of Medicine, Zhejiang University, China. All participants provided written informed consent for the collection of samples and subsequent analyses. For each donor, 10–30 ml blood was drawn according to the need of different assays. Bloods from at least three donors were used to repeat the same assay. Cell suspensions contained >96% neutrophils, as determined by Wright–Giemsa staining, with 98% cell viability as determined by Trypan blue staining. The cells (4×10^5/ml) were re-suspended in RPMI 1640 medium supplemented with bovine serum albumin (2%).

Neutrophils Stimulation

Neutrophils (2×10^5 cells/well in 500 μl) were stimulated with PMA (50 nM) or *S. aureus* (multiplicity of infection = 10) and placed in a humidified incubator at 37°C with CO_2 (5%) for 120 min. In some experiments, neutrophils were first incubated for 120 min with DXM (10 μM), TLR2 agonist (HKLM, 10^8 CFU/ml), TLR4 agonist (LPS, 1 μg/ml), TLR5 agonist (FSL-ST, 1 μg/ml), TLR6 agonist (FSL-1, 1 μg/ml), TLR neutralizing antibodies as antagonists (TLR2, 4, 5, 6 antibody, 1 μg/ml), or vehicle (controls). Stock solutions of DXM, TLR agonists, and TLR antagonists were prepared in DMSO and were further diluted in RPMI 1640 medium. The final DMSO concentration (0.1% v/v) did not have a toxic effect. All drugs were freshly prepared for each experiment.

NETs Formation Assay

After stimulation, cells were fixed with 4% PFA, blocked with 3% normal donkey serum and 0.05% Tween 20 in phosphate-buffered saline (PBS), and incubated with the primary antibodies anti-H2B and anti-neutrophil elastase, which were detected with secondary antibodies coupled to AF488 or AF555. Isotype-matched controls were used. For DNA detection, 4′, 6′-diamidino-2-phenylindole (DAPI) was used. Specimens were mounted and analyzed under a confocal microscope (Olympus IX-50).

Neutrophil extracellular traps were also examined using the membrane-impermeable DNA-binding dye SYTOX green (Molecular Probes, Invitrogen Life Technologies). SYTOX green (5 μM) was added to the cultures after specific periods of incubation, and the cultures observed 5 min later. In one case, DNase I (100 U/ml) was added for 10 min to degrade the NETs structure as control. To visualize NETs, live-cell cultures were imaged with an inverted fluorescence microscope (Olympus IX-50).

Bacterial Culture

Staphylococcus aureus (ATCC 25923) was cultured overnight in Luria–Bertani (LB) broth (37°C, 200 rpm), harvested by centrifugation, washed, and suspended in PBS. Bacterial growth was quantified at A600 and the cell number determined using a standard curve based on colony counts. Stationary-phase bacteria were used for all experiments.

Quantification of Extracellular DNA

The levels of extracellular DNA in supernatants were quantified using Quant-iT PicoGreen dsDNA assay kit according to the manufacturer's instructions. PicoGreen is a cell-impermeable dye that binds to extracellular dsDNA without staining live cells. Fluorescence intensity was measured on a SpectraMax M3 (Molecular Devices) fluorescent plate reader at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, with a 515-nm emission cutoff filter. The calibration curve was constructed using a standard dsDNA of a known concentration.

Bacterial Survival Assay of NETs

A bacterial survival assay was performed as described in earlier studies (15). Neutrophils (1×10^6 cells/well in 200 µl) were pre-incubated with or without DXM for 2 h and then treated with 50 nM PMA or left untreated for another 2 h at 37°C and 5% CO_2. NETs killing was examined by inhibiting phagocytic killing by the addition of 100 µg/ml cytochalasin D for 15 min before the addition of bacteria. After 1 h at 37°C, neutrophils and clumped NETs were disrupted by the addition of 0.01% Triton X-100 and three passes through a 25-gauge needle. Following serial dilution, bacteria were plated on LB plates for colony counting. After overnight incubation at 37°C, the number of colony-forming units (CFU) was determined. Zero killing was defined by control samples consisting of RPMI 1640. Killing efficacy was determined by subtracting the CFU of indicated treatment from control group.

ROS Production

Neutrophils were incubated in PBS (Ca^{2+}- and Mg^{2+}-free) with 10 µM DCF-DA (Sigma) at 37°C for 20 min. Subsequently, they were pelleted, washed in PBS three times, and transferred to a 96-well plate (1×10^6 cells/well in 100 µl). They were then stimulated with *S. aureus* for 1 h (some cells were pretreated with DXM for 120 min), and fluorescence was measured using SpectraMax M3 fluorescent plate reader at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Immunoblotting

The neutrophils (3×10^6 cells/well in 500 µl) were pre-incubated with or without DXM for 2 h and then stimulated for another 2 h with *S. aureus*. Cell lysates were prepared using 1× loading buffer and boiled. Samples were then frozen at −80°C until use. Equal amounts of proteins were run on 12% sodium dodecyl sulphate-polyacrylamide gel and then electrotransferred onto polyvinylidenefluoride membranes. After blocking with 5% bovine serum albumin, membranes were incubated with phospho-NF-κBp65 and anti-GAPDH antibody overnight at 4°C, and then with HRP-conjugated secondary antibody for 2 h at room temperature. Protein bands were visualized by enhanced chemiluminescence. The gray degree of protein bands was detected by image J, and the value of p-NF-κB p65/GAPDH was calculated.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.1. Data are expressed as mean ± SE of individual samples. For two-group comparison, Student's *t*-test was applied for normally distributed data. The comparisons between multiple groups were performed using one-way ANOVA, followed by a Bonferroni's post-test. The significance threshold was set at 0.05.

RESULTS

NETs Formation in Response to PMA and Bacterial Stimulation

Microscopic observation clearly showed NETs structure, including neutrophil-derived proteins. Neutrophils were labeled with DAPI to identify DNA (blue) and with antibodies to identify neutrophil histone (green) and elastase (red) (**Figure 1A**). This confirmed PMA- or *S. aureus*-triggered NETs formation. SYTOX green staining further showed that bacteria were trapped in the web like structure and could be released when this NETs formation was degraded by Dnase I (**Figure 1B**).

DXM Inhibits NETs Formation Induced by *S. aureus* But Not That Induced by PMA

Fluorescence microscopy showed that DXM did not have any effect on the NETs formation induced by PMA but markedly inhibited that induced by *S. aureus* (**Figure 2A**). To further corroborate these, NETs formation was measured by quantifying the extracellular DNA in the supernatants. This experiment confirmed that *S. aureus*-induced formation of extracellular traps was significantly decreased by DXM ($p < 0.05$). In contrast, the amount of NETs formed after PMA induction was similar in controls and in DXM-treated neutrophils ($p > 0.05$) (**Figure 2B**). In addition, DMSO (0.1% v/v) in the solution of stimulates had no effect on NETs formation.

DXM Decreases the Bactericidal Efficacy of NETs

Dexamethasone significantly decreased the bactericidal efficacy of NETs, following abrogating phagocytic killing by the addition of cytochalasin D ($p < 0.05$; **Figure 3**). However, if neutrophils were activated to form NETs by PMA, DXM treatment had no effect on the killing efficacy of NETs ($p > 0.05$).

ROS and NF-κB Activation Are Not Involved in DXM-Regulated NETosis

Reactive oxygen species generation was first evaluated in resting neutrophils by performing a DCF-DA fluorescence assay. DCF-DA is a non-fluorescent molecule that becomes fluorescent in the presence of a wide variety of ROS, including superoxide anion and hydroxyl radicals (16). NETs formation has previously been reported to be dependent on or independent of ROS (17). In order to examine if DXM-regulated NETs formation is

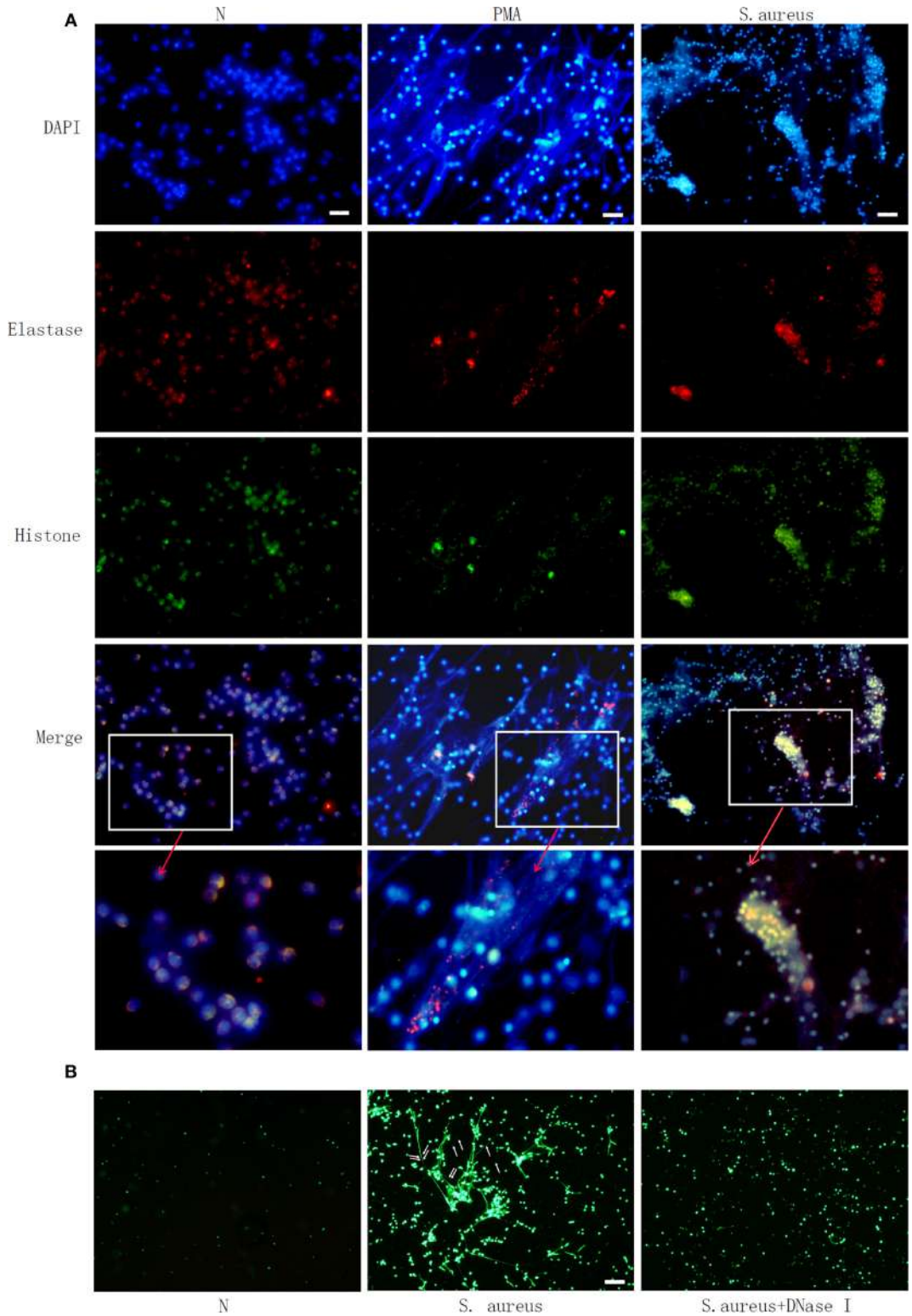

FIGURE 1 | **Phorbol 12-myristate 13-acetate (PMA) and *Staphylococcus aureus* (*S. aureus*) stimulate neutrophil extracellular traps (NETs) formation in human neutrophil**. Human neutrophil suspended in media were treated with PMA (50 nM) or *S. aureus* at MOI of 10. Human neutrophil without treatment (N) was used as control. NETs formation was measured at 2 h. **(A)** Neutrophils were labelled with 4′, 6′-diamidino-2-phenylindole (DAPI) to identify DNA (blue) and with antibodies to identify neutrophil histone (green) and elastase (red). PMA and *S. aureus*-induced NETs formation. **(B)** *S. aureus* (indicated with arrow) were trapped in NETs and released when NETs structure was degraded by DNase I, as observed by SYTOX green staining Bar: 50 μm.

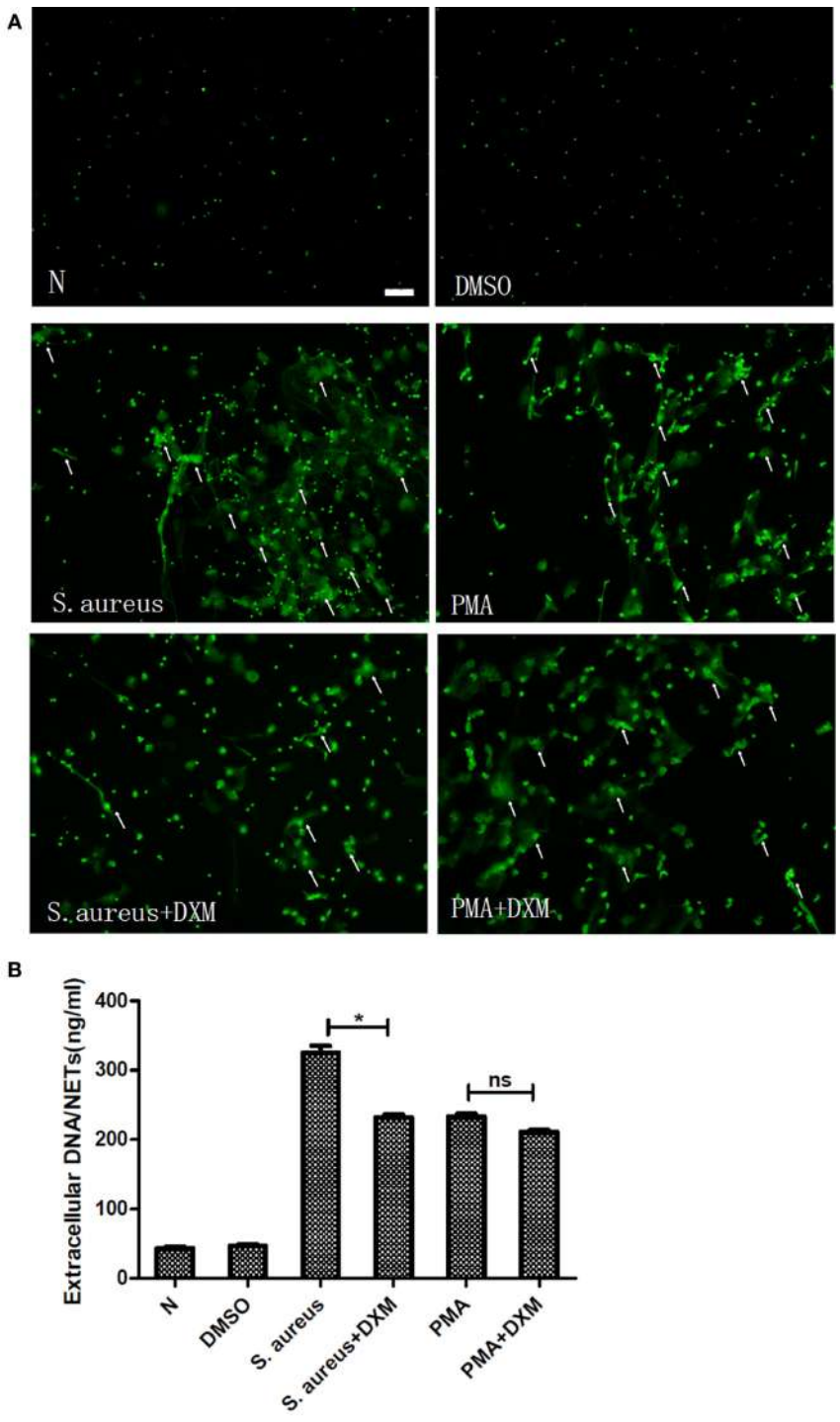

FIGURE 2 | **Dexamethasone (DXM) inhibited neutrophil extracellular traps (NETs) formation induced by *Staphylococcus aureus* (*S. aureus*) but not that induced by phorbol 12-myristate 13-acetate (PMA)**. Human neutrophils suspended in media were pretreated with or without DXM (10 μM) for 2 h, and then NETs formation 2 h after stimulation with PMA or *S. aureus* was examined using the membrane-impermeable DNA-binding dye SYTOX green and quantified by Quant-iTPicoGreen double-stranded deoxyribonucleic acid assay kit. Neutrophils without any stimulation or treated with DMSO (0.1% v/v) were used as control. **(A)** DMSO (0.1% v/v) did not affect NETs formation. While dexamethasone did not modify NETs formation induced by PMA, it markedly inhibited that induced by *S. aureus*. Several typical NETs were indicated with arrows. Bar: 50 μm. **(B)** Quantification of extracellular DNA confirmed that it inhibited NETs formation induced by *S. aureus* but not that induced by PMA. Neutrophils (1×10^5cells/well in 100 μl) were stimulated to form NETs, and the mean value of NETs amount in five replicated wells was adopted. The assay was repeated for three times with bloods from three different donors; error bars represent SEM. *$p < 0.05$ by ANOVA with Bonferroni's post-test.

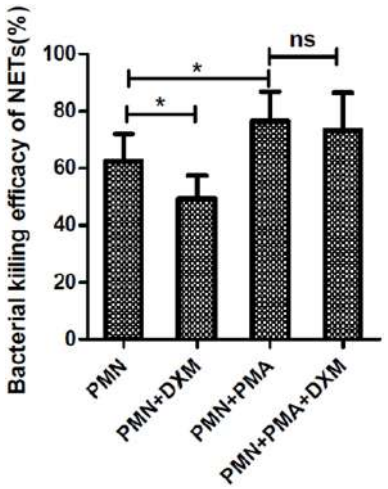

FIGURE 3 | Dexamethasone (DXM) inhibited the bactericidal efficacy of neutrophil extracellular traps (NETs). Neutrophils were pre-incubated with or without DXM for 2 h and then treated with 50 nM phorbol 12-myristate 13-acetate (PMA) or left untreated for another 2 h. One hour after addition of bacteria, colony-forming units (CFU) were determined by overnight incubation at 37°C following serial dilution. Zero killing was defined by control samples consisting of only media. Killing efficacy was determined by subtracting the CFU of indicated treatment from control groups. By using cytochalasin D to abrogate phagocytic killing, dexamethasone was found to significantly inhibit the bactericidal efficacy of NETs. However, dexamethasone could not inhibit PMA-activated bactericidal efficacy of NETs. The assay was repeated for nine times, each case in three wells; error bars represent SEM. *$p < 0.05$ by ANOVA with Bonferroni's post-test.

ROS-dependent, ROS production by activated neutrophils with or without DXM stimulation was measured. *S. aureus* infection elicited significant neutrophil oxidative burst, but DXM treatment neither increased nor decreased this response noticeably (**Figure 4A**).

The transcription factor NF-κB is a key regulator of inflammation and therefore plays a pivotal role in a wide range of inflammatory diseases (18). The phosphorylation of NF-κB has been believed to be involved in NETs generation (19). Therefore, we explored the role of DXM in the activation of NF-κB induced by *S. aureus*. The expression of p-NF-κB (p65) was significantly higher when the cells were stimulated with *S. aureus*, but this effect was not modified by DXM (**Figures 4B,C**).

TLRs Are Involved in NETs Formation Induced by *S. aureus* But Not That Induced by PMA

Toll-like receptors are key PRRs, which are important in innate immune responses. Thus, we explored the role of TLRs in the formation of NETs. None of TLR2 agonist, TLR4 agonist, TLR5 agonist, and TLR6 agonist could induce NETs formation. However, TLR2 and TLR4 agonists significantly enhanced NETs formation induced by *S. aureus* but not that induced by PMA, as shown by the quantification of extracellular DNA. Moreover, blocking TLR2 and TLR4 with neutralizing antibodies significantly reduced the NETs formation induced by *S. aureus* but not that induced by PMA, as shown by quantification of extracellular DNA (**Figure 5**). Furthermore, neither the TLR5/TLR6 agonist nor the antagonist could modulate the formation of NETs.

DXM May Modulate *S. aureus*-Induced NETs Formation through TLR2 and TLR4

To explore the mechanism of DXM-modulated NETs formation, we first pre-incubated the cells with TLR agonists to examine the effect of TLRs on DXM-inhibited NETs formation. As expected, both HKLM (TLR2 agonist) and LPS (TLR4 agonist) rescued DXM-reduced NETs formation (**Figure 6A**). Moreover, neither TLR2 nor TLR4 antagonist could further decrease DXM-induced NETosis reduction (**Figure 6B**). While these findings suggested that DXM may modulate *S. aureus*-induced NETs formation through TLR2 and TLR4, further research is required to understand the precise mechanism.

DISCUSSION

NETosis, a recently identified mechanism of pathogen killing, helps in isolating and preventing the spread of invading bacteria, but the persistent formation or insufficient degradation of NETs can also cause injury to the host (8, 20). Since regulation of NETs formation is essential for tissue homeostasis, we aimed to determine the mechanisms and molecules underlying the regulation of this process.

A variety of stimuli promote NETs formation. In our study, NETs formation could be induced in neutrophils by both pharmacologic (PMA) and pathogenic (bacterial) stimuli, a finding that is in agreement with those of previous studies (21, 22). Although several signaling mechanisms responsible for NETs formation have been reported, critical regulatory elements remain unidentified. Since the findings from different studies often vary, it is possible that more than one mechanism exists. In this study, we observed that DXM-inhibited NETs formation induced by bacteria but not that induced by PMA. In addition, it markedly decreased the bactericidal ability of NETs. Thus far, DXM has not been reported to affect NETs formation induced by *S. aureus*. Lapponi reported that treatment of neutrophils with DXM had no effect on NETs formation induced by PMA or TNF-α (19). This is consistent with our observation that DXM was not required for the regulation of PMA-induced NETs formation. Other studies have suggested that NETs formation induced by different stimuli have distinct mechanisms. For example, Riyapa et al. (23) reported that when compared to the neutrophils of diabetic patients, those of normal individuals produced less PMA-induced NETs but the same amount of *S. aureus*-induced NETs. Parker et al. (24) hypothesized that whether NADPH oxidase and myeloperoxidase are required in NETs formation depends on the stimulus. These results prompted us to investigate whether different stimuli indeed have different underlying mechanisms. Our findings strongly suggested that bacteria and PMA regulate NETs formation through different pathways and that DXM may

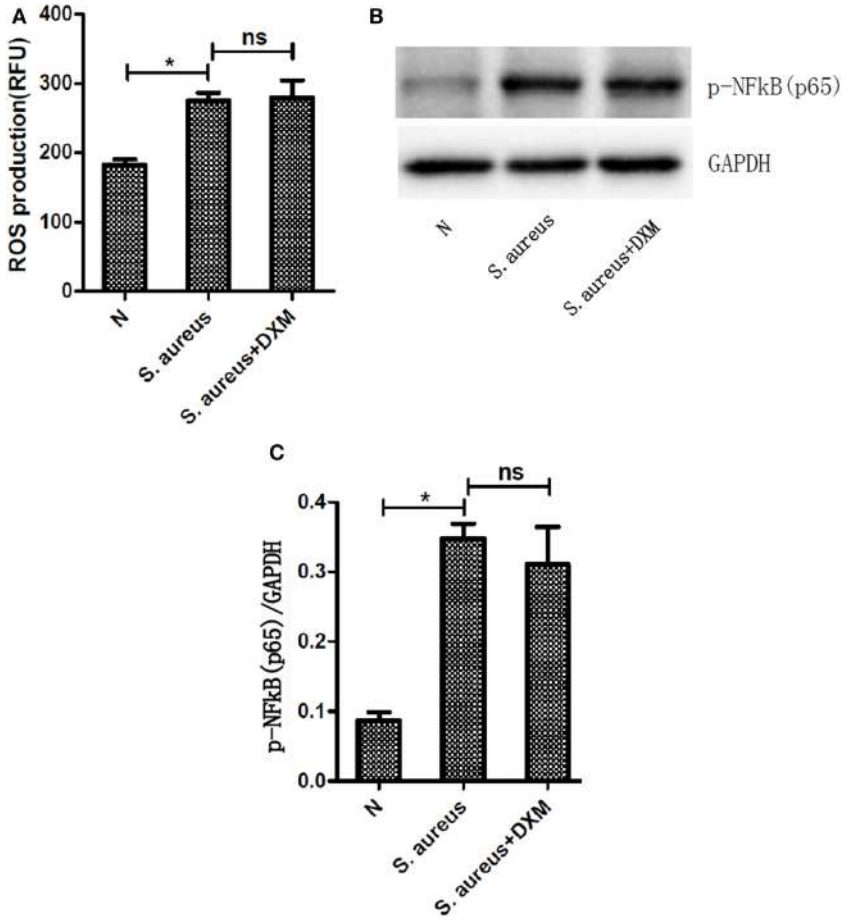

FIGURE 4 | **Activation of reactive oxygen species (ROS) or nuclear factor (NF)-κB was not involved in dexamethasone-regulated NETosis**. Neutrophils were pretreated with or without dexamethasone (DXM) for 2 h and then stimulated with *Staphylococcus aureus* (*S. aureus*) for 1 h. ROS production were determined by dichlorofluorescein diacetate fluorescence and NF-κB activation were determined by Western blot. **(A)** *S. aureus* infection elicited significant neutrophil oxidative burst, but DXM treatment neither increased nor decreased this response. Data represent mean ± SEM of triplicate experiments. **(B)** NF-κB was activated when stimulated with *S. aureus* but not modified by dexamethasone. **(C)** Quantification showed that p-NF-κB (p65) expression was significantly higher when the cells were stimulated with *S. aureus*, but this effect was not modified by dexamethasone. Data represent mean ± SEM of triplicate experiments, $^*p < 0.05$ by Student's *t*-test.

have an effect on NETs formation induced by bacteria but not on that induced by PMA.

Neutrophil extracellular traps formation has been shown to require NADPH oxidase activity as well as NF-κB activation. Our results verified the involvement of NADPH oxidase activity and NF-κB activation in the process of NETs formation. However, in contrast to our expectation, no change in ROS or pNF-κB levels was observed in DXM-treated neutrophils stimulated by *S. aureus*, which indicated that ROS and NF-κB signaling pathways were not involved in DXM-regulated NETs formation. NETosis was previously reported to be of two types: ROS dependent and ROS independent. Our study shows that DXM may modulate ROS-independent NETosis. Interestingly, DXM has been reported to inhibit calcium mobilization, which was shown to increase in LPS-treated cells (25). Therefore, DXM may regulate NETosis by modulating calcium mobilization, which is ROS independent. Moreover, our study showed that the phosphorylation of NF-κB, which has been shown to participate in NETs formation (19), is not involved in DXM-modulated NETosis. It may be because different stimuli were used, with bacteria in ours and PMA in others. Nevertheless, as we only detected the phosphorylation of NF-κB in whole cell, it could not be excluded that there were NF-κB shifting from plasma to nucleus.

The specific detection of microorganisms by innate cells is mediated by PRRs—germline-encoded receptors that recognize microbial structures referred to as pathogen-associated molecular patterns (26). TLRs are essential PRRs that mediate the recognition of microbial structures, such as those of bacteria, as well as the subsequent inflammatory and adaptive responses (27–30). Because neutrophils and TLRs are, respectively, the prototypical cells and receptors involved in innate immune responses, the effect of TLRs on NETosis was investigated. Our findings suggested that TLRs involved in inflammatory response could be key regulatory factors in NETs formation. Our results

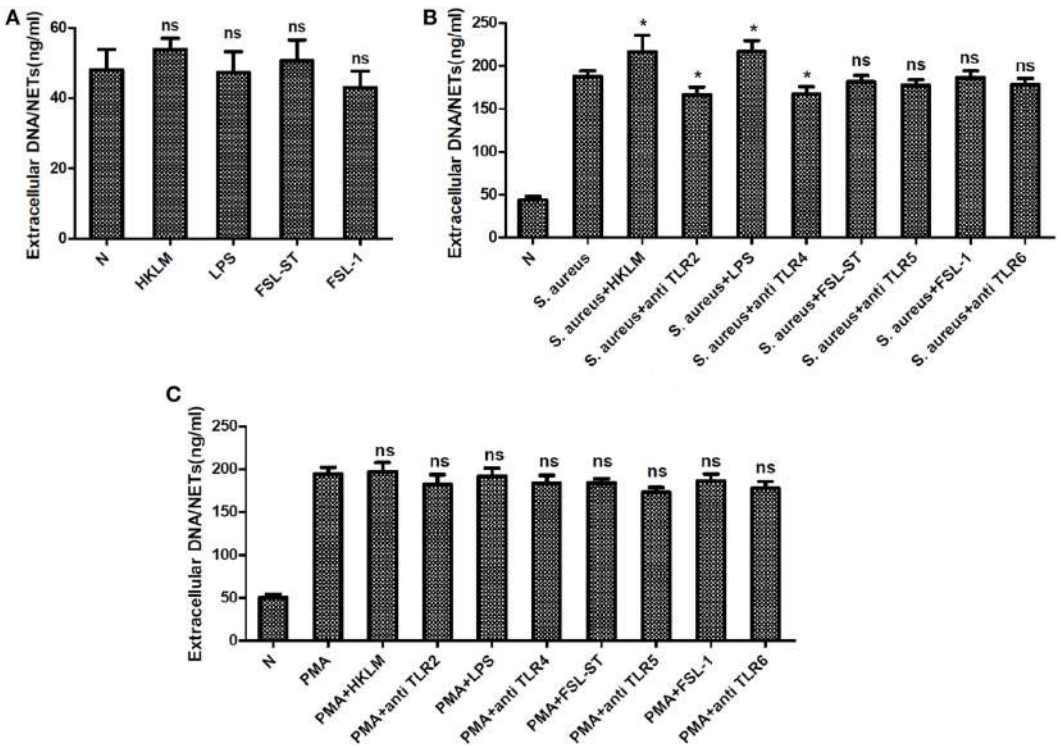

FIGURE 5 | Toll-like receptors (TLRs) were involved in *Staphylococcus aureus* (*S. aureus*)-induced but not phorbol 12-myristate 13-acetate (PMA)-induced neutrophil extracellular traps (NETs) formation. Neutrophils were pretreated with TLRs agonist or antagonist, followed by PMA or *S. aureus* stimulation. NETs formation was quantified by Quant-iT PicoGreen double-stranded deoxyribonucleic acid assay kit. **(A)** None of TLR2 agonist (HKLM), TLR4 agonist (LPS), TLR5 agonist (FSL-ST), and TLR6 (FSL-1) agonist could induce NETs formation. **(B)** Treatment with TLR2 agonist (HKLM) and TLR4 agonists (LPS) significantly enhanced NETosis, and blocking TLR2 and TLR4 with neutralizing antibodies significantly reduced *S. aureus*-induced NETs formation. None of TLR5 agonist (FSL-ST), TLR6 agonist (FSL-1), and TLR5 and TLR6 neutralizing antibodies was involved in *S. aureus*-induced NETs formation. **(C)** TLRs were not involved in PMA-induced NETs formation. The assay was repeated for three times, each case in five wells, error bars represent SEM. Compared to *S. aureus* or PMA stimulation, $^*p < 0.05$, ns = $p > 0.05$ by Student's *t*-test.

showed that *S. aureus*-induced NETosis was markedly inhibited by TLR2 and TLR4 antagonists and enhanced by TLR2 and TLR4 agonists. This strongly supports the role of TLR2 and TLR4 in the biogenesis of NETs, but these effects were not observed in PMA-induced NETosis. Furthermore, neither TLR5 nor TLR6 agonists/antagonists had any effect on bacteria-induced NETosis. As TLR2 is the main receptor for Gram positive, and TLR4 is for Gram-negative bacteria, respectively, it is reasonable that both of them may directly or indirectly participate in the process of NETosis triggered by *S. aureus* through the whole inflammatory network. It is further confirmed by the following results. The addition of TLR2 and TLR4 agonists (HKLM and LPS) rescued DXM-inhibited NETs formation induced by *S. aureus*, but to a lower extent than in the control group stimulated by *S. aureus*. Therefore, we believe that both TLR2 and TLR4 were involved in DXM-modulated NETosis, which is consistent with the observation in other studies that multiple receptors may together regulate NETs formation (31). Besides, we were unable to conclude whether other TLRs that mediated the interaction of neutrophils and other pathogens like viruses could also be involved.

In addition, we aimed to determine the relationship between DXM and TLRs. Both HKLM and LPS rescued DXM-reduced NETs formation. Moreover, neither TLR2 nor TLR4 antagonist could further decrease DXM induced NETosis reduction. This indicated the involvement of TLRs in DXM-reduced NETosis. A previous study showed that DXM down-regulates TLR4 mRNA expression in neutrophils (32), which implies that it may regulate NETosis by modulating TLR expression (33).

Our study has a limitation: we examined neutrophil function only *in vitro*; further *in vivo* studies are needed to characterize the fate of neutrophils. It is also not clear how DXM and TLRs cooperatively modulate NETs formation. Further research is needed to clarify these points.

In conclusion, we have demonstrated that NETs formation can be induced in neutrophils by different stimuli but not by a common mechanism. The mechanism of how DXM modulates bacteria-induced NETs formation was found to be unrelated to oxidant production and phosphorylation of NF-κB. TLR2 and TLR4 are involved in the formation of NETs. Although the specific mechanisms of how DXM regulates

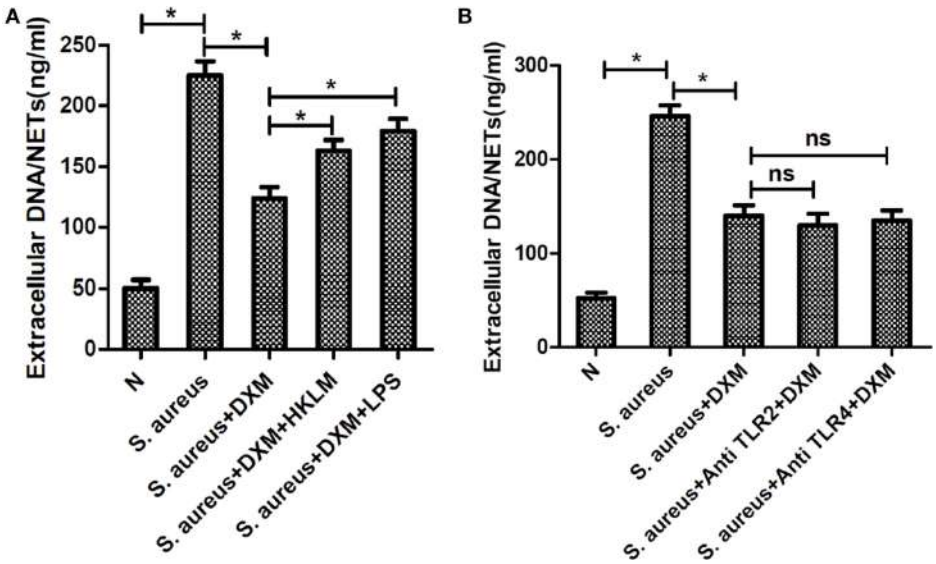

FIGURE 6 | Dexamethasone (DXM) may modulate *Staphylococcus aureus* (*S. aureus*)-induced neutrophil extracellular traps (NETs) formation through toll-like receptor (TLR)2 and TLR4. (A) Neutrophils were pretreated with HKLM (TLR2 agonist) and LPS (TLR4 agonist), followed by DXM treatment and *S. aureus* stimulation. NETs formation was quantified by Quant-iTPicoGreen double-stranded deoxyribonucleic acid (dsDNA) assay kit. Both HKLM and LPS rescued dexamethasone-reduced NETs formation. **(B)** Neutrophils were pretreated with TLR2 and TLR4 antagonist, followed by DXM treatment and *S. aureus* stimulation. NETs formation was quantified by Quant-iTPicoGreen dsDNA assay kit. Neither TLR2 nor TLR4 antagonist could further decrease DXM induced NETosis reduction. The assay was repeated for three times, each case in five wells, error bars represent SEM. $^*p < 0.05$ by Student's *t*-test.

NETs formation are unclear, it is possible that DXM regulates NET formation induced by *S. aureus via* a TLR-dependent mechanism.

AUTHOR CONTRIBUTIONS

TW and YZ wrote the main manuscript text. YZ, TW, FF, and RH performed the experiments. YZ and TW prepared **Figures 1–6**. XJ designed the study and provided advice on the discussion.

ACKNOWLEDGMENTS

This study was funded by National Natural Science Foundation of China (No.81270974; 81500694); Zhejiang Provincial Natural Science Foundation of China (LQ13H120003).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SS and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Immunology

REVIEW
published: 25 January 2017
doi: 10.3389/fimmu.2017.00022

Applications of Genetically Modified Immunobiotics with High Immunoregulatory Capacity for Treatment of Inflammatory Bowel Diseases

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Inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn's disease, are chronic inflammatory diseases characterized by dysregulated immune responses of the gastrointestinal tract. In recent years, the incidence of IBDs has increased in developed nations, but their prophylaxis/treatment is not yet established. Site-directed delivery of molecules showing anti-inflammatory properties using genetically modified (gm)-probiotics shows promise as a new strategy for the prevention and treatment of IBD. Advantages of gm-probiotics include (1) the ability to use bacteria as a delivery vehicle, enabling safe and long-term use by humans, (2) decreased risks of side effects, and (3) reduced costs. The intestinal delivery of anti-inflammatory proteins such as cytokines and enzymes using *Lactococcus lactis* has been shown to regulate host intestinal homeostasis depending on the delivered protein-specific machinery. Additionally, clinical experience using interleukin 10-secreting *Lc. lactis* has been shown to be safe and to facilitate biological containment in IBD therapy. On the other hand, some preclinical studies have demonstrated that gm-strains of immunobiotics (probiotic strains able to beneficially regulate the mucosal immunity) provide beneficial effects on intestinal inflammation as a result of the synergy between the immunoregulatory effects of the bacterium itself and the anti-inflammatory effects of the delivered recombinant proteins. In this review, we discuss the rapid progression in the development of strategies for the prophylaxis and treatment of IBD using gm-probiotics that exhibit immune regulation effects (gm-immunobiotics). In particular, we discuss the type of strains used as delivery agents.

Keywords: probiotics, immunobiotics, IBD, gmLAB, gm-immunobiotics

OPEN ACCESS

Edited by:
Julio Villena,
Reference Centre for Lactobacilli (CERELA-CONICET), Argentina

Reviewed by:
Graciela Liliana Garrote, CIDCA (CONICET-UNLP), Argentina
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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *17 November 2016*
Accepted: *05 January 2017*
Published: *25 January 2017*

Citation:
Shigemori S and Shimosato T (2017) Applications of Genetically Modified Immunobiotics with High Immunoregulatory Capacity for Treatment of Inflammatory Bowel Diseases. Front. Immunol. 8:22. doi: 10.3389/fimmu.2017.00022

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that occurs in the gastrointestinal tract (GIT); IBDs are largely classified as ulcerative colitis (UC) and Crohn's disease (CD). There has been an increase in the number of cases of IBD in recent years, mainly in Western countries (1). IBD causes inflammatory obstruction of the GIT, resulting in symptoms such as

stomach cramps, pain, diarrhea, constipation, and vomiting over an extended period of time. These symptoms cause considerable reduction in quality of life. While IBD is not a direct cause of mortality, the disease can increase the risk of colorectal cancer (2). The precise etiology of IBD has yet to be clarified, but causal factors are thought to include the environment, genetics, and microorganisms (3). The chronic inflammation seen in IBD is characterized by dysregulated immune response of the host as a result of marked changes in the intestinal environment (3). Consequently, favorable regulation of the compromised immune homeostasis is effective in the prognosis and treatment of IBD. Corticosteroids, thiopurines, and anti-tumor necrosis factor (TNF) antibody (Ab), which exhibit immune-regulatory effects, can control IBD to a certain extent, and these treatments are widely used in clinical settings as therapeutic drugs (4). However, there are individual-specific differences in the effectiveness of these drugs, and there are also issues such as the possibility of serious side effects and high costs (4, 5).

There is currently a great deal of interest in the use of probiotics that have been genetically modified (gm) to produce proteins with IBD therapeutic potential as novel drug substitutes. Probiotics, defined as "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host" (6), have been reported to attenuate inflammation in the host GIT through immune system regulation, strengthening of barrier function, and improvement of the changed intestinal microbiota (7). Probiotics comprise primarily lactic acid bacteria (LAB) and bifidobacteria, and also include non-pathogenic *Escherichia coli*. Probiotics have been used in food for a long time, and many of the bacteria included in probiotics fall under the Generally Recognized As Safe assessment designated by the United States Food and Drug Administration and meet the Qualified Presumption of Safety designation of the European Food Safety Authority. Genetic modification technology has undergone considerable advances in recent years, and *Lactococcus* (*Lc.*) *lactis* in particular has been established as an efficient expression system for recombinant proteins (RPs) (8) (**Figure 1A**). Thus, probiotics, which have excellent safety and health advantages, are likely to be very useful as producers of IBD therapeutic proteins and as agents for delivering such proteins to the GIT (**Figure 1B**). gm-Probiotics that produce or secrete various different anti-inflammatory proteins have been constructed in recent years, and their anti-inflammatory effectiveness when administered orally has been verified using

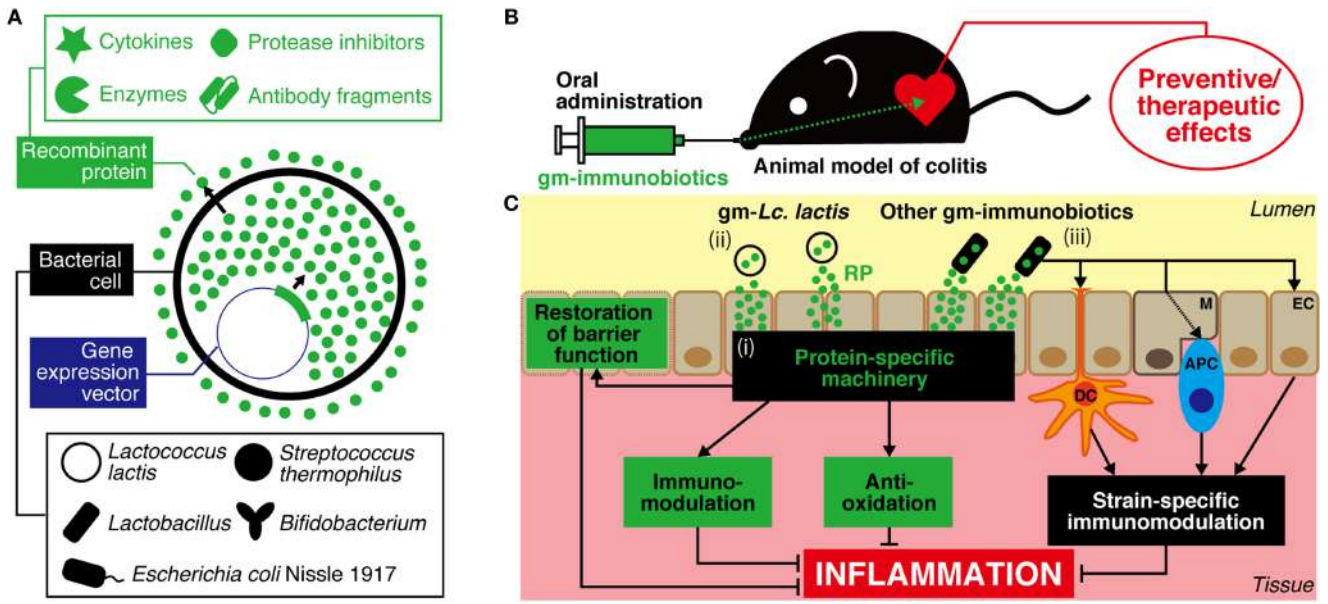

FIGURE 1 | A strategy for prevention and treatment of IBD using genetically modified (gm)-immunobiotics. **(A)** Different bioactive proteins such as cytokines, enzymes, protease inhibitors, and antibody fragments can be produced/secreted by gm-strains. **(B)** After oral administration, viable cells of gm-immunobiotics transit through the gastric environment and reach the intestine. Then, gm-immunobiotics provide preventive/therapeutic effects against experimental colitis in animal as a result of the exertion of anti-inflammatory effects *in situ*. **(C)** General mechanisms of action of gm-immunobiotics on anti-inflammatory effects in the intestine. Physiologically meaningful amounts of recombinant proteins are yielded by gm-immunobiotics *via* secretion or cell lysis, and exert host anti-inflammatory effects through a protein-specific machinery including immunomodulation, anti-oxidation, and restoration of epithelial barrier functions (i). *Lactococcus* (*Lc.*) *lactis* has been most widely used as a safe and effective vector in this strategy (ii). *Lc. lactis* has little or no effect on either the improvement or aggravation of the intestinal inflammation and does not colonize the intestine. Other gm-immunobiotics (including some strains of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus salivarius* subsp. *thermophilus*, and *Escherichia coli* Nissle 1917) provide beneficial effects on intestinal inflammation as a result of the synergy between the immunoregulatory effects of the bacterium itself and the anti-inflammatory effects of the delivered recombinant proteins (iii). Immunobiotics interact with pattern recognition receptors of host epithelial cells and antigen-presenting cells such as dendritic cells and macrophages to exert strain-specific immunomodulatory effects. Some strains of immunobiotics may colonize the intestine. IBD, inflammatory bowel disease; RP, recombinant protein; EC, epithelial cell; M, microfold cell; DC, dendritic cell; APC, antigen-presenting cell.

TABLE 1 | Selected preclinical evidence showing beneficial effects of gm-immunobiotics in treatment of gastrointestinal tract inflammation.

Strains	Recombinant protein	Disease model	Outcome	Efficacy	Potential mechanisms	Reference
Lc. lactis MG1363/ NZ9000	IL-10	mDAC, mTAC, m*IL-10*$^{-/-}$	Reduction in MS, HS, and IM (MPO, Cox-2, SAA) Modulation of P/AICy	CC = WT/VC < Objects	Immunomodulation	(33–37)
Lc. lactis MG1363	IL-27	mTTC, mDAC	Reduction in Mo, MS, and HS Modulation of P/AICy and PTc	CC = Systemic IL-27 = VC < Object MG1363-IL-10 < Object	Immunomodulation	(38)
Lc. lactis NZ9000	Elafin/SLPI	mDAC, mDCC, mTTC, hIEC	Reduction in MS, HS, CT, IIP, and IM (PL, MPO, PICy, PIL)	CC ≤ WT < NZ9000-IL-10/ TGF-β < Objects	Reduction in elastolytic activity	(33, 39)
Lc. lactis NZ9000	HO-1	mDAC	Reduction in MS, HS, and CS Modulation of P/AICy	CC = VC < Object	Immunomodulation	(40)
Lb. casei BL23	Cat/SOD	mDAC, mTAC	Reduction in MS, HS, and LMT Modulation of P/AICy	CC ≤ WT/VC < Objects	Reduction in oxidative stress Immunomodulation	(15, 17, 18)
Lb. casei BLS	α-MSH	mDAC	Reduction in Mo, MS, HS, CS, and IM (MPO, NF-κB) Modulation of P/AICy	CC ≤ WT < Object	Immunomodulation	(23)
S. thermophilus CRL807	Cat/SOD	mTAC	Reduction in Mo, MS, HS, and LMT Modulation of CPIc	CC < WT < Objects	Reduction in oxidative stress Immunomodulation	(13)
B. longum NCC2705	IL-10	mDAC	Reduction in Mo, MS, HS, CS, and IM (MPO, NF-κB) Modulation of PTc and P/AICy	CC < WT/VC < Object	Immunomodulation	(21, 22)
EcN	AvCys	mDAC, pPWD, hIEC	Reduction in MS, HS, CS, IIP, and IM (PIM, PICh, PICy) Increase in Treg, TER	CC ≤ WT < Object	Immunomodulation Improvement of intestinal barrier function	(24)

Lc., Lactococcus; Lb., Lactobacillus; S. thermophilus, Streptococcus salivarius subsp. thermophilus; B., Bifidobacterium; EcN, Escherichia coli Nissle 1917; IL-10, interleukin 10; IL-27, interleukin 27; SLPI, secretory leukocyte protease inhibitor; HO-1, heme oxygenase-1; SOD, superoxide dismutase; Cat, catalase; α-MSH, α-melanocyte-stimulating hormone; AvCys, cystatin from Acanthocheilonema viteae; mDAC, murine dextran sulfate sodium-induced acute colitis; mTAC, murine 2,4,6-trinitrobenzene sulfonic acid-induced acute colitis; mIL-10$^{-/-}$, spontaneous colitis in IL-10-deficient mice; mTTC, murine T-cell transfer-induced enterocolitis; mDCC, murine dextran sulfate sodium-induced chronic colitis; hIEC, human intestinal epithelial cells; pPWD, porcine post-weaning diarrhea; MS, macroscopic symptoms; HS, histological symptoms; IM, mediators of inflammation; MPO, myeloperoxidase activity; Cox-2, cyclooxygenase-2 activity; SAA, serum amyloid A; P/AICy, pro-/anti-inflammatory cytokines; Mo, mortality; PTc, phenotypes of T-cell; CT, colon thickening; IIP, intestinal epithelial permeability; PL, proteolytic activity; PICy, pro-inflammatory cytokines; PIL, pro-inflammatory leukocytes; CS, colon shortening; LMT, liver microbial translocation; NF-κB, nuclear factor-κB; CPIc, cytokine phenotypes of immune cells; PIM, pro-inflammatory macrophages; PICh, pro-inflammatory chemokines; Treg, regulatory T-cell; TER, transendothelial electrical resistance; CC, colitis control; WT, wild-type strain; VC, vector control; MG1363-IL-10, IL-10-secreting Lactococcus lactis MG1363; NZ9000-IL-10/TGF-β, IL-10- or TGF-β-secreting Lactococcus lactis NZ9000.

in vivo experiments in animal models of IBD (9, 10) (**Table 1**; Table S1 in Supplementary Material). In this context, it is important to note that the delivery of IBD therapeutic proteins to the GIT using gm-probiotics is expected (1) to allow the therapeutic protein to act locally, with greater effectiveness and decreased risk of medical error or side effects compared to conventional systemic administration of the molecule by injection, and (2) to be considerably cheaper than refined drugs (10, 11). It is of particular interest that many of the molecules selected as anti-inflammatory proteins target the host immune system. Many studies to date have used *Lc. lactis* as a model strain, but methods using lactobacilli, bifidobacteria, streptococci, and *E. coli* Nissle 1917 (EcN), bacteria that have more beneficial health effects than *Lc. lactis*, as delivery agents have been attempted in recent years (**Figure 1A**). Many of these studies (12–26) employ bacteria that have been termed "immunobiotics," which have been defined as probiotic strains that are able to beneficially regulate mucosal immunity (27, 28). Immunobiotics are recognized by the pattern recognition receptors of epithelial and antigen-presenting cells such as dendritic cells and macrophages, and these immunobiotics are known to beneficially regulate innate and adoptive immune responses (**Figure 1C**); there have been tremendous advances in the clarification of strain-specific immune regulation functions at the cellular and molecular levels (28–32).

In this review, we describe recent developments in preventive and therapeutic strategies for the treatment of IBD using gm-probiotics. In particular, our discussion focuses on gm-probiotics that exhibit immune regulation effects (gm-immunobiotics) and bacterial species that are used as protein delivery agents.

Lactococcus lactis

Lactococcus lactis is a species of LAB used universally in cheese and other fermented dairy products. To date, *Lc. lactis* MG1363 (MG1363) and its derivatives have been widely used to produce RPs and as carriers for delivery to mucous membranes (**Figure 1**). *Lc. lactis* was the first LAB species to have its whole genome sequenced, and there exists a wealth of genetic data on this species (8, 41, 42). In addition, *Lc. lactis* genetic modification is straightforward, and there are a great number of useful gene expression systems for this organism (8). Furthermore, *Lc. lactis* is able to pass through the GIT alive but does not establish itself in the GIT and is easy to control pharmacokinetically (43, 44). It is important to note that *Lc. lactis* itself has little or no effect on either the improvement or aggravation of GIT inflammation in animals and humans and is therefore highly safe for use against IBD (14, 33–35, 38–40, 45–52) (http://ClinicalTrials.gov Identifier: NCT00729872). The research to date into gm-*Lc. lactis* has been compiled into a number of review articles (9–11, 53). In the present review, we will deal with a series of landmark studies that showed the usefulness and practicality of the present strategy, and we will examine the latest findings.

The strategy of reducing intestinal inflammation by using gm-probiotics for delivery of RPs to the GIT was first proposed in 2000 by Steidler et al. (35), who created a MG1363 strain that secreted interleukin (IL)-10 (LL-mIL10). IL-10 is a cytokine that plays a central role in the suppression of inflammation (54), and mutation of the endogenous gene has been shown to be involved in the onset of murine enterocolitis (55, 56) and infantile-onset IBD (57, 58). Steidler et al. showed that daily oral administration of LL-mIL10 resulted in a dramatic reduction of colitis onset and progression in a murine IBD model (35). Notably, the effective amount of IL-10 was 1/10,000th of the amount used in conventional systemic administration. This enhancement may be regarded as the greatest advantage of the present strategy. The reduction in the amount administered has also been demonstrated in the delivery systems of other RPs (38, 49, 50). Next, Steidler et al. constructed LL-Thy12, in which the thymidylate synthase gene (*thyA*) of the *Lc. lactis* genome was replaced by the human IL-10-encoding gene (59). The results of a phase 1 clinical study in CD patients confirmed the safety, biological containment, and significant therapeutic effect of LL-Thy12 (52). However, no statistically significant therapeutic effect was found in the subsequent phase 2a clinical study (http://ClinicalTrials.gov Identifier: NCT00729872). The authors suggested that the lack of therapeutic effect was due to low concentration of IL-10 in the intestine. Nonetheless, bearing in mind that this first clinical study using gm-LAB suggested the safety and usefulness of this delivery system, the results were remarkable.

IL-27 is an anti-inflammatory cytokine belonging to the IL-12 family, a group of molecules that has been shown to attenuate murine experimental colitis by suppressing the development of T helper 17 (Th17) cells (60). In addition, the involvement of low-expressing variants of the IL-27-encoding gene in early-onset IBD has been demonstrated (61). In 2014, Hanson et al. showed that daily oral administration of MG1363 that secretes IL-27 (LL-IL-27) almost completely cured murine T-cell transfer-induced enterocolitis and reduced the associated mortality rate (38). LL-IL-27 treatment caused a reduction in the level of inflammatory cytokines that had increased in the GIT as a result of enterocolitis and a reduction in the number of colitis pathogenic IL-17-producing T-cells. In addition, the results indicated that increased local production of IL-10 by LL-IL-27 in the GIT was effective in providing a therapeutic effect. It is important to note that oral administration of LL-IL-27 demonstrated a notably greater therapeutic effect than systemic administration of IL-27 or oral administration of IL-10-secreting MG1363.

In 2015, a study comparing *Lc. lactis* NZ9000 (NZ9000) that secreted serine protease inhibitors (elafin or secretory leukocyte protease inhibitor) to NZ9000 that secreted the anti-inflammatory cytokines IL-10 or transforming growth factor-β showed that the former significantly attenuated the symptoms of dextran sodium sulfate (DSS)-induced colitis (33). Prior to that study, Motta et al. showed that the expression of elafin was lower in IBD patients than in healthy people, and that this decreased expression correlated with the increased elastolytic activity of the colonic mucosa in IBD patients (39). Also, delivery of elafin to the GIT using a gm-NZ9000 resulted in marked improvement of acute and chronic colitis in murine models (39). Elafin-secreting NZ9000 restored the colonic elastolytic homeostasis that had broken down as a result of colitis, reduced the number of immune cells infiltrating the colon, and repaired the barrier function of the intestinal epithelium (39).

In 2015, we successfully constructed a gm-NZ9000 strain (designated NZ-HO) that secretes biologically active heme oxygenase-1 (HO-1). HO-1 is an enzyme that catalyzes heme catabolism *in vivo*. HO-1 is induced endogenously by stimuli such as inflammation or oxidative stress, and the enzyme exhibits anti-inflammatory and cytoprotective effects mediated by the generation of heme breakdown products (62, 63). We showed that daily oral administration of NZ-HO markedly attenuated the symptoms of DSS colitis (40). Interestingly, NZ-HO increased the production of IL-10, decreased inflammatory cell infiltration, and decreased expression of IL-6 and IL-1α in the colonic tissue of murine colitis models (40). In 2014, Zhang et al. showed that intraperitoneal injection of an HO-1 inducer-induced IL-10-producing regulatory T cells (Treg) (rather than IBD pathogenic Th17) by inhibiting IL-6/IL-6 receptor signaling, thus ameliorating DSS colitis (64). This result suggested that NZ-HO regulates the immune responses of the inflamed colon in a beneficial fashion to ameliorate DSS colitis.

In 2015, Aubry et al. found that preventive oral administration of MG1363 that secreted thymic stromal lymphopoietin caused a transient increase in the number of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells in the mesenteric lymph node and attenuated DSS colitis in mice (45). Quevrain et al. found that MG1363 that secreted an anti-inflammatory protein (MAM) isolated from a strain of *Faecalibacterium prausnitzii*, a species that is deficient in CD patients and alleviated dinitrobenzene sulfonic acid-induced colitis in mice (47). MAM-secreting MG1363 markedly reduced the production of pro-inflammatory cytokines (IL-17A and interferon-γ) in the colonic tissue of colitis mice (47).

IL-6 is an important pathogenic factor in various different inflammatory diseases, including IBD. By regulating the function

and proliferation of T cells, IL-6 exacerbates GIT inflammation in IBD (65). In addition, studies using murine models of colitis and CD patients showed that inhibition of IL-6 signaling using antibodies improved the symptoms (66, 67). However, the cost of Ab drugs is very high. We therefore created a NZ9000 derivative that secretes a single-chain variable fragment Ab against IL-6 (IL6scFv) (68). Importantly, we showed that the recombinant IL6scFv produced by gm-NZ9000 is immunoreactive, as demonstrated by binding to IL-6 (68). Thus, IL6scFv-secreting NZ9000 is an attractive gm-LAB for research and development of a low-cost IBD therapeutic drug that can yield site-directed delivery of anti-IL-6 antibodies.

Lactobacillus

Bacteria of the genus *Lactobacillus*, which are classified as LAB, are the best-known type of probiotics. Several strains belonging to this genus are commensal bacteria that reside within the human GIT. To date, many preclinical studies have indicated that strains belonging to genus *Lactobacillus* regulate GIT inflammation in a favorable fashion through strain-specific, health-beneficial mechanisms (9). In addition, clinical research to date has shown that a probiotic mixture containing four species of *Lactobacillus* (VSL#3) and *Lactobacillus reuteri* ATCC 55730 exhibits benefits in the treatment of active UC (69–72). Bacteria belonging to genus *Lactobacillus* are used predominantly in probiotic formulations that are useful for the prevention and drug therapy of GIT-related diseases selected by the World Gastroenterology Organization (73).

In 2007, Rochat et al. showed that daily oral administration of *Lactobacillus casei* BL23 (BL23) attenuated murine DSS colitis (17). The same year, Foligne et al. demonstrated that BL23 induced an immune reaction with dominance of anti-inflammatory IL-10 over pro-inflammatory IL-12 in human peripheral blood mononuclear cells and reduced the symptoms of murine 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis (74). In 2010, Watterlot et al. orally administered superoxide dismutase (SOD)-producing and SOD-non-producing BL23 to mice and found that the former resulted in marked amelioration of DSS-induced histological damage to the colon, while the latter gave only slight amelioration (18). An excess of reactive oxygen species causes considerable tissue damage, which suggests a link to IBD development, and the use of antioxidative enzymes to eliminate reactive oxidative species is expected to have potential as an IBD treatment strategy (75). Oral delivery of SOD using gm-LAB has actually been shown to reduce colitis in rodents (12, 14). In 2011, LeBlanc et al. orally administered BL23 that produced an antioxidative enzyme (SOD or catalase) to mice, and their results showed that the mortality rate, weight loss, histological colon damage, and liver microbial translocation induced by TNBS administration were markedly reduced (15). However, in the studies performed by Watterlot et al. (18) and LeBlanc et al. (15), wild-type (WT) BL23 had only mild anti-inflammatory properties and did not induce marked IL-10 production in colon tissue, indicating that the amelioration effects on murine colon inflammation are limited. In 2014, Hou et al. showed that oral administration of SOD-producing *Lactobacillus fermentum* I5007 (I5007) improved lipid peroxidation and immune parameters in the colon, thus ameliorating murine TNBS colitis (26). A partial, but significant, improvement effect was also observed with WT-I5007. I5007 was isolated from healthy porcine intestinal mucosa and has been used as a growth stimulator for livestock. The above series of studies proposed a novel IBD preventive strategy combining the two different intestinal inflammation amelioration mechanisms: the immunobiotic effects of lactobacilli and the antioxidative effects of delivered proteins (**Figure 1C**).

In 2008, α-melanocyte-stimulating hormone (α-MSH)-secreting *Lb. casei* BLS (BLS) was created (23). α-MSH is a neuropeptide with immunosuppressant effects that has been reported to exhibit anti-inflammatory effects in animal models of various diseases, including IBD (76). Orally administered gm-BLS shows curative effects for the symptoms of murine DSS colitis (23). This improvement involves decreased secretion of inflammatory cytokines (TNF-α, IL-1β, and IL-6) and increased secretion of immune-regulatory cytokines (IL-4 and IL-10) in *ex vivo* cultures of colonic tissue (23). It is interesting to note that gm-BLS brought about considerable improvement in a number of parameters when compared to the WT strain (23).

Streptococcus salivarius subsp. *thermophilus* (*S. thermophilus*)

Streptococcus thermophilus is a LAB that has traditionally been used as a yogurt starter. Preclinical studies to date have clarified the roles of specific *S. thermophilus* strains as immunobiotics (77–82). For example, Ogita et al. showed that *S. thermophilus* ST28 (ST28) derived from milk regulated IL-17 production in murine splenocytes in Th17-skewed conditions by induction of counteracting interferon-γ (82). Moreover, oral administration of ST28 to mice markedly decreased DSS-induced intestinal lesions, and this treatment markedly decreased IL-17 secretion and the frequency of accumulation of Th17, the numbers of which had increased in the lamina propria as a result of DSS (81). *S. thermophilus* is a component of a probiotic mixture agent (VSL#3) that has been found to be effective for induction and maintenance of remission in UC and prevention and maintenance of remission in pouchitis (73). It is interesting to note that several *S. thermophilus* strains are known to be autolytic, a useful trait for strains used as gm-immunobiotics (83).

In 2014, an immunobiotic strain, *S. thermophilus* CRL807 (CRL807), which exhibits immunosuppressant action *in vitro* and *in vivo*, was selected from a mixed yogurt starter; CRL807's usefulness as a delivery agent for SOD and catalase then was investigated (13). CRL807 significantly increased the ratios of IL-10:inflammatory cytokine (IL-12, IL-17, or interferon-γ) in human peripheral blood mononuclear cells and the digestive tract of healthy mice. Oral administration of antioxidative enzyme-producing gm-CRL807 and WT-CRL807 to mice markedly potentiated the ratio of IL-10-positive:IL-17-positive cells, a ratio that had been reduced by TNBS administration, and provided amelioration of colitis. Notably, administration of either or both SOD-producing and catalase-producing CRL807 improved antioxidative enzyme activity in the colon, demonstrating greater

anti-inflammatory action than WT-CRL807 administration. Experimental long-term (30-day) oral administration of gm-CRL807 and WT-CRL807 in healthy mice showed the safety of CRL807 (84).

Bifidobacterium

The genus *Bifidobacterium* comprises indigenous bacteria that make up the intestinal flora and in particular are present in significant numbers in healthy infants. In IBD patients, on the other hand, it is known that there is a decreased number of *Bifidobacterium* and an increase in pro-inflammatory *E. coli* and *Bacteroides* in the intestinal mucosa (85–89). Preclinical studies to date have shown that various strains of genus *Bifidobacterium* bring about beneficial effects in the prevention and treatment of colitis, mediated by different effects [immunoregulation effects (90, 91), improvement of the barrier function of intestinal epithelium (92, 93), and improvement of the intestinal flora (94, 95)]. It is interesting that *Bifidobacterium longum* subsp. *infantis* 35624 has been shown to selectively drive specialization of FoxP3$^+$ Treg cells and/or induce IL-10 production in animal disease models and in humans (96–99). In addition, clinical studies of patients with UC and other inflammatory diseases showed that, compared to placebo, oral administration of this immunobiotic strain resulted in a marked decrease in the level of plasma C-type protein, an inflammatory biomarker that increases with the disease (100). It has also been shown that the symptoms of UC patients are ameliorated by a single Bifidobacteria strain (101), probiotic mixtures that include Bifidobacteria (69, 71, 72, 102, 103), and symbiotics (probiotic/prebiotic mixtures) in which Bifidobacteria is the main constituent (104–106).

In 2011, an immunobiotic strain, *B. longum* NCC2705 (NCC2705), was engineered to secrete biologically active IL-10, and the strain's curative effects in DSS colitis were investigated (21). Improvement of the symptoms of DSS colitis (aggravation of gross symptoms, colon shortening, histopathological changes accompanying tissue damage, and myeloperoxidase activation) was observed with oral administration of WT-NCC2705 alone. Considerable improvement was found with IL-10-secreting gmNCC2705 when compared to WT-NCC2705 treatment (21). In addition, this study found that WT-NCC2705 and gm-NCC2705 reduced the expression of nuclear factor-κB and pro-inflammatory cytokines in the colon and the peripheral blood, and restored the proportion of CD4$^+$ CD25$^+$ FoxP3$^+$ Treg cells (21). These effects were markedly stronger with gm-NCC2705. In 2015, Zhang et al. showed that the Treg/Th17 balance that had broken down as a result of DSS colitis was fully restored by gm-NCC2705 through the inhibition of two intracellular signaling pathways for Th17 induction (22). In 2016, the intestinal inflammation amelioration action of different strains of *B. longum* that produced human α-MSH was reported (19, 20). In the first of these reports, preventive daily oral administration of α-MSH-secreting *B. longum* HB15 (HB15) markedly reduced histopathological damage, increased myeloperoxidase activity, corrected an inflammatory/anti-inflammatory cytokine imbalance, and induced production of the pro-inflammatory factor nitrogen monoxide, overcoming effects caused by DSS colitis in rats. Administration of WT-HB15 improved all the parameters with the exception of nitrogen monoxide production, but to a considerably lower degree than that seen with the recombinant strain (19). In the second report, α-MSH-secreting *B. longum* HB25 (HB25) was created. Therapeutic daily oral administration of this recombinant strain markedly improved murine DSS colitis. Interestingly, no curative effects were observed from oral administration of the vector control strain (20). The two serial studies above indicated that immunobiotic Bifidobacteria that secrete proteins exhibiting immunomodulatory effects beneficial to IBD amelioration (IL-10 or α-MSH) are capable of stronger prevention/cure of UC-like colitis in mice than are WT strains, with effects presumably mediated through synergistic effects on various functions (**Figure 1C**).

Escherichia coli Nissle 1917

Escherichia coli Nissle 1917 has no pathogenic factors (adhesion molecules, invasiveness, enterotoxin, cytotoxins, etc.). This strain's genetics, physiology, and biological activities as a probiotics were largely characterized some time ago; as an alternative medicine (Mutaflor) for IBD and other GIT-related diseases, EcN currently serves as one of the most useful bacterial strains (104). In randomized controlled trials of UC remission maintenance, oral administration of EcN was as effective as treatment with mesalazine in preventing relapse of the disease (105–107). In studies using IBD model animals, EcN was proven to ameliorate colitis symptoms by regulation of the immune system and intestinal barrier function (108–111). In addition, the utility of this immunobiotic strain as a production platform for vaccines and pharmaceutics and as an intestinal delivery system continues to grow (112). Studies of gm-EcN that produces pathogenic bacteria/virus antigens (113–115) and immunomodulatory molecules such as cytokines and proteins derived from parasites (24, 25) have been reported, and disease preventive/curative effects have been verified in animals.

In 2012, Gardlik et al. developed IL-10-secreting EcN and verified this strain's anti-inflammatory effects using DSS colitis (25). Oral administration of IL10-secreting EcN was shown to improve inflammation parameters (reduced stool consistency, colon shortening, decreased oxidative and carbonyl stress), but these effects were of the same degree as obtained with WT-EcN or IL-10-secreting MG1363. In 2014, EcN that secretes a protease inhibitor protein derived from nematodes (AvCys) was created (24). AvCys' immune-regulatory action is mediated mainly by targeting macrophages, and this inhibitory protein exhibits anti-inflammatory action in murine models of IBD and allergies (116–119). Oral administration of AvCys-secreting EcN (EcN-AvCys) on alternate days attenuated DSS colitis by beneficial regulation of the immune system in the inflamed colon (regulation of the proportion and function of pro-inflammatory macrophages, increase in the proportion of FoxP3$^+$ Treg cells, and decrease in inflammatory cytokines and chemokines). In addition, in experiments using pigs (whose GITs closely resemble those of humans), oral administration of EcN-AvCys on alternate days to post-weaning piglets reduced spontaneous colon inflammation. Interestingly, the results of that study suggested that EcN-AvCys ameliorates

inflammation in this piglet model by improving intestinal barrier function rather than by regulating the intestinal immune system. WT-EcN shows some benefits in ameliorating murine intestinal inflammation, inducing Treg cells, and increasing transepithelial resistance in a culture of a human colonic epithelial cell strain, but the efficacies were significantly milder than those obtained with EcN-AvCys.

CONCLUSION AND FUTURE PERSPECTIVES

Site-directed delivery of proteins that exhibit anti-inflammatory effects using gm-immunobiotics is extremely attractive as an effective preventive/curative strategy for IBD (**Figure 1**). A series of studies using IL-10-secreting *Lc. lactis*, ranging from basic to clinical, established a milestone by indicating the effectiveness and the feasibility of clinical application of this concept. Subsequently, gm-*Lc. lactis* strains that efficiently produce cytokines, enzymes, and protease inhibitors with a range of anti-inflammatory properties have been developed, and anti-inflammatory properties of these strains have been verified using rodent models of IBD (**Table 1**; Table S1 in Supplementary Material). Recent research into intestinal delivery of serine protease inhibitors and IL-27 has shown that these strains provide markedly more beneficial amelioration of murine intestinal inflammation than do strains that deliver IL-10. In addition, the research strongly implies that MG1363 and its derivatives do not have any negative impact on GIT inflammation or health maintenance, regardless of whether the strains are WT or recombinant. It may therefore be concluded that *Lc. lactis* is the bacterium that holds the most promise as a delivery agent for proteins with IBD therapeutic potential. In addition, work has also advanced to verify the potential for application of immunobiotics in this strategy. Interestingly, these studies show marked amelioration of GIT inflammation in animals as a result of the synergy between the immunoregulatory effects of the immunobiotic bacterium itself and the anti-inflammatory effects of the delivered RPs (**Figure 1C**; **Table 1**; Table S1 in Supplementary Material). This observation implies that the strategy of using immunobiotics is an effective means toward the development of IBD therapeutics with greater efficacy. For future work, it would be desirable to carry out comparative investigations of the therapeutic effects on GIT inflammation of different gm-strains that produce the same RP.

Clinical trials that include verification of safety and efficacy will be essential for developing gm-immunobiotics as therapeutic drugs for IBD. To date, there have been no findings that demonstrate any danger in the use of gm-probiotics including gm-immunobiotics. At the same time, there is little evidence to prove the safety of these agents in clinical use, and it remains possible that gm-probiotic organisms may be spread into the environment. Thus, there is some skepticism regarding the use of these agents. However, two clinical studies using IL-10-secreting *Lc. lactis* have demonstrated tremendous breakthroughs (59, 120, 121). In addition, in a recent phase 1b trial, oral administration of AG013 (an oral rinse containing trefoil factor 1-secreting MG1363 as the main component) was shown to be safe and well tolerated in cancer patients while also exhibiting efficacy against oral mucositis (122). Guidelines toward clinical use of gm-*Lc. lactis* have been proposed (123), and the feasibility of the clinical application of gm-*Lc. lactis* is strongly implied. With other probiotics, aspects such as the time for passage through the GIT, establishment in the GIT, health benefits, or the danger of side effects will differ from those of *Lc. lactis*, so safety evaluations will be needed and biological containment strategies will have to be developed. The establishment of effective gm-immunobiotics for prevention and treatment of IBD is near at hand, and it is to be hoped that this strategy will be facilitated by advances in the scientific understanding of gene recombination techniques in the future.

AUTHOR CONTRIBUTIONS

SS and TS conceived, designed, and wrote the manuscript.

FUNDING

The study was funded by a Grant-in-Aid for the Japan Society for the Promotion of Science Fellows (No. 14J06317) to SS and by a grant from Kato Memorial Bioscience Foundation to TS.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00022/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SA and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Microbiology

REVIEW
published: 09 May 2017
doi: 10.3389/fmicb.2017.00800

Use of Wild Type or Recombinant Lactic Acid Bacteria as an Alternative Treatment for Gastrointestinal Inflammatory Diseases: A Focus on Inflammatory Bowel Diseases and Mucositis

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OPEN ACCESS

Edited by:
Julio Villena, Centro de Referencia para Lactobacilos (CONICET), Argentina

Reviewed by:
Maria Guadalupe Vizoso Pinto, National University of Tucumán, Argentina
Cammie Lesser, Massachusetts General Hospital, USA

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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

Received: *03 February 2017*
Accepted: *19 April 2017*
Published: *09 May 2017*

Citation:
Carvalho RDDO, do Carmo FLR, de Oliveira Junior A, Langella P, Chatel J-M, Bermúdez-Humarán LG, Azevedo V and de Azevedo MS (2017) Use of Wild Type or Recombinant Lactic Acid Bacteria as an Alternative Treatment for Gastrointestinal Inflammatory Diseases: A Focus on Inflammatory Bowel Diseases and Mucositis. Front. Microbiol. 8:800. doi: 10.3389/fmicb.2017.00800

The human gastrointestinal tract (GIT) is highly colonized by bacterial communities, which live in a symbiotic relationship with the host in normal conditions. It has been shown that a dysfunctional interaction between the intestinal microbiota and the host immune system, known as dysbiosis, is a very important factor responsible for the development of different inflammatory conditions of the GIT, such as the idiopathic inflammatory bowel diseases (IBD), a complex and multifactorial disorder of the GIT. Dysbiosis has also been implicated in the pathogenesis of other GIT inflammatory diseases such as mucositis usually caused as an adverse effect of chemotherapy. As both diseases have become a great clinical problem, many research groups have been focusing on developing new strategies for the treatment of IBD and mucositis. In this review, we show that lactic acid bacteria (LAB) have been capable in preventing and treating both disorders in animal models, suggesting they may be ready for clinical trials. In addition, we present the most current studies on the use of wild type or genetically engineered LAB strains designed to express anti-inflammatory proteins as a promising strategy in the treatment of IBD and mucositis.

Keywords: inflammatory bowel diseases, mucositis, lactic acid bacteria, *Lactococcus lactis*, genetic engineering

INTRODUCTION

The gastrointestinal tract (GIT) is colonized by a complex community of microorganisms, known as the intestinal microbiota, consisting mainly of bacteria that are classified as indigenous or transient. Symbiotic bacteria, such as short chain fatty acid (SCFA)-producing species from the Lactobacillales order and *Faecalibacterium prausnitzii*, contribute to host metabolism and immune system function while occupying a protected environment rich in nutrients (Hooper and Macpherson, 2010; de Vos and de Vos, 2012; Chang and Lin, 2016). Pathobionts of the GIT, consisting mainly of Proteobacteria such as *Escherichia coli* and *Clostridium difficile*, present a

potential risk to the GIT by disrupting the integrity of tissues if, for instance, they are allowed to grow in number (Lebeer et al., 2010; Vangay et al., 2015).

Therefore, the host contains several biological structures that are essential for controlling bacterial overgrowth and invasion. In this context, the mucous layer protecting the intestinal epithelial cells (IECs) plays an important role by restricting the contact of harmful bacteria with host cells (Johansson et al., 2013; Peterson and Artis, 2014). In addition, specialized IEC, such as Paneth cells, secrete several antimicrobial peptides to eliminate microbes that eventually penetrate into the mucus (Salzman et al., 2007; Carlsson et al., 2013). When pathobionts translocate into the intestinal epithelium, the host immune response is activated to eliminate them by producing pro-inflammatory mediators. However, the overproduction of these compounds represents a risk, as they can inflame the tissue, causing intestinal barrier disruption and mucosal dysfunctions in the host (Hidalgo-Cantabrana et al., 2014; Kashyap et al., 2014). Therefore, to maintain intestinal homeostasis, specialized immunological structures, known as the gut-associated lymphoid tissue (GALT), must be able to specifically recognize and eliminate the pathogenic species while tolerating the commensals (Izcue et al., 2009; Carlsson et al., 2013).

Under normal conditions, GALT generates tolerance to commensals mainly through the action of regulatory T (Treg) cells. When the dynamic balance between Treg and activated effector T cells is broken, homeostasis is compromised and may lead to the development of mucosal inflammation in the gut (Strober et al., 2007). In addition to microbiota composition impairment, known as dysbiosis, other factors can influence the proper functioning of the GIT immune system, including individual genetic susceptibility, diet, use of drugs and environmental stress (Ananthakrishnan, 2015). The intersection of these factors may generate an exaggerated pro-inflammatory reaction against the microbiota that causes inflammatory bowel diseases (IBDs), a group of idiopathic and chronic inflammatory conditions of the GIT, which primarily includes ulcerative colitis (CD) and Crohn's disease (UC) (Vangay et al., 2015; Velasquez-Manoff, 2015). In addition, other factors, such as the use of some medications, can also contribute to the breakdown of this immunological tolerance against commensals. It has been reported that chemotherapeutic agents, such as 5-fluoracil, that are widely used in the treatment of advanced solid tumors, may also lead to the development of another inflammatory condition of the GIT known as mucositis, a disease characterized by painful inflammation and ulceration of the mucosal membranes (Soares et al., 2013; Pedroso et al., 2015).

CD and UC are associated with severe intestinal inflammation, and patients have reported gastrointestinal (GI) symptoms such as abdominal pain, diarrhea, rectal bleeding, and weight loss (Lennard-Jones, 1989; Stepaniuk et al., 2015). IBD represent a global health issue, as its incidence has increased in several countries, while safe and efficient therapies are still in development (Molodecky et al., 2012; Ananthakrishnan, 2015). Mucositis induced by 5-FU is of great clinical significance as well, as it might result in cancer therapy being adjusted, affecting a patient's chances of survival (de Vasconcelos Generoso et al., 2015; Antunes et al., 2016). Thus, the scientific community has sought novel therapeutic alternatives to fight both IBD and mucositis. As dysbiosis plays a key role in the pathogenesis of both diseases, the modulation of the patient microbiota via the administration of probiotic bacteria has been proposed.

USE OF PROBIOTIC LACTIC ACID BACTERIA IN THE TREATMENT OF GASTROINTESTINAL INFLAMMATION

Over a century ago, Elie Metchnikoff was the first to propose the rationale for using host-friendly bacteria found in yogurt to manipulate the intestinal microbiome. He also predicted the existence of bacterial translocation, from the intestinal lumen to inner layers of the mucosa and also to systemic organs, and described theories associating the microbiota with intestinal inflammation and other diseases (Mackowiak, 2013). Currently, several research groups have confirmed his hypothesis, demonstrating that the administration of certain bacterial species in several animal models actually provides health benefits to alleviate inflammation, including the containment of inflammatory mediators, stimulation of the immune system and microbiota restoration by competitive exclusion of potentially pathogenic species (Ljungh and Wadström, 2006; Luerce et al., 2014; Quinto et al., 2014; Santos Rocha et al., 2014; Thomas, 2016). These microorganisms are considered to be probiotics, a term defined by the World Health Organization (WHO) as "live microorganisms administered in adequate amounts that confer a beneficial health effect on the host" (FAO/WHO, 2002).

Probiotics are live bacteria and yeasts; however, the majority of strains are gram-positive bacteria belonging to the *Lactobacillus*, *Bifidobacterium*, *Streptococcus,* and *Lactococcus* genera. These genera are included in a diverse group of microorganisms entitled lactic acid bacteria (LAB), as they are able to convert sugars into lactic acid (Holzapfel et al., 1998; Carr et al., 2002). With regards to Gram-negative bacteria, some strains of *E. coli* are also considered to promote health, for instance, *E. coli* Nissle 1917 (EcN1917) was originally isolated from the feces of a soldier during the First World War who did not develop infectious diarrhea during an outbreak of contagious Shigella (Westendorf et al., 2005; Henker et al., 2007).

Although Metchnikoff introduced the concept of probiotics in 1907, some of these microorganisms have been used for centuries to prepare yogurt, sourdough bread, sauerkraut, cucumber pickles and olives, as they are able to produce lactic acid, as previously mentioned (Mackowiak, 2013; Vikhanski, 2016). In the latter half of the 20th century, probiotics have gained visibility as there has been increasing interest in applying them to other areas, such as the pharmaceutical industry. Thus, the selection of new probiotic strains, the development of new food products based on probiotics and freeze-dried probiotic pharmaceutical formulations has increased in importance. There are many studies being conducted that focus on the development of probiotic-based pharmaceutical formulations that can be administered to either the gastrointestinal, nasal, or vaginal

mucosa, as well as to the skin of patients (Guglielmetti et al., 2010; Iannitti and Palmieri, 2010; Vicariotto et al., 2012).

The Lactic Acid Bacteria Group

The LAB group includes a heterogeneous group of ubiquitous microorganisms that obtain energy through the conversion of sugars into lactic acid. Morphologically, LAB bacteria can resemble cocci, rods, or bacilli. They are gram-positive microorganisms with a low genomic GC content (54%) and are facultative anaerobes that are non-spore-forming, immotile and do not produce catalase (Stiles and Holzapfel, 1997; Carr et al., 2002). Species of this group can be naturally found in different environments that are rich in nutrients, such as decomposing vegetables and fruits, and even in the oral, urogenital and intestinal tracts of mammals and other animals. They can also be found in several kinds of dairy foods, as some strains are used to produce them (Holzapfel et al., 1998; Liu et al., 2014). LAB species found in the human GIT can be autochthonous as indigenous GI microflora, especially those belonging to the *Lactobacillus* and *Streptococcus* genera, or allochthonous as transients of the GIT, such as *Lactococcus* sp. and some strains of *Lactobacillus* used to produce yogurts. Some species, especially those belonging to the *Streptococcus* genera are pathogenic; however, the vast majority of LAB strains have a positive impact on human health and are generally regarded as safe (GRAS) by the United States Department of Agriculture (USDA) (Felis and Dellaglio, 2007).

After the pioneering work of Elie Metchnikoff, who first suggested that the ingestion of dairy foods produced by LAB fermentation could prevent intestinal infections and promote both health and human longevity, the scientific community is continuously exploring in more detail the positive effects promoted by these bacteria (Johnson and Klaenhammer, 2014; Vikhanski, 2016). Among all LAB species described that exert probiotic effects, *Lactobacillus* spp., *Streptococcus* spp., and *Lactococcus* spp. stand out for use in therapeutic applications for both the treatment and prevention of various intestinal disorders (Majamaa and Isolauri, 1997; Ouwehand et al., 2002; Prescott and Björkstén, 2007; Ohland and MacNaughton, 2010; Luerce et al., 2014; Santos Rocha et al., 2014). This topic has been widely studied, and certain immunological aspects of LAB anti-inflammatory properties have been described.

Effects of Probiotic Lactic Acid Bacteria in Animal Models of Gastrointestinal Inflammation

Lactic acid bacteria probiotic strains can alleviate intestinal inflammation through several mechanisms (**Figure 1**). Accumulating evidence has revealed that probiotic LAB are able to protect the host against potentially pathogenic species that inhabit the GIT of animals, including humans. It seems that lactobacilli strains, such as *L. acidophilus* LA1, can prevent the colonization of the intestine by pathogenic bacteria, such as *Staphylococcus aureus, Salmonella typhimurium*, and *Pseudomonas aeruginosa*, by competitive exclusion (Bernet-Camard et al., 1997; Adolfsson et al., 2004). Apparently, these LAB compete for nutrients and adhesion sites in the intestinal epithelium with these potentially pathogenic bacteria that transit in the GIT and are consequently eliminated. The secretion of lactic acid and bacteriocins (natural antibiotics) by probiotic species has also been implicated in the mechanism of the elimination of pathogens (Ogawa et al., 2001; Moal et al., 2007).

Another manner by which LAB strains may protect the host from pathogen invasion is by boosting the intestinal epithelial barrier. Some LAB microbe-associated molecular pattern (MAMPs) are capable of interacting with epithelial pattern recognition receptors, mainly the Toll-like receptor-2 (TLR2), TLR6 and nod-like receptors (Ren et al., 2016). This activation induces several protective mechanisms that restore tissue damage, such as modulation of the stability of tight junctions (Lebeer et al., 2010; Ohland and MacNaughton, 2010; Villena and Kitazawa, 2014; Bajaj et al., 2015). Species such as *B. infantis*, *L. plantarum*, and *L. casei* have been shown to increase the expression of proteins involved in tight junction barrier function, such as occludins and zonula occludens-1 (ZO-1) (Ewaschuk et al., 2008; Anderson et al., 2010; Eun et al., 2011).

Some *Lactobacillus* strains are capable of increasing the production of other proteins involved in the maintenance of epithelial barrier homeostasis, such as mucin-2 (MUC2), the most abundant glycoprotein in mucus. *In vitro* studies showed that increased MUC2 expression in intestinal epithelial Caco-2 cells blocked the adhesion of pathogenic *E. coli* (Mattar et al., 2002; Mack et al., 2003). Furthermore, an *in vivo* study demonstrated that mice treated with a VSL#3 probiotic-mixture consisting of *S. thermophilus*, four strains of lactobacilli (*L. delbrueckii*, *L. casei*, *L. acidophilus*, and *L. plantarum*) and three species of *Bifidobacterium* (*B. longum*, *B. infantis*, and *B. breve*) for 7 days exhibited an approximate 60-fold increase in the production of MUC2 in treated animals (Gaudier et al., 2005).

Other studies have suggested that some LAB strains are able to induce the secretion of defensins by enterocytes, which are related to the biological control of potentially pathogenic species in the lumen. Administration of certain species of lactobacilli or the VSL#3 probiotic-mixture in mice resulted in an increase in the production of β-defensin-2, which has microbicidal activity against important opportunistic pathogens, such as *P. aeruginosa*, *E. coli*, and *Candida albicans* (Harder et al., 2004; Schlee et al., 2008).

The stimulation of the host immune system and the suppression of pro-inflammatory responses are well-established probiotic effects. One of the major mechanisms of these processes is the stimulation of immunological tolerance to GIT microbiota through an increase in IL-10 secretion and a significant reduction in IFNγ and IL-12 expression. This probiotic effect is caused due to the interaction of "good" bacteria with intestinal dendritic cells that drives the development of T regulatory cells and IgA-producing B cells (Fedorak et al., 2000; Ng et al., 2009). Administration of *B. lactis*, *B. bifidum*, and *B. infantis* in mice previously infected with rotavirus or enterohemorrhagic

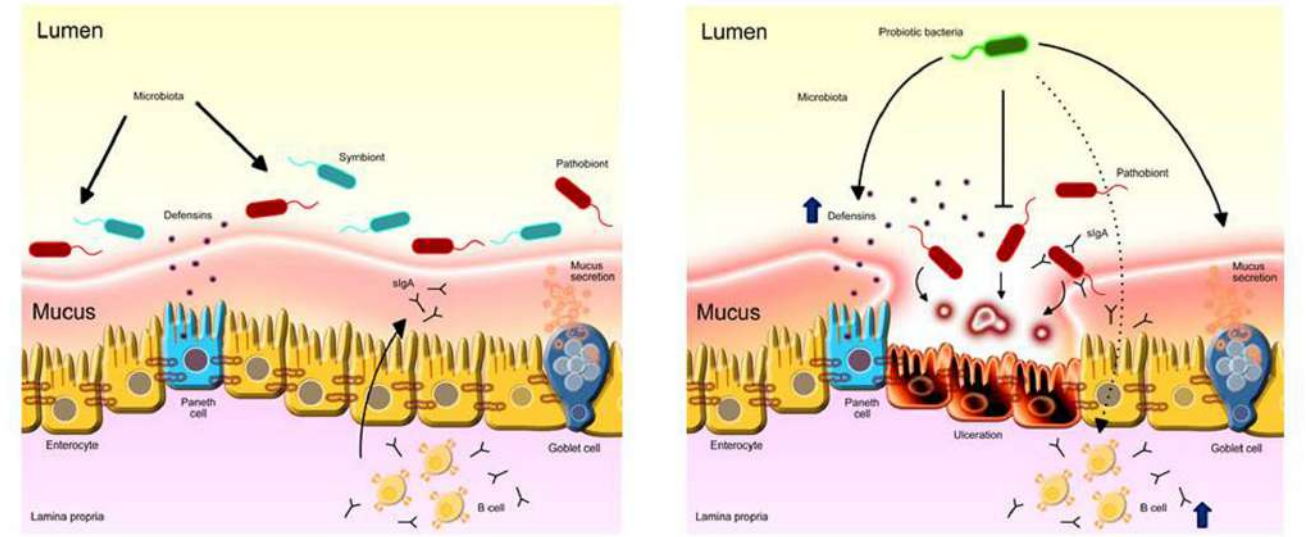

FIGURE 1 | Probiotic LAB anti-inflammatory mechanisms on the intestinal mucosa. (A) Intestinal homeostasis provided by the healthy microbiota role in stimulating ephitelial barrier components such as mucus, Paneth cells activity and eliciting protective immune responses such as IgA. **(B)** The overgrowth of pro-inflammatory mucin-degrading pathobionts induces inflammation in the mucosa. Administration of probiotics prevents inflammatory responses by inhibiting the growth of pathogens directly; increases mucus secretion by goblet cells and the secretion of defensins by Paneth cells; fortificates tight junction stability; stimulates mucosal immunity by inducing IgA production by B cells to the intestinal lumen, limiting harmful microbe adherence and colonization.

E. coli has been shown to increase the titers of specific IgA against the rotavirus (Shu and Gill, 2001; Qiao et al., 2002). For instance, Santos Rocha et al. (2014) showed that the probiotic effect of *L. delbrueckii* strain CNRZ327 was related to an expansion of Treg cells and an increase of total IgA in Dextran sulfate sodium (DSS)-induced colitis in mice. This effect was shown to be enough to prevent inflammation in mice (Santos Rocha et al., 2014). Recently, it was reported that a *Lactococcus lactis* ssp. *lactis* NCDO2118 strain prevented DSS-induced colitis in mice and the protective effect was related to increased IL-10 levels in the colon and the induction of Treg cells in the mesenteric lymph nodes (Luerce et al., 2014). In another study using a similar colitis model, *L. lactis* FC ssp. *cremoris* demonstrated a protective role in treating inflammation in mice, by preventing the NF-kB activation and in decreasing IL-8 expression in epithelial cells (Nishitani et al., 2009).

Lactic acid bacteria have also been studied and has generated promising results, both *in vitro* and *in vivo*, in other models of intestinal inflammation, such as preclinical mucositis models (Tooley et al., 2006; Bowen et al., 2007; Smith et al., 2008; Southcott et al., 2008; Whitford et al., 2009; Tooley et al., 2011; Prisciandaro et al., 2012). *In vitro*, it was observed that IECs previously treated with 5-FU presented reduced levels of cytotoxicity and apoptosis through the inhibition of caspase-3 and caspase-7 when co-cultured with *L. rhamnosus* (Prisciandaro et al., 2012). *In vivo*, *L. fermentum* BR11 administered to mice injected with 5-FU exhibited reduced levels of intestinal inflammation and myeloperoxidase enzyme activity, a marker of eosinophilic inflammation (Smith et al., 2008). In another study, VSL#3 was used in the treatment of mucositis that was induced in rats through the injection of a chemotherapy drug known as irinotecan. The administration of probiotics has been shown to prevent weight loss and reduce diarrhea in these rats. These findings were associated with significant improvement in the integrity of crypts in the jejunum and a reduction in apoptosis levels in both the small and large intestines of irinotecan-treated rats (Bowen et al., 2007). Whitford et al. (2009) compared the efficiency of live *S. thermophilus* TH-4 strain (TH-4), dead TH-4 and TH-4 culture supernatants in rats treated with 5-FU. They showed that live TH-4 significantly reduced disease severity scores as well as crypt fission indices, which is an indicator of longitudinal intestinal growth and stem cell proliferation, suggesting that this strain may be useful for treating diseases characterized by increased crypt fission, such as colorectal carcinoma. However, Tooley et al. (2011) ascertained the effects of live TH-4 on small intestinal damage generated by the injection of methotrexate (MTX), a chemotherapy drug that induces mucositis and tumor progression in tumor-bearing rats. This study verified that although TH-4 did not protect animals from chemotherapy-induced mucositis, the progression of mammary adenocarcinoma was unaffected (Tooley et al., 2011).

The efficacy of cow's milk yogurt containing *L. johnsonii* and sheep's milk yogurt containing *L. bulgaricus* and *S. thermophilus* was assessed in an MTX-induced model of mucositis in rats. It was shown that both types of yogurt reduced intestinal permeability, revealing them to be useful in restoring intestinal barrier function (Southcott et al., 2008).

THE USE OF RECOMBINANT LACTIC ACID BACTERIA FOR THE TREATMENT OF GIT INFLAMMATORY DISEASES

As probiotics have been shown to be capable of acting on many diverse biological processes within the host, they have been experimented with as an alternative therapy against GIT inflammatory disorders. To enhance probiotic properties, research is focusing on the development of genetically modified bacterial strains expressing heterologous proteins of medical interest, such as anti-inflammatory molecules. Recently, the use of recombinant LAB strains with natural probiotic activities have shown promising results in pre-clinical studies as an alternative therapy to treat cancer, obesity, and especially GI tract inflammation (Bermúdez-Humarán et al., 2007; Cortes-Perez et al., 2007; Bahey-El-Din et al., 2010; Bermúdez-Humarán et al., 2013; Wang et al., 2016).

Since 1960, molecular biologists have developed several sophisticated techniques to identify, isolate, and manipulate the genetic components of the bacterial cell. This knowledge enabled the construction of different LAB recombinant strains with increased anti-inflammatory properties. Well-reported examples include the construction of *L. casei*, *L. plantarum*, *S. thermophilus*, and *L. lactis* strains capable of expressing anti-inflammatory molecules, thus increasing the benefitial effects of the above-mentioned strains (**Table 1**) (Han et al., 2006; LeBlanc et al., 2011; Del Carmen et al., 2014). Thus, several studies have focused on the use of recombinant anti-inflammatory LAB as an interesting alternative treatment for GIT inflammatory diseases (de Moreno de LeBlanc et al., 2015; Wang et al., 2016).

Lactic acid bacteria have been proven to successfully express proteins of interest in different cell compartments (in the cytoplasm, anchored to the cell membrane or secreted into the extracellular medium) (Miyoshi et al., 2010; Pontes et al., 2011; Pereira et al., 2014). It has been shown that LAB can be administered orally, making the need for clean needles and syringes unnecessary. In fact, the WHO recommends that immunization or treatment be orally administered due

TABLE 1 | Heterolgous proteins with anti-inflammatory properties produced in different strains of lactic acid bacteria.

Organism	Heterologous protein	Expression system	Inflammatory condition	Anti-inflammatory effects	Reference
L. casei BL23	Superoxide dismutase A from *L. lactis* MG1363	SodA native promoter from *L. lactis* MG1363	Mouse model of DSS-induced colitis	Protection against ROS	Watterlot et al., 2010
L. fermentum I5007	Superoxide dismutase from *B. subtilis*	Constitutive promoter from *L. casei* ATCC334	Mouse model of TNBS-induced colitis	Inhibition of NF-κB pathway	Hou et al., 2014
S. thermophilus CRL807	Superoxide dismutase A from *L. lactis* MG1363	SodA native promoter from *L. lactis* MG1363	Mouse model of TNBS-induced colitis	Reduction of intestinal permeability and histological damage	Del Carmen et al., 2014
L. lactis NCDO2118	Human 15-lipoxygenase-1	XIES	Mouse model of DSS-induced colitis	Decreased IFN-γ and IL-4. Increased IL-10	Carvalho et al., 2016
L. lactis NZ3900	Mouse cathelicidin	NICE	Mouse model of DSS-induced colitis	Reduced tissue damage and MPO activity	Wong et al., 2012
L. lactis NZ9000	Human elafin	NICE	Mouse model of DSS-induced colitis	Inhibition of elastase and proteinase-3	Bermúdez-Humarán et al., 2015
L. lactis NZ9000	Mouse leukocyte protease inhibitor	NICE	Mouse model of DSS-induced colitis	Reduced tissue damage and MPO activity	Bermúdez-Humarán et al., 2015
L. lactis NZ9000	Mouse TGF-β	NICE	Mouse model of DSS-induced colitis	Reduced granulocytes infiltration	Bermúdez-Humarán et al., 2015
L. casei CECT 5276	Human IL-10 combined with 5-aminosalicylic acid (5-ASA)	Lactose inducible promoter	Mouse model of DSS-induced colitis	Inhibition of NF-κB pathway	Qiu et al., 2013
L. lactis MG1363	Mouse IL-10	TREX1	Mouse model of DSS-induced colitis and IL-10 knockout mice	Reduced tissue damage	Steidler et al., 2000
L. lactis MG1363	Mouse IL-10	SICE	Mouse model of DNBS-induced colitis	Reduced tissue damage	Benbouziane et al., 2013
L. lactis AG013	Human IL-10	ThyA native promoter from *L. lactis*	Clinical trial with Crohn's disease patients	No significant improvement comparing to placebo	Steidler et al., 2003
L. lactis NZ9000	Human pancreatitis-associated protein (Reg3A)	NICE	Mouse model of 5-fluoracil – induced intestinal mucositis	Villous architeture preservation and improved Paneth cells activity	Carvalho et al., 2017
L. lactis AG013	Human trefoil factor I	ThyA native promoter from *L. lactis*	Hamsters model of radiation-induced oral mucositis	Reduced clinical scores of oral mucosits	Rottiers et al., 2009
L. lactis AG013	Human trefoil factor I	ThyA native promoter from *L. lactis*	Clinical trial with oral mucositis patients	Reduced the severity and course of radiation-induced oral mucositis	Limaye et al., 2013

to economic, logistical and security reasons. Furthermore, this route offers important advantages over systemic administration, such as reducing side effects, as the molecules are administered locally and have the ability to stimulate the GALT immune responses (Levine and Dougan, 1998; Neutra and Kozlowski, 2006; Bermúdez-Humarán et al., 2011).

The majority of studies in the literature describe the genetic engineering of *L. lactis* because it is the best-characterized member of the LAB group, both physiologically and genetically, and a large number of genetic tools are available for its genetic manipulation. Additional features that make *L. lactis* one of the most extensively studied bacteria are related to its economic importance in cheese production, as it is easy to grow and manipulate and was the first LAB to have its genome completely sequenced (de Vos, 1999; Bolotin et al., 2001; Felis and Dellaglio, 2007; Wells and Mercenier, 2008; Bermúdez-Humarán et al., 2011). In addition, it does not produce endotoxins such as lipopolysaccharide (LPS) and secretes few proteins, facilitating the purification of heterologous proteins. In fact, only the unknown secreted protein of 45 kDa (Usp45) is detectable after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue (van Asseldonk et al., 1990; Bahey-El-Din et al., 2010).

Lactococcus lactis, the Model Lactic Acid Bacteria for the Expression of Anti-inflammatory Molecules

Properties of *L. lactis*

Lactococcus lactis is a mesophilic, facultative heterofermentative bacterium with an optimum growth temperature of approximately 30°C that is important in dairy industry, especially for cheese production. There are two reported subspecies (ssp.) of *L. lactis*, ssp. *lactis* and ssp. *cremoris*. Both can be found naturally in plants, especially grass. As they are used in the food industry for milk fermentation, both species can also be found in dairy products, such as cheeses, yogurts, and some breads and wines (Carr et al., 2002). *L. lactis* subsp. *cremoris* MG1363 is the most commonly used strain for cloning and protein expression, as it has no plasmids and does not produce any extracellular proteases. In addition, this strain was cataloged by the FDA and the European Food Safety Authority (EFSA) as a safe microorganism (GRAS), non-invasive and non-pathogenic, reinforcing its use as a factory for the production of anti-inflammatory molecules. Although it is considered GRAS, *L. lactis* spp. *lactis* was reported to cause an infection in two individuals who had been diagnosed with cardiac abnormalities. Afterward, they were treated with antibiotics, and the infection was cleared. Both patients did not develop any further infection by *L. lactis* (Mercenier, 1999; Bermúdez-Humarán et al., 2011). As *L. lactis* does not colonize the human GIT, most studies have focused on the beneficial effects of LAB strains in the *Lactobacillus* genus, which is autochthonous. However, recent studies have demonstrated that some allochthonous lactococci strains have anti-inflammatory properties. Ballal et al. (2015) found that *L. lactis* I-1631 prevents colitis in T-bet-/- Rag2-/- mice. Two additional studies have shown that NCDO2118 sub. *lactis* or FC sub. *cremoris* are anti-inflammatory when inoculated in inflamed mice receiving the chemical agent DSS (Nishitani et al., 2009; Luerce et al., 2014). Moreover, *L. lactis*, was used for the treatment of eosinophilic esophagitis in mice. It was demonstrated that the administration of NCC2287 in mice decreased esophageal eosinophilia, which was elicited by epicutaneous sensitization with protein extract from the fungi *Aspergillus fumigatus*, highlighting the beneficial effects of *L. lactis* in another severe inflammatory disease (Holvoet et al., 2016).

As mentioned previously, there are several expression systems available for heterologous protein production in *L. lactis* (Miyoshi et al., 2010). This has allowed the cloning and expression of different heterologous anti-inflammatory proteins by the use of both cloning and expression vectors designed for *L. lactis* (**Table 1**) (Langella and Le Loir, 1999; Le Loir et al., 2005; Bermúdez-Humarán et al., 2011).

Heterologous Protein Expression Systems in *L. lactis*

The first expression systems for use in *Lactococcus lactis* were based on the classic bacterial lactose operon. This operon is activated when the lac promoter is induced in the presence of lactose, while the transcriptional repressor gene (*lacR*) is suppressed in the same condition. Therefore, lactococci strains harboring a plasmid carrying this operon fused to a target gene allow recombinant proteins to be expressed in a tightly controlled fashion (van Rooijen et al., 1992). Wells et al. (1993) improved this system by integrating it with a strong phage promoter that allowed for high levels of heterogous protein production. It consisted of three plasmids containing the lac operon elements and two elements from the T7 bacteriophage found in *E. coli*. In this system, the presence of lactose induces the lac promoter in the first plasmid, promoting expression of the T7 RNA polymerase. Afterward, the T7 RNA polymerase activates expression of the gene of interest controlled by the T7 promoter in the second plasmid. The third plasmid coded for the functional lac operon, allowing the cell to be capable of metabolizing soluble lactose in an artificial medium. This system and other complex systems based on phage promoters have allowed for the strict control of gene expression, although they require many antibiotic resistance markers, making them unsuitable for use in the food and pharmaceutical industry (Wells et al., 1993; Nauta et al., 1996; O'Sullivan, 2001).

In this context, several studies have been carried out to develop safer and more simple vectors. One of the most powerful expression systems already developed for use in the food industry is based on genes involved in the biosynthesis and regulation of the antimicrobial nisin, a peptide naturally secreted by several strains of *Lactococcus lactis*. In brief, the Nisin-Controlled Gene Expression system (NICE) is based on the expression of three genes involved in the production and regulation of the the peptide nisin, which is naturally secreted by various *L. lactis* strains, in a genetically engineered *L. lactis* strain. The *nisR* and *nisK genes* encode a two-component regulatory system (NisRK), which controls the expression of the nisin

operon through the activation of signal transduction pathways (Kuipers et al., 1993). The strain used in this system is a genetically modified version of a *L. lactis* MG1363 strain, *L. lactis* NZ9000, in which both *nisR* and *nisK* regulatory genes were inserted into its chromosome. The expression vector contains the nisin promoter P*nisA*, followed by multiple cloning sites (MCSs) for the insertion of heterologous genes coding for anti-inflammatory molecules or antigens (Kuipers et al., 1993; Mierau and Kleerebezem, 2005). Because NICE system expression vectors exist in different versions, heterologous proteins can be expressed in different cellular compartments. In addition to the cytoplasm, recombinant protein can be anchored to the bacterial cell wall by means of a cell wall anchor (CWA) peptide, composed of 30 amino acids located in the carboxy-terminal portion (C-terminus) of the protein. CWA is recognized by the cell anchoring machinery and is usually covalently attached to the peptidoglycan from the cell membrane. Furthermore, recombinant proteins may be coupled with a short (5–30 amino acid long) peptide present at the N-terminus region of the heterologous protein, allowing its translocation across the cell membrane and secretion to the extracellular medium (Le Loir et al., 1994; Piard et al., 1997).

The NICE system has been successfully used to express and address a variety of heterologous proteins of medical and biotechnological interest, and according to some authors, it is considered as one of the best genetic tools already developed for gene cloning and expression in *L. lactis* (Nouaille et al., 2003; Le Loir et al., 2005).

Miyoshi et al. (2004) developed the xylose-inducible expression system (XIES) based on the xylose permease gene promoter (PxylT) from *Lactococcus lactis* NCDO2118. In the presence of glucose, fructose and/or mannose, PxylT was shown to be repressed; otherwise, PxylT is transcriptionally activated by xylose in *Lactococcus lactis* (Miyoshi et al., 2004). Therefore, this system could be successively turned on by adding xylose and turned off by washing the cells and growing them on glucose. The system combines the use of PxylT, the ribosome-binding site (RBS) and the signal peptide (SP) of the lactococcal secreted protein Usp45 and the *Staphylococcus aureus* nuclease gene (*nuc*) as the reporter (Shortle, 1983; Le Loir et al., 1994). This system was successfully used for the production of highlevels of Nuc, which was tested for correct protein targeting in the *Lactococcus lactis* subsp. *lactis* strain NCDO2118. These systems are considered less expensive and safer for laboratory use compared to many available expression methods (de Azevedo et al., 2015).

Most heterologous protein expression systems used in *L. lactis* are based on inducible promoters, which allows for the controlled expression of the protein of interest. In this context, they prevent protein aggregation and degradation within the bacterial cytoplasm. However, the majority of the expression vectors present inherent safety drawbacks due to the necessity to add chemical compounds into the bacterial culture to induce heterologous protein expression prior to *in vivo* administration. Other food grade expression systems that do not require the pre-induction of the cultures to allow the expression of a given recombinant protein have been reported (Derre et al., 1999; Ruiz et al., 2012; Benbouziane et al., 2013). Benbouziane et al. (2013) developed the stress-inducible controlled expression system (SICE), based on the use of the heat shock protein groESL operon promoter (pGroESL) from *L. lactis*, to deliver proteins of health interest *in situ*. Heat-shock proteins play an essential role under different stress conditions such as heat-shock, low pH, UV-irradiation, and salt stress. Indeed, upon administration into the host, recombinant bacteria should find very different conditions from culture conditions and likely suffer different types of stress (Benbouziane et al., 2013). In the case of oral administration, heat stress can be accompanied by an acid stress during passage through the stomach as well as bile stress in the duodenum. SICE system represents an interesting alternative for the treatment of GI inflammatory diseases, since it allows for the local delivery of therapeutic proteins in the GIT during the passage of the bacteria, allowing for the localized action of the protein and thus a greater efficiency. This system is an interesting alternative for proof of concept studies because it does not require the presence of regulatory genes or the pre-induction of the cultures. However, it still presents a bottleneck, since antibiotic resistance markers could be horizontally transferred to harmful microbes in the human GIT in clinical studies. In this context, the scientific community has been trying to develop biological confinement strategies, which are discussed later in this review (Vandermeulen et al., 2011).

Therapeutic Interventions Using Recombinant *L. lactis* Strains to Alleviate GI Inflammation

Since *L. lactis* can be genetically modified to efficiently produce and secrete different anti-inflammatory proteins, recombinant strains of *L. lactis* have been tested in pre-clinical and clinical experimental trials to treat or prevent various human diseases, including intestinal inflammation (**Table 1**) (Steidler et al., 2000; Rochat et al., 2007; LeBlanc et al., 2011; Bermúdez-Humarán et al., 2013; Del Carmen et al., 2014; Carvalho et al., 2016, 2017). The oral administration of *L. lactis* expressing anti-inflammatory proteins is a very interesting strategy to fight GIT inflammation, as this species is non-invasive and allochthonous, as commented on earlier. As it is unable to colonize the GIT, the potential to elicit adverse effects on host microbiota related to its long-term administration is reduced (Nouaille et al., 2003). It has been shown that the oral administration of a recombinant *L. lactis* strain expressing the enzyme SOD, naturally produced by *Bacillus subtilis*, reduced inflammation scores in animals treated with trinitrobenzenesulfonic acid (TNBS). This therapeutic effect was tied to the antioxidant properties of the recombinant SOD (Rochat et al., 2005). Later, the same strain was able to prevent the development of colorectal cancer cells in mice.

In another proof-of-concept study, the anti-inflammatory strain *L. lactis* NCDO 2118 was engineered to produce the oxidative enzyme, 15-lipoxygenase-1 (15-LOX-1), which catalyzes the formation of several anti-inflammatory mediators, such as lipoxins, resolvins and protectins. The 15-LOX-1 produced by *L. lactis* was effective in treating DSS-induced

colitis in mice during the remission period and decreased pro-inflammatory cytokines such as IFN-γ and IL-4 while increasing the anti-inflammatory IL-10 (Carvalho et al., 2016). Another strategy has been the use of *L. lactis* to secrete either regulatory cytokines involved in the regulation of inflammation processes, or antibodies that neutralize pro-inflammatory cytokines. *L. lactis* strains able to secrete anti-TNFα antibodies that bind to TNF-α, one of the most important mediators of inflammation, were described (Yoshida and Miyazaki, 2008; Strukelj et al., 2014). It was demonstrated in a DSS-induced colitis mouse model that the oral administration of *L. lactis* expressing murine anti-TNFα showed reduced inflammation, and work by Bermúdez-Humaran and collaborators demonstrated that a recombinant *L. lactis* strain expressing the cytokine TGF-β was able to ameliorate clinical symptoms, such as weight loss and diarrhea in the same DSS model of intestinal inflammation (Yoshida and Miyazaki, 2008; Bermúdez-Humarán et al., 2015). Another strain that is presenting good results in pre-clinical trials expresses IL-10, an anti-inflammatory cytokine capable of suppressing proinflammatory responses of both innate and adaptive immune cells. The effect of the recombinant IL-10 producing *L. lactis* has been tested in several IBD animal models, such as IL-10 knockout mice and TNBS or DSS models (Schotte et al., 2000; Steidler et al., 2000, 2003; Braat et al., 2006; Del Carmen et al., 2014). The recombinant IL-10 producing *L. lactis* strain demonstrated promising results in pre-clinical. Indeed, a large clinical trial using recombinant *L. lactis* secreting the human IL-10 was conducted in patients with Crohn's disease approximately 10 years ago. Its use in humans was allowed by regulatory agencies, such as the Genetically Modified Organisms (GMOs) European Commission, because of a biological containment strategy that was developed. A gene encoding the essential protein thymidylate synthase (ThyA), located on the *L. lactis* chromosome, was exchanged for the human IL-10 gene. Therefore, the strain was only able to survive in the presence of thymine or thymidine that was artificially provided in the culture medium, making *L. lactis*-IL-10 critically dependent on this compound. Inside the human body, the strain could survive and deliver IL-10, since thymine or thymidine is available. Outside of the body, the GMO strain was unable to survive, avoiding its spread into the environment (Steidler et al., 2003). Clinical results showed no significant improvement between patients receiving the IL-10 producing *L. lactis* strain and those who received a placebo (Braat et al., 2006).

Few studies regarding the treatment of mucositis using recombinant *L. lactis* strains expressing therapeutic molecules have been reported. Most pre-clinical studies found in the literature describe the use of purified anti-inflammatory compounds intended to eliminate disease. An example is the systemic administration of either IL-11 or TGF-β regulatory cytokines in patients. The authors noted that this alternative treatment was not able to contain oral mucositis. The possible causes for this failure were linked to an inadequate dosage, route of administration and drug stability (Antin et al., 2002; de Koning et al., 2006). Other clinical studies have tested growth factors that stimulate cell proliferation, thereby maintaining epithelial barrier integrity, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and epidermal growth factor (EGF). However, their use was associated with an increased risk and progression of tumors (Hong et al., 2009). Rottiers et al. (2009) evaluated the effect of *L. lactis* secreting trefoil factor I (TFF-1), naturally involved in the repair of the epithelial barrier, administered to hamsters with oral mucositis. It was observed that recombinant *L. lactis* was able to reduce mucosal inflammation (Rottiers et al., 2009; Caluwaerts et al., 2010). Furthermore, as undesired reactions were not detected in pre-clinical trials, another genetically modified *L. lactis* strain (AG013), capable of secreting human TFF1, was engineered based on the ThyA biological confinement system. A phase 1 clinical trial was performed in patients with oral mucositis who tolerated the treatment well, and administration of the AG013 strain was shown to be more efficient in ameliorating clinical syntoms than placebo (Limaye et al., 2013). Several molecules with anti-inflammatory properties have sought to be cloned and expressed in *L. lactis*, which has proven to be a safe vehicle for the treatment of GI intestinal disorders. Anti-inflammatory cytokines, anti-oxidant enzymes, epithelial growth factor and especially antimicrobial peptides produced by *L. lactis* are the focus of future research efforts for the development of a possible treatment for GI tract inflammation.

Mammalian Antimicrobial Peptides Produced by *L. lactis* as a Possible Treatment for Intestinal Inflammation

Antimicrobial peptides that are involved in the maintenance of the epithelial barrier could represent an interesting candidate to prevent microbiota-driven inflammatory signaling. Various antimicrobial peptides, such as defensins, cathelicidins and histatins, that are produced by Paneth cells seem to play a critical role in intestinal homeostasis, and their biological activity has been reported to be compromised in IBD patients (Clevers and Bevins, 2013; Peterson and Artis, 2014). Different research groups are investigating whether the administration of these peptides could have a protective effect against intestinal inflammation. In a study conducted by Seo et al. (2012), α-defensin (HD5) and human β-defensin 2 (HBD2), which have been purified from the probiotic *E. coli* Nissle 1917, inhibithed the growth of pathogenic *E. coli*, *S. typhimurium,* or *L. monocytogenes* when co-incubated, *in vitro*, with these bacterial species (Seo et al., 2012).

Another antimicrobial peptide, cathelicidin, was expressed in *L. lactis* and the efficacy of this strain in decreasing intestinal inflammation was evaluated in a DSS murine model. The authors observed a reduced number of bacteria in the feces from animals that received the *L. lactis*-cathelicidin strain, suggesting an anti-microbial effect of the strain. According to the study, these findings were correlated to reduced tissue damage and MPO activity (Wong et al., 2012).

Among the antimicrobial peptides, the C-type lectin, Reg3A has been extensively studied due to its protective effect in the intestines of humans and animals during the inflammation process. This peptide, also known as pancreatitis-associated protein (PAP), belongs to the Reg family, which encodes a diverse group of proteins called secreted C-type lectins that contain a carbohydrate recognition domain (CRD). The Reg3A protein

is predominantly produced in the small intestine of mammals, mainly by Paneth cells, where the density of microorganisms is higher (Christa et al., 1996). Several studies revealed that Reg3A exerts a bactericidal activity against Gram-positive bacteria. Furthermore, it appears that its activation in the intestinal mucosa is required to generate a protective response against intestinal microbiota during bacteria-driven inflammatory events (Christa et al., 1999; Malka et al., 2000). In fact, the PAP protective effect in GI inflammation models has been demonstrated for the first time in a DSS-induced colitis rat model. This work used an adenovirus strategy to deliver PAP cDNA into host cells to increase the expression of PAP (Lv et al., 2012). Recently, Breyner et al. (2017, personal communication) have shown that the use of *L. lactis* expressing human PAP could prevent colitis in a DNBS-chemically induced murine model. Interestingly, as it was shown to be useful in the treatment of IBD, another study sought to investigate a protective role of *L. lactis* secreting human PAP in mucositis using the 5-FU-induced intestinal mucositis experimental mouse model. The authors showed that the PAP antimicrobial peptide, cloned into *L. lactis*, has an inhibitory effect against the opportunistic commensal *E. faecalis*. Moreover, *L. lactis* NZ9000 by itself was able to prevent histological damage and reduce neutrophil and eosinophil infiltration in mice injected with 5-FU. In addition, the recombinant lactococci producing PAP improved villous architecture preservation and increased Paneth cell activity in response to 5-FU inflammation (Carvalho et al., 2017).

CONCLUSION

The efficacy of probiotic LAB, especially in the context of using recombinant *L. lactis* strains designed to deliver anti-inflammatory proteins *in situ*, has been demonstrated for treating IBD in many studies in the past decades. Moreover, as highlighted in this review, the same therapeutic approach is being successfully transposed for treating mucositis. Thus, this work reiterates that probiotic LAB, wild type or genetically modified, could also be used as an alternative for treating other GI inflammatory diseases in which dysbiosis has been shown to be implicated. As most of the beneficial effects of recombinant *L. lactis* strains have been demonstrated in proof-of-concept studies, further translational aproaches are needed to make them safe for testing in humans. In this context, biological confinement strategies that prevent recombinant lactococci from escaping into natural ecosystems should be considered.

AUTHOR CONTRIBUTIONS

RC contributed to conception of the work, bibliographic survey, and manuscript writing. FdC contributed to bibliographic survey and drafting or the work. AdO was responsible for creating the figure and contributed to the bibliographic survey. PL was involved in the critical revision of the article. J-MC was involved in the critical revision of the article. LB-H was involved in manuscript correction and drafting of the work. VA contributed to critical revision of the article and conception of the work. MdA contributed to manuscript writing and correction.

FUNDING

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 October 2016
doi: 10.3389/fimmu.2016.00408

Supplementation with *Lactobacillus plantarum* WCFS1 Prevents Decline of Mucus Barrier in Colon of Accelerated Aging *Ercc1*$^{-/\Delta 7}$ Mice

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Although it is clear that probiotics improve intestinal barrier function, little is known about the effects of probiotics on the aging intestine. We investigated effects of 10-week bacterial supplementation of *Lactobacillus plantarum* WCFS1, *Lactobacillus casei* BL23, or *Bifidobacterium breve* DSM20213 on gut barrier and immunity in 16-week-old accelerated aging *Ercc1*$^{-/\Delta 7}$ mice, which have a median lifespan of ~20 weeks, and their wild-type littermates. The colonic barrier in *Ercc1*$^{-/\Delta 7}$ mice was characterized by a thin (< 10 μm) mucus layer. *L. plantarum* prevented this decline in mucus integrity in *Ercc1*$^{-/\Delta 7}$ mice, whereas *B. breve* exacerbated it. Bacterial supplementations affected the expression of immune-related genes, including Toll-like receptor 4. Regulatory T cell frequencies were increased in the mesenteric lymph nodes of *L. plantarum*- and *L. casei*-treated *Ercc1*$^{-/\Delta 7}$ mice. *L. plantarum*- and *L. casei*-treated *Ercc1*$^{-/\Delta 7}$ mice showed increased specific antibody production in a T cell-dependent immune response *in vivo*. By contrast, the effects of bacterial supplementation on wild-type control mice were negligible. Thus, supplementation with *L. plantarum* – but not with *L. casei* and *B. breve* – prevented the decline in the mucus barrier in *Ercc1*$^{-/\Delta 7}$ mice. Our data indicate that age is an important factor influencing beneficial or detrimental effects of candidate probiotics. These findings also highlight the need for caution in translating beneficial effects of probiotics observed in young animals or humans to the elderly.

Keywords: aging, probiotics, immunity, mucus, intestinal barrier, microbiota

OPEN ACCESS

Edited by:
Julio Villena,
CERELA-CONICET, Argentina

Reviewed by:
Jennelle Maree Kyd,
Swinburne University of Technology, Australia
Susana Salva,
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Specialty section:
This article was submitted to Mucosal Immunity, a section of the journal Frontiers in Immunology

Received: *04 August 2016*
Accepted: *22 September 2016*
Published: *07 October 2016*

Citation:
van Beek AA, Sovran B, Hugenholtz F, Meijer B, Hoogerland JA, Mihailova V, van der Ploeg C, Belzer C, Boekschoten MV, Hoeijmakers JHJ, Vermeij WP, de Vos P, Wells JM, Leenen PJM, Nicoletti C, Hendriks RW and Savelkoul HFJ (2016) Supplementation with Lactobacillus plantarum WCFS1 Prevents Decline of Mucus Barrier in Colon of Accelerated Aging Ercc1$^{-/\Delta 7}$ *Mice. Front. Immunol. 7:408. doi: 10.3389/fimmu.2016.00408*

INTRODUCTION

Aging is accompanied by multiple age-related diseases (1), posing a major burden to public health care (2). With age, a decline in the regenerative potential of tissues due to stem cell exhaustion occurs (3). Turnover in epithelial cells is rapid, and mounting evidence indicates that intestinal stem cells are compromised with aging (4). For example, a crucial component of the intestinal barrier is mucus secreted by goblet cells (5). The Muc2 glycoprotein regulates immunity by inducing tolerogenic signals in mucosal dendritic cells (6) and is important in host–microbe interactions (7). Thus, changes in mucus quantity and integrity influence immunity (6, 8).

Aging is accompanied by the development of a low-grade inflammation ("inflammaging"), which is characterized by elevated IL-6 and TNF serum levels in elderly (9). Involution of the thymus and the bone marrow (BM) leads to decreased T and B cell production (10, 11). By contrast, the production of myeloid cells is enhanced with aging, characterized by a progressive increase of neutrophil frequencies in the circulation (12).

Probiotics are defined as live bacteria that confer health benefits to the host, for example, by competing with pathogens, enhancing intestinal barrier function, and regulating immunity (13, 14). They might, therefore, prevent some of the undesired age-related intestinal barrier and immune effects. Probiotic supplementation of elderly subjects led to changes in fecal microbiota composition (15–17), and affected the distribution and function of NK cells, macrophages, granulocytes, and T cells in the circulation (18, 19). Supplementation of aged mice with *Lactobacillus paracasei* resulted in increased IgG2a serum titers after antigenic challenge (20). Middle-aged mice that were supplemented with *Bifidobacterium animalis* showed decreased colon permeability, extended lifespan, and improved quality of life (21). Besides these studies, little is known about how exposure to probiotics impacts on the aging intestinal barrier and immune system. Moreover, it is unknown whether the beneficial effects of probiotics are age dependent.

In this report, we have used an accelerated aging mouse model to evaluate the effects of candidate probiotics in aging. Based on a variety of histological, functional, metabolomic, and proteomic data, it has been concluded that *Ercc1*$^{-/\Delta 7}$ mice resemble normal murine aging (22). Recently, we have shown that the immune system of *Ercc1*$^{-/\Delta 7}$ mice resembles the immune system of aged WT mice. For instance, we showed a similar decrease in B cell precursors and naïve T cells, and a similar increase in memory T cells and regulatory T cells (23). The ERCC1 protein is involved in multiple DNA repair pathways. *Ercc1*$^{-/\Delta 7}$ mice (median lifespan ~20 weeks) are deficient for fully functional ERCC1 protein. The expression of ERCC1-XPF (excision repair cross-complementation group 1-xeroderma pigmentosum group F) DNA repair endonuclease is reduced to ~5% compared with *Ercc1*$^{+/+}$ mice. Moreover, the residual ERCC1-XPF protein present is expressed from a truncated allele, and lacks the last seven amino acids. A reduction of ERCC1 protein activity leads to increased accumulation of DNA damage and, hence, results in an accelerated aging phenotype (24, 25).

The aim of this study was to investigate the potential of supplementation with candidate probiotic strains to ameliorate the effects of aging on the intestinal barrier and the immune system. Previously, probiotic activity was documented for *Lactobacillus plantarum* WCFS1 (26–28), *Lactobacillus casei* BL23 (29, 30), and relatives of *Bifidobacterium breve* DSM20213 (31). We selected these strains on the basis of induced IL-10/TNF ratios in young and aged immune cells *in vitro* (32). The three strains can be classified as potential pro-inflammatory (*L. plantarum*), regulatory (*L. casei*), or anti-inflammatory (*B. breve*), based on low, intermediate, or high IL-10/TNF ratios, respectively.

For this study, we supplemented 6-week-old *Ercc1*$^{+/+}$ mice and *Ercc1*$^{-/\Delta 7}$ mice with *L. plantarum*, *L. casei*, or *B. breve* for 10 weeks. Mucus barrier, microbiota composition, and gene regulation in the colon were analyzed, as well as the distribution of immune cells in various mucosal and peripheral lymphoid organs. We determined immune competence by antigenic challenge.

MATERIALS AND METHODS

Mice

The generation and characterization of *Ercc1*$^{+/\Delta 7}$ and *Ercc1*$^{-/+}$ mice has been previously described (25). *Ercc1*$^{-/\Delta 7}$ mice were obtained by crossing *Ercc1*$^{+/\Delta 7}$ with *Ercc1*$^{-/+}$ mice of pure C57Bl6/J and FVB backgrounds to yield *Ercc1*$^{-/\Delta 7}$ with an F1 C57Bl6J/FVB hybrid background. Genotyping was performed as described previously (33). Wild-type littermates (C57Bl6J/FVB F1) were used as controls. Four-month-old and 18-month-old C57Bl6/J mice were purchased from Harlan (Horst, The Netherlands; only used in **Figure 1**).

Animals were housed in individual ventilated cages under SPF conditions. Experiments were performed in accordance with the Principles of Laboratory Animal Care and with Dutch legislation. This study was carried out in accordance with the recommendations of the Dutch Ethical Committee of Wageningen that approved the work. Blood was taken from mice being sacrificed, and serum was frozen in −80°C for later use. After mice ($n = 4$–6) were sacrificed, feces from colon was collected and snap-frozen. Distal ileum and proximal colon sections were isolated and fixed in Carnoy or snap-frozen in liquid nitrogen. BM, thymus, spleen, mesenteric lymph nodes (MLN), and Peyer's patches (PPs) were isolated.

Bacterial Cultures and Supplementation

L. plantarum WCFS1, *L. casei* BL23, and *B. breve* DSM20213 were grown on MRS medium (Merck, Darmstadt, Germany) until stationary phase, frozen in glycerol, and stored in −80°C until use. Upon use, bacteria were thawed and 10× diluted in NaHCO3/PBS buffer. Around 2×10^8 CFU in 200 µL were administered to mice by gavage, three times per week. Treatment of mice started at 6 weeks of age until 1 day before sacrifice at 16 weeks or until death.

Histology and Fluorescence *In Situ* Hybridization

Carnoy-fixed proximal colon sections were embedded in paraffin. Paraffin sections (5 µm) were attached to poly-L-lysine-coated glass slides (Thermo Scientific, Germany). After overnight

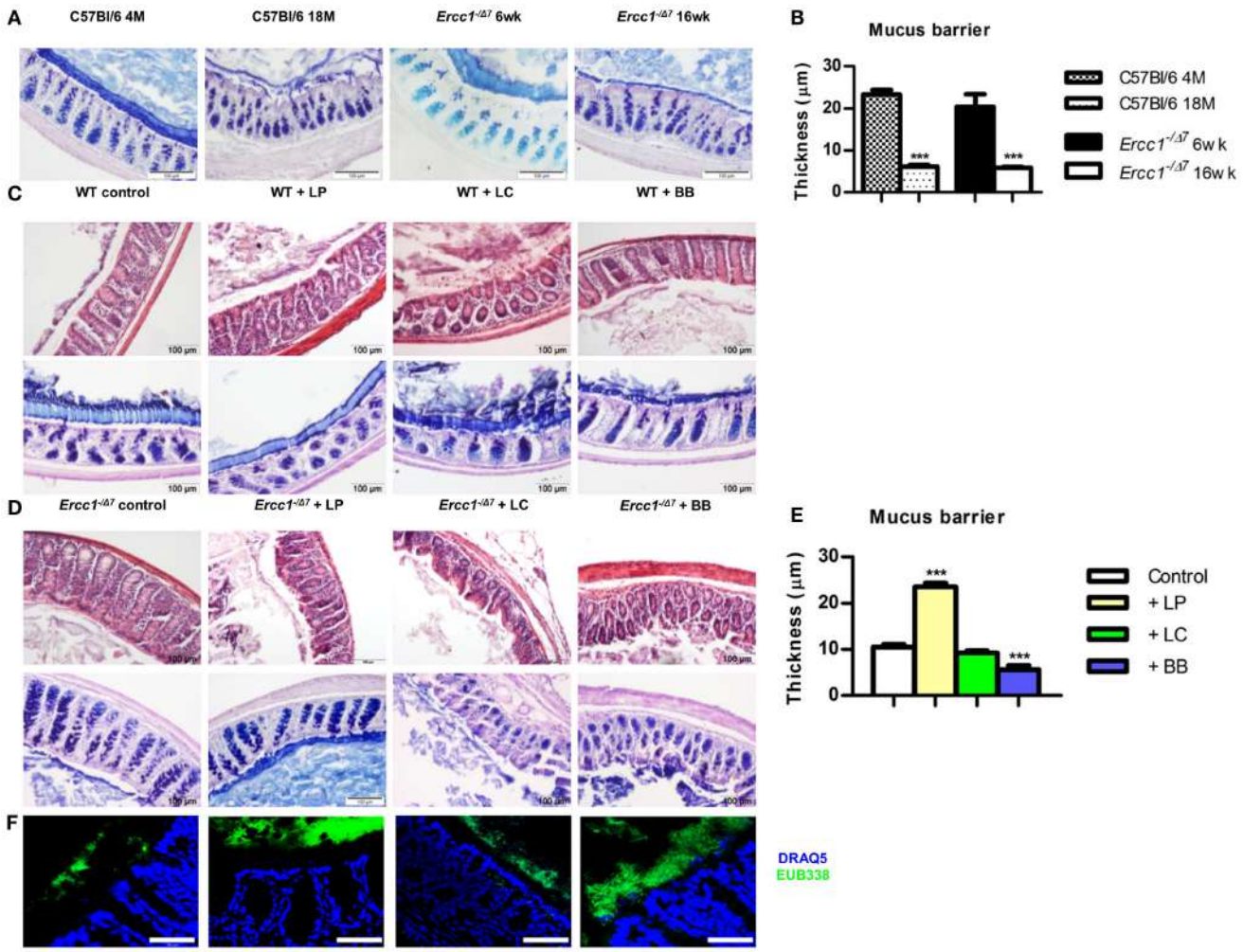

FIGURE 1 | Treatment with *L. plantarum* prevented the age-related decline in colonic mucus barrier of accelerated aging *Ercc1*$^{-/\Delta 7}$ mice. **(A)** Representative pictures of proximal colon stained with PAS/Alcian Blue of 4-month-old (4 M) or 18-month-old (18 M) WT mice and 6-week-old (6 weeks) or 16-week-old (16 weeks) *Ercc1*$^{-/\Delta 7}$ mice. **(B)** Quantitative measurement of mucus thickness in young and old WT and *Ercc1*$^{-/\Delta}$ mice by ImageJ. **(C)** Representative pictures of proximal colon stained with H&E or PAS/Alcian Blue of *Ercc1*$^{+/+}$ mice supplemented with control treatment, *L. plantarum* WCFS1 (LP), *L. casei* BL23 (LC), or *B. breve* DSM20213 (BB). **(D)** Representative pictures of proximal colon stained with H&E and PAS/Alcian Blue of *Ercc1*$^{-/\Delta 7}$ mice supplemented with control treatment, LP, LC, or BB. **(E)** Quantitative measurement of mucus thickness in *Ercc1*$^{-/\Delta 7}$ mice by ImageJ. **(F)** Fluorescence *in situ* hybridization (FISH) of proximal colon from *Ercc1*$^{-/\Delta 7}$ mice. Data represent the mean + SEM from four to six animals per group. ***$p < 0.001$. Scale bars histological pictures: 100 μm; scale bars FISH: 50 μm.

incubation at 37°C, slides were de-waxed and rehydrated. Sections were stained with hematoxylin and eosin (H&E) and PAS/Alcian blue. Mucus layer thickness was measured using ImageJ software (NIH, MD, USA), as previously published (34). For detection of bacteria, tissue sections were used for fluorescence *in situ* hybridization (FISH), as previously published (8).

MIT-Chips/16S Sequencing

Microbiota composition in colonic content was analyzed by Mouse Intestinal Tract Chip (MITChip), as described previously (35). The data were normalized and analyzed using a set of R-based scripts in combination with a custom-designed relational database, which operates under the MySQL database management system. For the microbial profiling, the Robust Probabilistic Averaging signal intensities of 2667 specific probes for the 94 genus-level bacterial groups detected on the MITChip were used (36). Diversity calculations were performed using a microbiome R-script package (https://github.com/microbiome). Multivariate statistics, redundancy analysis (RDA), and principal response curves were performed in Canoco 5.0 and visualized in triplots or a principal response curves plot (37).

RNA Isolation and Transcriptome Analysis

Total RNA was isolated from proximal colon ($n = 3$–6 per group) using the RNeasy kit (Qiagen) with a DNase digestion step according to the manufacturer's protocol. Transcriptome analysis

on individual samples was performed as previously described (8). The gene expression datasets were deposited in NCBI's Gene Expression Omnibus (GEO) and are accesible through GEO Series accession number: GSE87368.

General Flow Cytometry Procedures

Single-cell suspensions of BM were obtained by crushing femurs, tibias, iliac crests, and sternum with mortar and pestle. BM cells were then filtered on a 40-μm cell strainer. A proportion of the BM cells was frozen for later use in *in vitro* cultures. Spleen, MLN, PP, thymus, and peritoneal cavity single-cell suspensions were obtained by gently pushing cells through a 40-μm cell strainer with a syringe. All cells were stained for extracellular markers and dead cells were identified with fixable live/dead stain (Ebioscience, San Diego, CA, USA), after which intracellular staining was enabled by fixing and permeabilizing cells with Fix/Perm buffer (Ebioscience) according to manufacturer's instructions. Antibodies used for flow cytometric measurements are listed in Supplementary Table 1 in Data Sheet 1. All flow cytometric measurements were performed on a Canto II flow cytometer (BD Biosciences, Erembodegem, Belgium). FlowJo vX.07 software (Tree Star) was used for data analysis. Gating of all presented immune cell populations was based on single live cells.

Spleen Cell Cultures

Splenic cells were cultured at 10^6 cells/mL for 4 days in the absence or presence of 5 μg/mL concanavalin A (ConA). Proliferation was measured by Ki-67 (Ebioscience). Supernatants were stored at −20°C. After thawing, levels of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF were measured with the Cytometric Bead Array Th1/Th2/Th17 Kit (BD Biosciences), according to manufacturer's instructions. Samples were acquired on a Canto II flow cytometer. Data were analyzed using FCAP Array version 3.0 (BD Biosciences) software.

Antibody Titers in Serum

Levels of IgM, IgG1, IgG2a, IgG2b, IgG3, IgE, and IgA were analyzed in serum using ProcartaPlex Mouse Antibody Isotyping Panel kit on the Luminex platform (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Data were acquired on a BioPlex 200 (Bio-Rad, Hercules, CA, USA) and analyzed with BioPlex software (version 5.0, Bio-Rad).

In Vivo Immunization and Antibody Detection

Primary and secondary T cell-dependent (TD) immune responses against TNP-KLH were measured 7 days after primary i.p. immunization and 7 days after i.p. booster immunization. The primary immunization was performed at 8 weeks of age (TNP-KLH in alum), booster doses were injected at 12 weeks of age (TNP-KLH in PBS). Total and TNP-specific Ig subclasses were determined by sandwich ELISA as previously described (38).

Statistical Analysis

Values are expressed as mean + SEM. Normal distribution of the data was confirmed using the Kolmogorov–Smirnov test. Statistical comparisons were performed using the two-sided Student's *t*-test. Where non-Gaussian distribution was demonstrated, we applied the non-parametric Mann–Whitney U test. Where no equal variances were observed, we applied the two-sided Student's *t*-test with Welch's correction. Statistical comparisons for lifespan data were performed using the log-rank (Mantel–Cox) test. Statistical comparisons for serum immunoglobulins were performed using two-way ANOVA, with subsequent Bonferroni posttests. Values of $p < 0.05$ were considered to be statistically significant. Values between $p > 0.05$ and $p < 0.10$ were considered as a trend.

RESULTS

The Mucus Layer in the Colon Declines with Age

To assess the mucus barrier in normal and accelerated aging, we compared the proximal colon of 4-month-old (young) with 18-month-old (aged) C57Bl/6 mice, and of 6-week-old (young) with 16-week-old (aged) *Ercc1*$^{-/\Delta 7}$ mice. We observed that in aged C57Bl/6 and *Ercc1*$^{-/\Delta 7}$ mice, a thinner mucus layer was present, compared with young C57Bl/6 and *Ercc1*$^{-/\Delta 7}$ mice (**Figure 1A**). With ImageJ, we measured the thickness of the mucus layer. In young C57Bl/6 and *Ercc1*$^{-/\Delta 7}$ mice, a mucus layer of ~20 μm was present, whereas in normal and accelerated aged mice, a significantly thinner mucus layer of less than 10 μm was observed ($p < 0.001$; **Figure 1B**).

Bacterial Supplementations Do Not Change the Mucus Layer in Colon of Young WT Mice

To determine the effects of the three selected bacterial strains in the young intestine, we analyzed proximal colon tissues of WT mice that were treated with *L. plantarum* WCFS1, *L. casei* BL23, or *B. breve* DSM20213 for 10 weeks. No change in tissue integrity (H&E) or mucus layer (PAS/Alcian Blue) was observed in the colon after supplementation with bacterial strains (**Figure 1C**).

Age-Related Decline in the Mucus Barrier is Prevented by Supplementation of *Ercc1*$^{-/\Delta 7}$ Mice with *L. plantarum*

Because the mucus layer declines with age, we questioned whether bacterial supplementation of *Ercc1*$^{-/\Delta 7}$ mice prevents the decline in mucus barrier. Colon tissue of 10-week treated *Ercc1*$^{-/\Delta 7}$ mice was checked for tissue integrity and mucus layer thickness. In contrast to our findings in WT mice, bacterial supplementation had significant effects on tissue integrity and the mucus layer. In *Ercc1*$^{-/\Delta 7}$ mice supplemented with *L. plantarum*, the colon showed a thicker mucus layer than their controls (**Figure 1D**). *L. plantarum* supplementation completely prevented age-related decline in the mucus layer compared with controls ($p < 0.001$; **Figure 1E**), resulting in a mucus thickness comparable to young WT mice. Spatial compartmentalization of bacteria in the colon was improved after *L. plantarum* supplementation (**Figure 1F**), as demonstrated by FISH analyses. On the contrary, *Ercc1*$^{-/\Delta 7}$ mice supplemented with *L. casei* or *B. breve* showed loss of tissue integrity (**Figure 1D**). No difference in mucus thickness was

observed after supplementation with *L. casei* (**Figure 1E**). *B. breve* supplementation resulted in a deteriorated mucus layer and a loss in mucus thickness ($p < 0.001$; **Figure 1E**). *B. breve* supplementation also resulted in less spatial compartmentalization of bacteria in the colon of *Ercc1*$^{-/\Delta 7}$ mice (**Figure 1F**).

Collectively, these data show that *L. plantarum* supplementation improves the mucus layer in the aged (but not young) colon. In addition, supplementation with *L. casei* or *B. breve* exacerbates the age-related decline of mucus barrier in the colon.

Bacterial Supplementation Associated with Minor Alterations in Colonic Microbiota Composition

As we introduced bacteria by bacterial supplementations into the intestinal microbial community, we investigate whether changes in the microbiota composition were underlying the observed changes in the mucus barrier of *Ercc1*$^{-/\Delta 7}$ mice. Microbiota composition was determined by performing 16S rRNA gene microbiota profiles of colonic content. The bacterial supplementations did not significantly alter microbial diversity nor richness (data not shown).

Redundancy analysis showed that 10.1% of the total variability of the gut microbiota can be related to the bacterial supplementations (**Figure 2**). No statistical significance was established. The first ordination axis explained 4.9% of the variability and separated *Ercc1*$^{-/\Delta 7}$ mice supplemented with either of the three bacterial strains from the control *Ercc1*$^{-/\Delta 7}$ mice. The second ordination axis explained 3.6% of the variability but did not result in a separation between groups. The third ordination axis explained an additional 1.6% of the variability (data not shown).

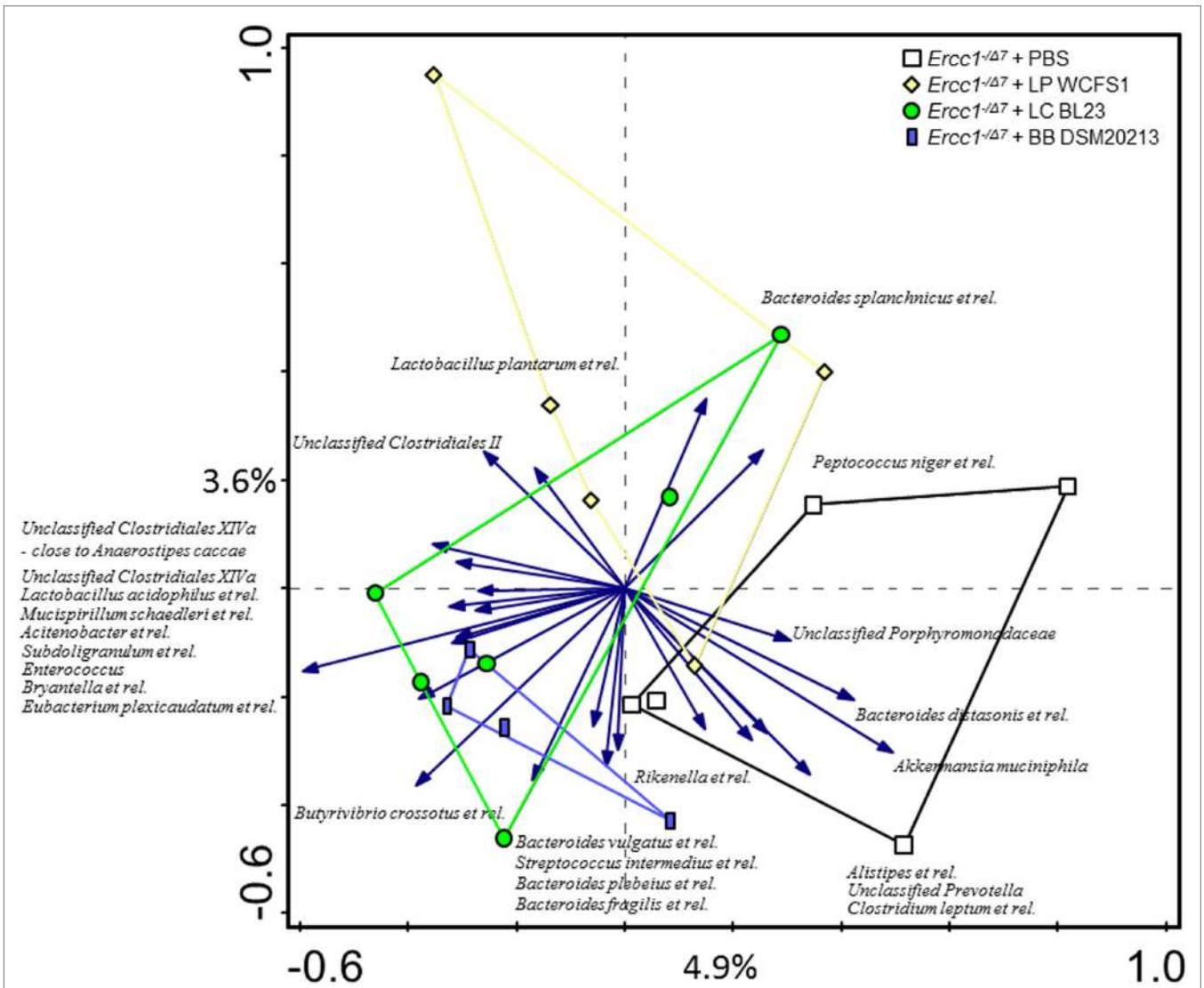

FIGURE 2 | The effect of bacterial supplementations on colonic microbiota composition in *Ercc1*$^{-/\Delta 7}$ mice. Redundancy analysis of the microbial composition after bacterial supplementations, on genus-like level of the MITChip analysis. Mice belonging to control-, LP-, LC-, and BB-treated groups are indicated by white squares, yellow diamonds, green circles, and blue rectangles, respectively. First and second ordination axes are plotted, showing 4.9 and 3.6% of the variability in the dataset, respectively. No significant changes were observed. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23; BB, *B. breve* DSM20213.

To assess whether significant changes in the microbial genus-like bacterial groups existed after different bacterial supplementations in *Ercc1*$^{-/\Delta 7}$ mice, we performed the Wilcoxon test. *Subdoligranulum* was higher in mice supplemented with *L. casei* ($p < 0.05$), whereas it tended to be higher in mice supplemented with *B. breve* ($p = 0.05$), as compared with control mice (**Figure 3**). *Akkermansia muciniphila* tended to be less present ($p = 0.06$) in mice supplemented with *L. plantarum* compared with control mice. *Eubacterium plexicaudatum* and a close relative to *Anaerostipes caccae* tended to be higher ($p = 0.06$) in *Ercc1*$^{-/\Delta 7}$ mice supplemented with *L. casei*.

These data demonstrate some differences in microbial species between control-treated *Ercc1*$^{-/\Delta 7}$ mice and *Ercc1*$^{-/\Delta 7}$ mice treated with bacterial supplementations.

Distinct Gene Expression Profiles in Colon after Each Bacterial Supplementation

To understand the mechanisms by which bacterial supplementation changes the mucus barrier, we performed transcriptome analysis on the proximal colon of *Ercc1*$^{-/\Delta 7}$ mice. Gene expression microarrays on total proximal colon samples from *Ercc1*$^{-/\Delta 7}$ mice treated with bacterial supplementations or control treatment revealed relatively low numbers of differentially expressed genes: 84 by *L. plantarum*, 238 by *L. casei*, and 384 by *B. breve*. Only a few genes were overlapping between two or three different bacterial supplementations, whereas most of the differentially expressed genes were distinctly regulated by one of the treatments (**Figure 4**).

Several growth- and immune-related genes were differentially expressed after bacterial supplementation. Apolipoprotein (APO) A-1, APOA-4, suppressor of cytokine signaling (SOCS) 3, and toll-like receptor (TLR) 4 were upregulated more than 1.2-fold after *L. plantarum* supplementation (Data Sheet 2 in the Supplementary Material). Several immunoglobulin variable genes and TLR13 were upregulated after administration of *L. casei*, whereas defensin 40β was 1.3-fold downregulated. Defensin 24α, amphiregulin, and keratinocyte growth factor 7 (FGF7) were upregulated more than 1.4-fold after administration of *B. breve*, while TLR6, TLR7, and CCL3 (MIP-1α) were more than 1.2-fold downregulated (Data Sheet 2 in the Supplementary Material). Remarkably, we found no significant up- or downregulation in any mucin.

Bacterial Supplementation Alters Growth- and Immune-Related Pathways in Colon

Because we found relatively low numbers of differentially expressed genes, we applied a gene set enrichment analysis (GSEA) (39) to gain insight into the regulated pathways by bacterial supplementations. Upstream regulators that can explain the observed changes in gene expression were identified using Ingenuity Upstream Regulator Analysis.

Gene set enrichment analysis revealed that *L. plantarum* supplementation significantly enhanced several processes involved in growth and cell cycle, and immunity (Supplementary Table 2 in Data Sheet 1), such as "Type II Interferon Signaling," "VIP pathway," and "IL8/CXCR1 pathway." Interestingly, in the top-10

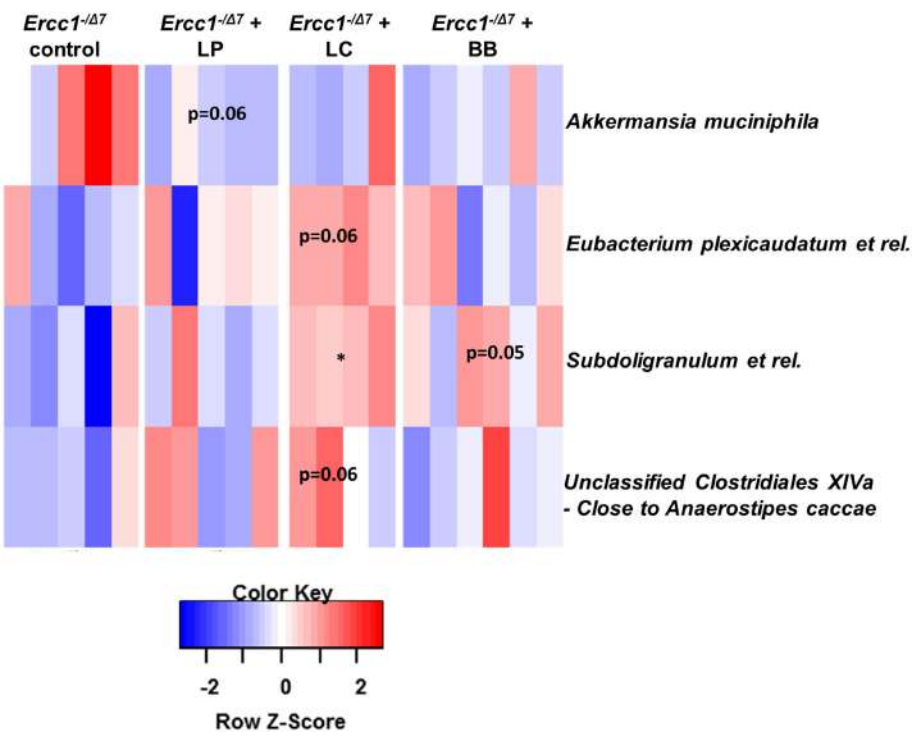

FIGURE 3 | Bacterial supplementation induced changes in bacterial taxa in the colon of *Ercc1*$^{-/\Delta 7}$ mice. Wilcoxon tests comparing mice treated with bacterial strains with control group. Data represent $n = 4$–6 mice per group. *$p < 0.05$.

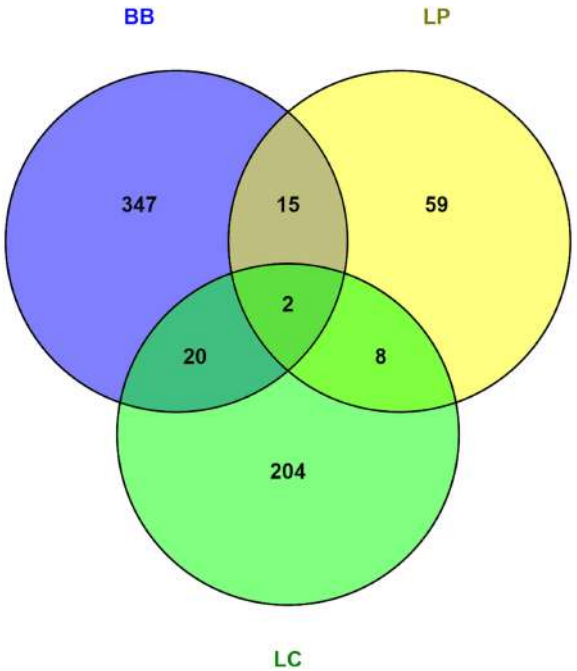

FIGURE 4 | Venn diagrams of differentially regulated genes in colon of *Ercc1*$^{-/\Delta 7}$ mice after bacterial supplementations. Total number of genes altered in the proximal colon of *Ercc1*$^{-/\Delta 7}$ mice treated with *L. plantarum* WCFS1 (LP), *L. casei* BL23 (LC), or *B. breve* DSM20213 (BB) compared with control-treated *Ercc1*$^{-/\Delta 7}$ mice. Venn diagram of the total number of genes upregulated and downregulated in the proximal colon of *Ercc1*$^{-/\Delta 7}$ mice treated with LP, BB, or LC ($p < 0.05$ and >1.2-fold difference).

TABLE 1 | Activation *z*-scores of upstream regulators in proximal colon of *Ercc1*$^{-/\Delta 7}$ mice after bacterial supplementations *L. plantarum* WCFS1 (LP), *L. casei* BL23 (LC), or *B. breve* DSM20213 (BB) as determined by Ingenuity.

Upstream regulator	LP	LC	BB
Leptin	2.41		
EGF	2.36		3.36
IL4	2.18		
IFN-γ	2.00		−1.35
PDGF BB	2.00		1.15
P38 MAPK	1.97		
CD40L	1.96		
Palmitic acid	1.96		
EGR1	1.95		
IGF1	1.82		
IL1β	1.77		
Ethanol	1.76		
CREB1	1.55		
CREBBP	1.54		
TNF	1.53		
KLF4		2.04	
Resistin-like β		2.00	
PML		−1.73	
miR-4800-5p		−1.98	
GATA3		−1.98	
MTOR		−2.00	
miR-4455		−2.22	
ADCYAP1			2.60
EDN1			2.17
WNT3A			2.16
VIP			1.95
FGF2			1.74
GLI1			1.63
miR-6967-5p			−1.58
Klra7 (includes others)			−1.87
IgG			−1.89
EZH2			−1.96
GATA2			−2.00
ANXA7			−2.00
miR-4707-5p			−2.16
ITK			−2.19
miR-4459			−2.63

Upstream regulators involved in growth and cell cycle are highlighted in blue; upstream regulators involved in immunity are highlighted in orange. Cut-off values for activation z-score ≥1.5 or ≤−1.5 combined with $p < 0.05$. Activated in blue, inhibited in red. ADCYAP, adenylate cyclase activating polypeptide; ANX, annexin; CREB(BP), cAMP-responsive element (binding protein); EDN, endothelin; EGF, epidermal growth factor; EGR, early growth response protein; EHZ, enhancer of zeste homolog; FGF, fibroblast growth factor; GLI, glioma-associated oncogene family zinc finger; IFN, interferon; IGF, insulin-like growth factor; ITK, IL-2-inducible T cell kinase; KLF, Kruppel-like factor; Klra, killer cell lectin-like receptor, subfamily A; LEP, leptin; MTOR, mechanistic target of rapamycin; PDGF, platelet-derived growth factor; PML, promyelocytic leukemia protein; RETNLB, resistin-like β; TNF, tumor necrosis factor; VIP, vasoactive intestinal peptide; WNT, wingless-type MMTV integration site family.

of upregulated pathways, three pathways were linked to DNA repair: the "Fanconi Pathway," "ATRBRCA pathway," and the "Fanconi anemia pathway." Several growth factors were activated after *L. plantarum* supplementation: leptin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) BB, early growth response protein (EGR) 1, and insulin-like growth factor (IGF) 1 (**Table 1**). Inflammatory cytokines (IFN-γ, IL-1β, IL-4, TNF) and CD40L (CD154) were activated in colon of mice supplemented with *L. plantarum*, compared with colon of mice supplemented with control treatment.

Lactobacillus casei supplementation enhanced several processes involved in growth and cell cycle, like "Mitotic G1-G1 S Phases," "DNA replication," "Synthesis of DNA," and "G1 S transition" (Supplementary Table 2 in Data Sheet 1). In addition, the "NOD-like receptor signaling pathway" was enhanced after *L. casei* supplementation, as well as and the "Unfolded protein response" (UPR), indicated endoplasmatic reticulum (ER) stress. Upstream regulators resistin-like β (RTNLB; activated) and GATA3 (inhibited) were regulated in the colon of mice supplemented with *L. casei* (**Table 1**).

Several metabolic pathways were enhanced in colon of *B. breve*-supplemented mice (Supplementary Table 2 in Data Sheet 1). Of note, "Protein folding" was upregulated. In contrast to *L. plantarum* and *L. casei* supplementation, *B. breve* supplementation significantly inhibited several processes involved in immunity, such as "IL2 STAT5 pathway," "Immunoregulatory interactions between lymphoid/non-lymphoid cells," "Type II Interferon signaling," "IL4 2 pathway," and "IL6 7 pathway" (Supplementary Table 3 in Data Sheet 1). *B. breve* supplementation activated EGF and inhibited fibroblast growth factor (FGF) 2. IgG, GATA2, and IL-2-inducible T cell kinase (ITK) were inhibited in colon of mice supplemented with *B. breve*. In line with GSEA, IFN-γ was inhibited as well after *B. breve* supplementation.

These data indicate that immune pathways in the colon are enhanced by *L. plantarum* and *L. casei*, but are inhibited by *B. breve* supplementation.

L. plantarum and *L. casei* Supplementation Induce Regulatory T Cells in MLN

Based on the regulation of immune genes by bacterial supplementations, we tested whether the distribution of immune cells was altered in mucosal immune organs of *Ercc1*$^{-/\Delta 7}$ mice.

First, we evaluated changes in distribution of immune cells in PPs and MLN. B cell frequencies were reduced in PP and MLN ($p < 0.05$) after *L. casei* supplementation in *Ercc1*$^{-/\Delta 7}$ mice (**Figure 5A**). By contrast, frequencies of T cells were increased in PP ($p < 0.01$) and MLN ($p < 0.05$; **Figure 5B**). The frequencies of regulatory T (Treg) cells in MLN were increased after *L. plantarum* and *L. casei* supplementation ($p < 0.05$; **Figures 5C,D**). No changes in distribution of B and T cells were observed upon bacterial supplementation in WT mice, except for a tendency to decreased Treg cells after *L. casei* supplementation ($p = 0.09$; Supplementary Figure 1 in Data Sheet 1).

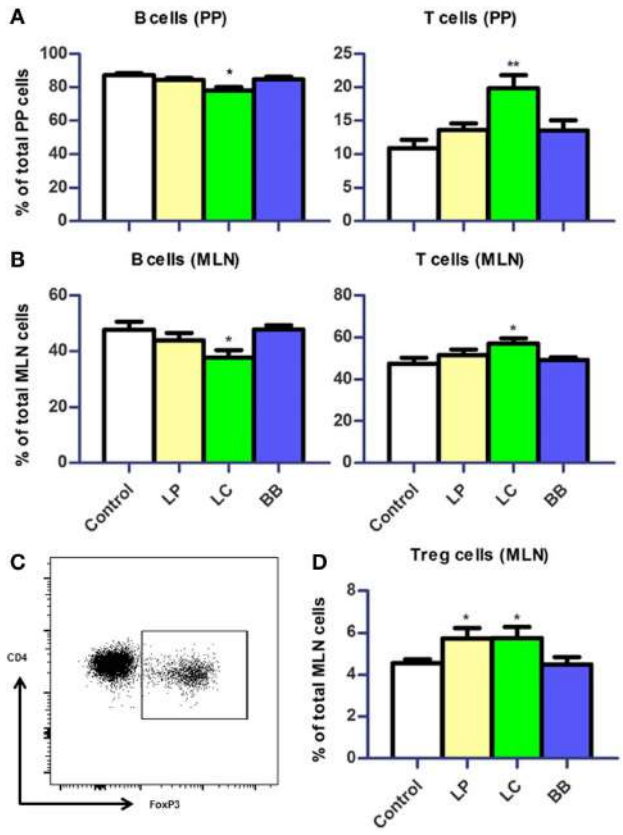

FIGURE 5 | Distribution of B cells and T cells in Peyer's patches (PP) and mesenteric lymph nodes (MLN) upon bacterial supplementation in *Ercc1*$^{-/\Delta 7}$ mice. **(A,B)** Mean frequencies of B and T cells in PP and MLN were determined by flow cytometry. B cells were defined as CD19$^+$, T cells were defined as CD3$^+$. **(C)** Flow cytometric analysis of CD3$^+$CD4$^+$CD8$^-$ regulatory T (Treg) cells in MLN. **(D)** Mean frequencies of Treg cells in MLN. Data represent the mean + SEM from four to six animals per group. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23; BB, *B. breve* DSM20213. *$p < 0.05$; **$p < 0.01$.

L. casei Elevates Systemic Inflammatory Markers

Next, we assessed distribution of immune cells in the spleen. We noted that the relative spleen weight increased after *L. casei* supplementation in *Ercc1*$^{-/\Delta 7}$ mice (Supplementary Figure 2A in Data Sheet 1). Absolute numbers of spleen cells were not affected by bacterial supplementations (data not shown). Splenic B cell frequencies tended to be decreased after *L. casei* supplementation ($p = 0.06$; **Figure 6A**), but no changes in T cell frequencies were observed (**Figure 6B**). Treg cell frequencies in the spleen were increased after *L. casei* supplementation in *Ercc1*$^{-/\Delta 7}$ mice ($p < 0.05$; **Figure 6C**).

Increased frequencies of CD11b$^+$Ly6G$^-$CD68$^+$Ly6C^{hi} monocytes ($p < 0.01$; **Figures 6D,E**) and a tendency to increased frequencies of CD11b$^+$CD68^{int}Ly6C^{int}Ly6G$^+$ neutrophils were observed after *L. casei* supplementation ($p = 0.07$; **Figure 6F**). In addition, the proportions of CD3$^+$CD4$^+$RORγt$^+$ Th17 cells (Supplementary Figure 2B in Data Sheet 1) were increased after *L. casei* supplementation ($p < 0.05$; **Figure 6G**). A 4-day culture of splenocytes stimulated with concanavalin A (ConA), also showed increased IL-17A production ($p < 0.01$; **Figure 6H**) and a decreased T cell proliferation in splenocytes derived from *L. casei*-treated mice ($p < 0.01$; **Figure 6I**). None of these changes were observed in *Ercc1*$^{-/\Delta 7}$ mice treated with *L. plantarum* or *B. breve*, and in WT mice treated with each of the bacterial supplementations (Supplementary Figure 3 in Data Sheet 1). These data suggest that *L. casei*, in contrast to *L. plantarum* and *B. breve*, raises several inflammatory markers in *Ercc1*$^{-/\Delta 7}$ mice.

Lymphocyte and Myeloid Development Affected after *L. plantarum* or *L. casei* Supplementation

We subsequently investigated the development of B cells and myeloid cells in BM and of T cells in thymus of *Ercc1*$^{-/\Delta 7}$ mice, as the observed changes in cell distribution in PP, MLN, and spleen might be explained by an altered migration or production. Absolute numbers in the BM were unchanged after bacterial supplementation (data not shown). In the BM, we observed significantly higher Lin$^-$CD117^{hi}CD11c$^-$CD135$^-$CD16/32$^+$ granulocyte–monocyte precursor (GMP), CD11b$^+$Ly6G$^+$ neutrophil, and Ly6C^{hi}CD31$^-$ monocyte frequencies after *L. casei* supplementation (**Figures 7A–C**). Frequencies of total CD19$^+$CD45R$^+$ B-lineage cells were decreased after *L. plantarum* ($p < 0.05$) and *L. casei* supplementation ($p < 0.001$), but not after *B. breve* supplementation (**Figure 7D**). We observed a reduction in all B-lineage subsets, except pro-B cells, after *L. casei* and *L. plantarum* supplementation (data not shown). In thymus, only *L. casei* supplementation caused changes in cell distribution, with significantly reduced CD3$^-$CD4$^+$CD8$^+$ double-positive (DP) cell numbers (**Figures 7E–H**).

In WT mice, we found no effect of bacterial supplementations on distribution of immune cells in BM or thymus, except for B-lineage cells after *L. plantarum* and *L. casei* supplementation (Supplementary Figure 4 in Data Sheet 1).

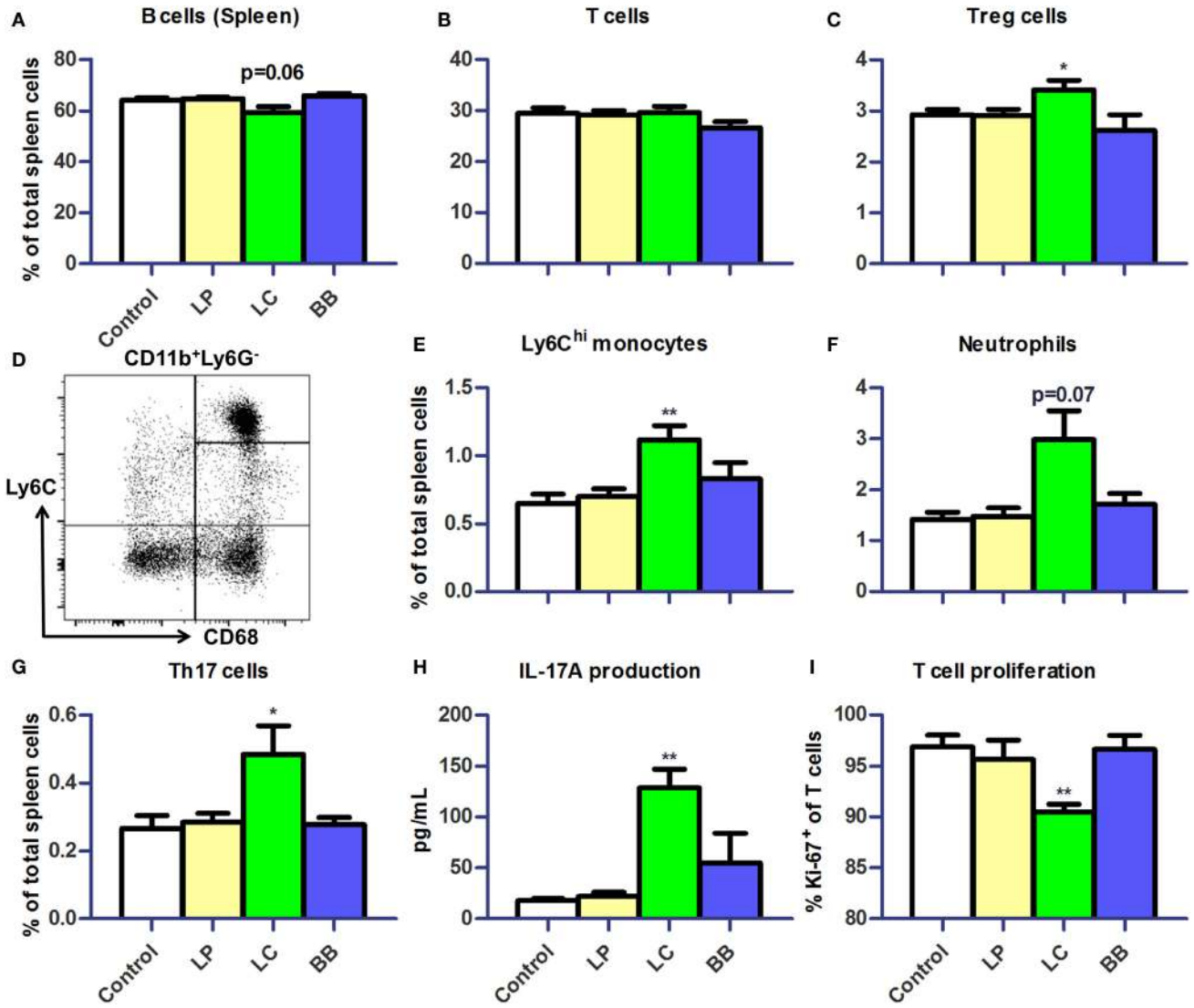

FIGURE 6 | ***L. casei* supplementation of *Ercc1*$^{-/\Delta7}$ mice raised inflammatory markers in spleen**. **(A,B)** Mean frequencies of B and T cells in spleen were determined by flow cytometry. B cells were defined as CD19$^+$, T cells were defined as CD3$^+$. **(C)** Mean frequencies of Treg cells in spleen. **(D)** Flow cytometric analysis of splenic monocytes. CD11b$^+$Ly6G$^-$CD68$^+$ cells were divided in Ly6C^{hi}, Ly6C^{int}, and Ly6C^{lo} monocytes. **(E–G)** Mean frequencies of Ly6C^{hi} monocytes, neutrophils, and CD3$^+$CD4$^+$CD8$^-$Rorγt$^+$ Th17 cells were determined by flow cytometry. **(H)** Mean concentration of IL-17A production by splenocytes stimulated with ConA for 4 days, as determined by Cytometric Bead Array. **(I)** Mean proliferating T cells (Ki-67$^+$) in splenocyte culture stimulated with ConA for 4 days, as determined by flow cytometry. Data represent the mean + SEM from four to six animals per group. *$p < 0.05$; **$p < 0.01$. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23; BB, *B. breve* DSM20213.

Bacterial Supplementations Do Not Alter Lifespan of *Ercc1*$^{-/\Delta7}$ Mice

The accelerated aging of *Ercc1*$^{-/\Delta7}$ mice enabled us to assess the potential life-extending properties of the bacterial strains. No significant change in lifespan of *Ercc1*$^{-/\Delta7}$ mice was observed after lifelong supplementation with *L. plantarum* or *L. casei* (Supplementary Figure 5 in Data Sheet 1).

L. casei Supplementation Increases IgG Serum Titers

Because *L. casei* supplementation led to decreased B cell proportions in several immune organs of *Ercc1*$^{-/\Delta7}$ mice, we tested whether serum antibody titers in *Ercc1*$^{-/\Delta7}$ mice were altered. Total IgG1 and IgG2b (but not IgG2a, IgG3, IgE, and IgA) titers were significantly increased after *L. casei* supplementation (**Figure 8**). *L. plantarum* and *B. breve* supplementation did not significantly change titers of any Ig subclass.

Immune Competence Improved by *L. casei* and *L. plantarum* Supplementation

To test whether changes in immune cell distribution also impact immune competence, we analyzed the B cell response of *Ercc1*$^{-/\Delta7}$ mice to the T cell-dependent antigen TNP-KLH. Specific anti-TNP-KLH Ig titers of the three tested isotype classes (IgM, IgG1,

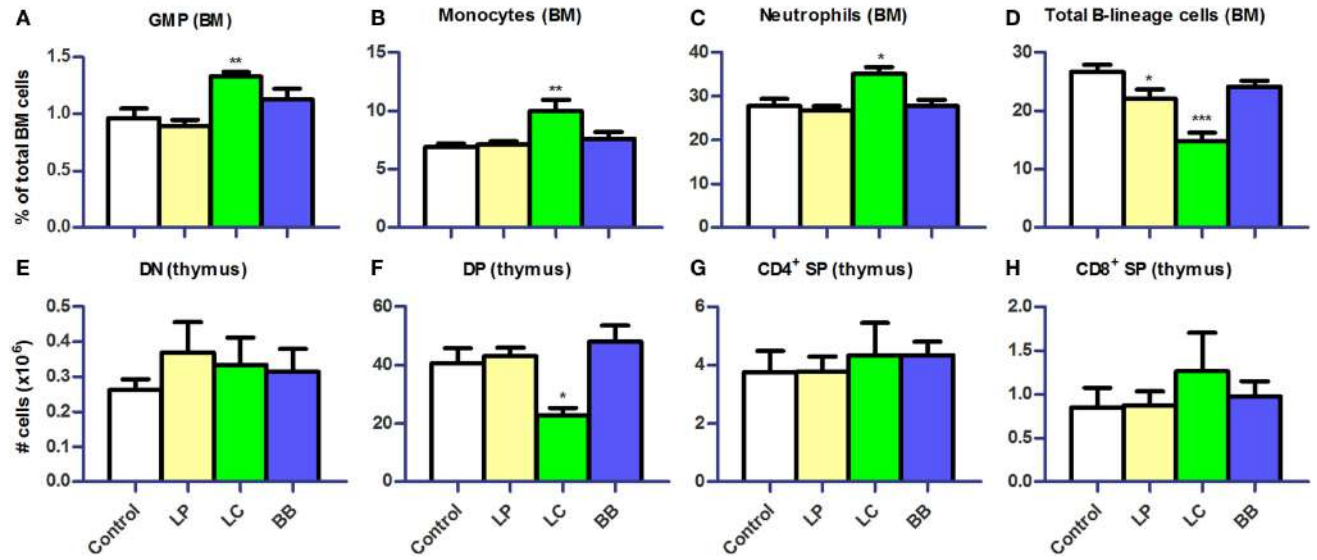

FIGURE 7 | *L. casei* or *L. plantarum* supplementation altered myeloid and lymphoid development in bone marrow and thymus of *Ercc1*$^{-/\Delta 7}$ mice. **(A–D)** Mean frequencies in bone marrow (BM) were determined by flow cytometry. Granulocyte–monocyte precursors (GMP) were defined as Lin$^-$CD117^{h}CD11c$^-$CD135$^-$CD16/32$^+$, neutrophils as CD11b$^+$Ly6G$^+$, monocytes as Ly6C^{h}CD31$^-$, and B-lineage cells as CD19$^+$CD45R$^+$. **(E–H)** Mean absolute numbers were determined by cell counts and flow cytometry. Double-negative (DN) cells were defined as CD3$^-$CD4$^-$CD8$^-$, double-positive (DP) cells as CD3$^-$CD4$^+$CD8$^+$, CD4$^+$ single-positive (SP) as CD3$^+$CD4$^+$CD8$^-$, and CD8$^+$ SP as CD3$^+$CD4$^-$CD8$^+$. Data represent the mean + SEM from four to six animals per group. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23; BB, *B. breve* DSM20213.

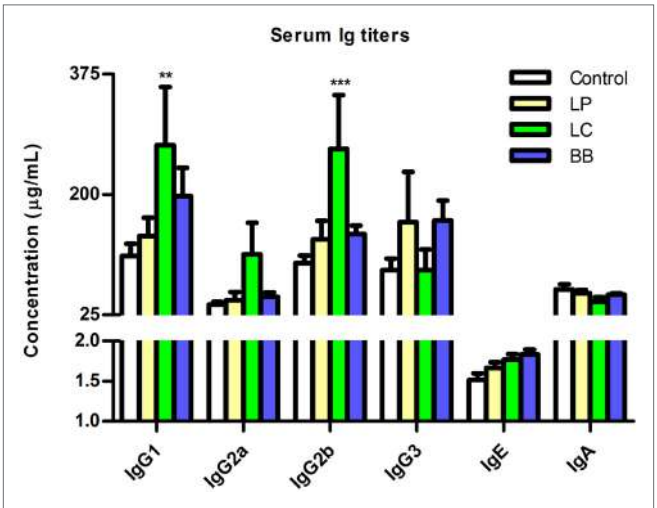

FIGURE 8 | *L. casei* supplementation increased IgG1 and IgG2b titers in *Ercc1*$^{-/\Delta 7}$ mice. Mean titers of IgG1, IgG2a, IgG2b, IgG3, IgE, and IgA in serum, as determined by Luminex. Data represent the mean + SEM from three to six animals per group. $^{**}p < 0.01$; $^{***}p < 0.001$. LP = *L. plantarum* WCFS1; LC = *L. casei* BL23; BB = *B. breve* DSM20213.

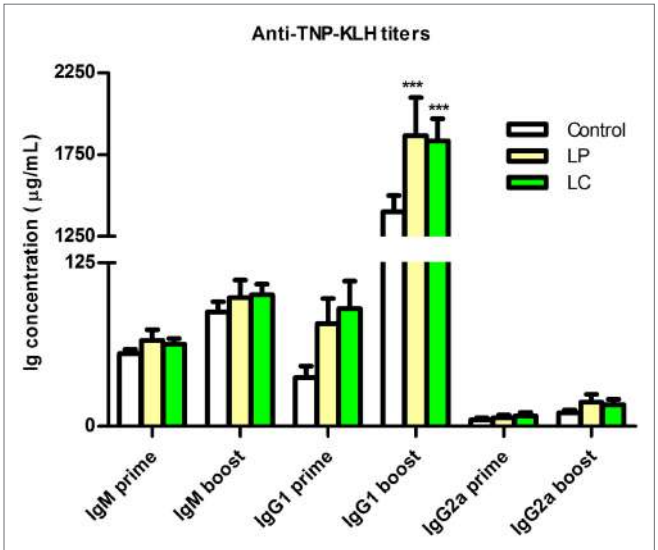

FIGURE 9 | Supplementation of *L. plantarum* and *L. casei* increased specific anti-TNP-KLH antibody responses of *Ercc1*$^{-/\Delta 7}$ mice. Mean TNP-specific IgM, IgG1, and IgG2a concentrations in serum were determined by ELISA, 7 days after primary immunization (prime, age 9 weeks), or 7 days after booster immunization (boost, age 13 weeks). Data represent the mean + SEM of 6–12 animals per group. $^{***}p < 0.001$. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23.

IgG2a) after primary and booster immunization were consistently higher after *L. plantarum* and *L. casei* supplementation (**Figure 9**). In particular, IgG1 titers after booster immunization increased in both *L. plantarum*- and *L. casei*-supplemented mice compared with control-treated mice ($p < 0.001$).

From these findings, we conclude that *L. plantarum* and *L. casei* enhance T cell-dependent B cell responses in *Ercc1*$^{-/\Delta 7}$ mice.

DISCUSSION

The effects of bacterial supplementations on the intestinal barrier and cellular parameters of immunity were studied in fast aging

Ercc1$^{-/\Delta 7}$ mice. We observed that the mucus layer in the colon declines with age and that bacterial supplementation may prevent or exacerbate the age-related decline in the mucus layer, dependent on the specific bacterial strain. Additionally, we demonstrated a marked difference in the response to bacterial supplementations between *Ercc1*$^{-/\Delta 7}$ mice and WT mice. Finally, supplementation with *L. casei* BL23 profoundly changed the distribution of immune cells and supplementation with *L. plantarum* WCFS1 or *L. casei* BL23 improved immune competence in *Ercc1*$^{-/\Delta 7}$ mice.

Recently, we showed the age-related decline in mucus barrier of C57Bl/6 mice as well (Sovran et al., unpublished data). Importantly, we report that the mucus barrier declines with age, in aged C57Bl/6 and *Ercc1*$^{-/\Delta 7}$ mice (**Figure 1**). This finding adds another age-related phenotype to the wide spectrum of age-related phenotypes observed in *Ercc1*$^{-/\Delta 7}$ mice (40). Moreover, we report that the age-related decline in mucus barrier can be modulated by bacterial supplementation. *L. plantarum* prevented the decline in mucus barrier. *L. plantarum* is able to bind to mucus with a mannose-specific adhesin, which is described as a potential probiotic feature (41). In total, *L. plantarum* harbors four mucus-binding proteins (42). Based on the improved spatial compartmentalization of bacteria after *L. plantarum* supplementation, we postulate that *L. plantarum* adheres to the mucus. In addition, we found that *L. plantarum* supplementation tended to decrease the abundance of *Akkermansia muciniphila* (**Figure 3**), which is known as a mucus degrader (43). Thus, it would be conceivable that mucus degradation is decreased after *L. plantarum* supplementation. By contrast, *B. breve* is known as a mucus degrader (44), and could, therefore, be directly responsible for the decrease in mucus thickness in the colon of *B. breve*-treated mice. Interestingly, several pathways involved in protein folding and the UPR were upregulated after *L. casei* and *B. breve* supplementation (Data Sheet 2 in the Supplementary Material). A high demand for synthesis of secretory proteins (such as mucins) induces ER stress, which in turn induces the UPR (45). The close proximity of bacteria to the epithelium in *L. casei*- and *B. breve*-treated mice might induce a high demand for mucin production and secretion, leading to induction of ER stress and UPR. There is indeed evidence that defects in MUC2 mucin and a subsequent defective mucus layer lead to ER stress and UPR (46).

Microbiota profiling showed that only few microbial species are slightly altered by bacterial supplementation (**Figures 2** and **3**). Therefore, most of the observed effects in the mucus barrier and immune system may be directly linked to the supplementation of each of the bacterial strains.

We found that the different bacterial strains elicited characteristically different responses in gene regulation in the colon (**Figure 4**). *L. plantarum* is known for its moderately pro-inflammatory profile, and relatively high IL-10 induction, when tested in human PBMC cultures (28, 47). In line with these studies, several upstream regulators predicted to be activated after *L. plantarum* supplementation included the inflammatory cytokines IFN-γ, IL-1β, IL-4, and TNF. The association between increased activation of inflammatory cytokines and the improved integrity of the colon after *L. plantarum* supplementation raises the possibility that it might be beneficial to locally increase inflammatory cytokine levels. This suggestion is corroborated by the absence of activation of these inflammatory cytokines after *L. casei* or *B. breve* supplementation, which did not improve or exacerbate the age-related decline in mucus integrity. A "tonic" level of constitutive TLR activation by commensal bacteria was previously shown to be crucial in the recovery from DSS-induced epithelial damage due to the role of NF-κB in epithelial repair processes (48). This notion that "physiological pro-inflammatory signals" is required for intestinal homeostasis is also supported by studies using epithelium-specific iκB kinase-γ (or NEMO) ablation in mice. These mice develop spontaneous colitis due to the failure of NF-κB to induce epithelial repair and steady-state production of innate effector mechanisms in the intestine (49). TLR2 signaling has been implicated in tight junction regulation *in vivo* and *in vitro* (13). Thus, it is possible that aged mice have sub-optimal level of TLR stimulation in the intestine to promote innate barrier defenses and that this is enhanced by *L. plantarum*, but not by *L. casei* and *B. breve*.

Remarkably, none of the significantly regulated genes were directly linked to mucus production. However, while performing Upstream Regulator Analysis, growth factors, such as EGF, IGF1, and EGR1, were predicted to be activated after *L. plantarum* supplementation. Together, these findings may indicate that mucus production by goblet cells is not directly enhanced, but is part of general epithelial integrity, supported by a number of growth factors.

Because many regulated genes involved immune-related genes, we additionally analyzed the makeup of the immune system after bacterial supplementation. Whereas supplementation with *B. breve* exacerbated the age-related decline in mucus barrier in colon, it did not cause any changes in mucosal or systemic immunity (**Figures 5**–**8**). Oppositely, *L. casei* supplementation caused various signs of inflammation, such as Ly6C^{hi} monocyte and neutrophil influx and production in spleen and BM, respectively. These inflammatory signs were coincided with the general decrease in B cell frequencies (also in the BM) and double-positive thymocytes. There is evidence that neutrophils in the BM are primed by microbial ligands (50). The effects of microbiota-derived signals on priming B and T cells in the BM have not been previously described. Our study suggests an, up to now, unknown link between microbiota, intestinal barrier, and B and T cell precursors. Specific precursor stages (i.e., small resting pre-B cells) were significantly decreased after *L. casei* supplementation, and to a lesser extent after *L. plantarum* supplementation. In the case of *L. plantarum* supplementation, we suggest that improved intestinal barrier function might alter circulating microbiota-derived products, such as peptidoglycan (PGN) and lipopolysaccharide (LPS). For instance, hematopoietic stem cells are damaged after chronic exposure to LPS (51). Interestingly, the decrease in small resting pre-B cells after *L. casei* supplementation (and to lesser extent by *L. plantarum*) was the only finding that could be reproduced in WT mice supplemented with these bacterial strains (Supplementary Figure 4 in Data Sheet 1). This may indicate that the effect of *L. casei* and *L. plantarum* supplementation on B cell development is independent of age.

A previous study showed lifespan extension after *B. animalis* supplementation (21). Therefore, we performed a lifespan study for *L. plantarum* and *L. casei*, which indicated that neither of them

is shortening or extending lifespan (Supplementary Figure 5 in Data Sheet 1).

Surprisingly, anti-TNP-KLH IgG1 titers in serum increased not only after *L. plantarum* but also after *L. casei* supplementation (**Figure 9**). This increase suggests that a demise in B cell development and B cell distribution does not necessarily translate into impaired B cell function. Previously, it has been shown that antigen-specific antibody titers can be enhanced by probiotic supplementation in aged mice (20), but data on B cell development are lacking.

The effects of the candidate probiotic strains were pronounced on the mucus barrier in the colon of *Ercc1*$^{-/\Delta 7}$ mice compared with WT mice. It has been shown in previous studies that strains, such as *L. casei* and *B. breve*, have beneficial effects on immunological parameters and intestinal barrier function in young mice (29–31). In our hands, *L. casei* and *B. breve* had no effect on mucus barrier or systemic immunity in young WT mice (except for the above-discussed finding on B cell development). A severe deteriorating effect, however, was observed on the mucus barrier or systemic immunity in *Ercc1*$^{-/\Delta 7}$ mice. These findings highlight the need for caution in translating beneficial effects of probiotics observed in young animals or humans to the elderly.

Our study has a number of limitations. We observed remarkable changes in the mucus layer, but could not pinpoint a single gene that is directly linked to the mucus layer. Furthermore, we did not include commercially available probiotic bacterial strains, such as *Lactobacillus rhamnosus* GG, or a non-probiotic bacterial strain. Nevertheless, our study reveals a previously unknown effect of age on the mucus barrier. We also show that it is possible to modulate this age-related decline in the mucus barrier by supplementation of bacterial strains, with coinciding effects on systemic immunity. More research is warranted to elucidate the interplay between bacteria, the aged gut epithelium, and the immune system.

Our data provide evidence that a comprehensive analysis of the intestinal barrier and immunity are needed in order to evaluate how bacterial supplementation contributes to the restoration of the age-related decline in intestinal barrier. A positive finding was that probiotic strains, such as *L. plantarum*, might contribute to maintenance of intestinal integrity by preventing age-related deterioration of the colonic mucus layer.

AUTHOR CONTRIBUTIONS

AB, BS, JHH, WV, PV, JW, PL, CN, RH, and HS conceived the study. AB, BS, FH, BM, CB, JAH, VM, CP, and MB performed the experiments. AB wrote the manuscript. BS, FH, BM, CB, JAH, JHH, WV, PV, JW, PL, CN, RH, and HS contributed to the revisions of the draft manuscripts.

ACKNOWLEDGMENTS

Authors thank Steven Aalvink, Marjolein de Jong-de Bruijn, and Jenny Jansen for technical help. We acknowledge the excellent help of all animal caretakers.

FUNDING

This work was funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00408

DATA SHEET 1

SUPPLEMENTARY TABLE 1 | Used antibodies in flow cytometry.

SUPPLEMENTARY TABLE 2 | Top-10 biological processes upregulated (as determined with GSEA) by bacterial supplementations in proximal colon of *Ercc1*$^{-/\Delta 7}$ mice treated with *L. plantarum* WCFS1 (LP), *L. casei* BL23 (LC), or *B. breve* DSM20213 (BB).

SUPPLEMENTARY TABLE 3 | Top-10 biological processes downregulated (as determined with GSEA) by bacterial supplementations in proximal colon of *Ercc1*$^{-/\Delta 7}$ mice treated with *L. plantarum* WCFS1 (LP), *L. casei* BL23 (LC), or *B. breve* DSM20213 (BB).

SUPPLEMENTARY FIGURE 1 | Distribution of B cells and T cells in Peyer's patches and mesenteric lymph nodes not changed upon bacterial supplementation in *Ercc1*$^{+/+}$ mice. **(A/B)** Mean frequencies were determined by flow cytometry. B cells were defined as CD19$^+$, T cells were defined as CD3$^+$. **(C)** Mean frequencies of CD3$^+$CD4$^+$CD8$^-$FoxP3$^+$ regulatory T (Treg) cells in MLN. Data represent the mean + SEM from four to six animals per group. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23; BB, *B. breve* DSM20213.

SUPPLEMENTARY FIGURE 2 | Increased relative spleen weight after *L. casei* supplementation of *Ercc1*$^{-/\Delta 7}$ mice. **(A)** Spleen weights relative to body weight. Data represent mean spleen weights + SEM of four to six animals per group. **(B)** Flow cytometric analysis of splenic Th17 cells. CD3$^+$CD4$^+$CD8$^-$ cells were gated for RORγt and FSC (forward scatter).

SUPPLEMENTARY FIGURE 3 | Bacterial supplementation of *Ercc1*$^{+/+}$ mice did not change splenic parameters. **(A)** Mean frequencies of Treg cells in spleen. **(B–D)** Mean frequencies of Ly6C^{hi} monocytes, neutrophils, and CD3$^+$CD4$^+$CD8$^-$Rorγt$^+$ Th17 cells were determined by flow cytometry. **(E)** Mean concentration of IL-17A production by splenocytes stimulated with ConA for 4 days, as determined by Cytometric Bead Array. **(F)** Mean proliferating T cells (Ki-67$^+$) in splenocyte culture stimulated with ConA for 4 days, as determined by flow cytometry. Data represent the mean + SEM from four to six animals per group. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23; BB, *B. breve* DSM20213.

SUPPLEMENTARY FIGURE 4 | *L. casei* supplementation altered B cell development in bone marrow of *Ercc1*$^{+/+}$ mice. **(A–E)** Mean frequencies in bone marrow (BM) were determined by flow cytometry. Granulocyte–monocyte precursors (GMP) were defined as Lin$^-$CD117^{hi}CD11c$^-$CD135$^-$CD16/32$^+$, neutrophils as CD11b$^+$Ly6G$^+$, monocytes as Ly6C^{hi}CD31$^-$, B-lineage cells as CD19$^+$CD45R$^+$, and small resting pre-B cells as sIgκ/λ$^-$cIgM$^+$CD2$^+$. (**F**) Mean absolute numbers were determined by cell counts and flow cytometry. Double-positive (DP) cells were defined as CD3$^-$CD4$^+$CD8$^+$. Data represent the mean + SEM from four to six animals per group. *$p<0.05$. LP, *L. plantarum* WCFS1; LC, *L. casei* BL23; BB, *B. breve* DSM20213.

SUPPLEMENTARY FIGURE 5 | Bacterial supplementations did not change lifespan of *Ercc1*$^{-/\Delta 7}$ mice. Data represent 11–12 animals per group (with an additional 6 animals per group censored at 16 weeks). LP, *L. plantarum* WCFS1; LC, *L. casei* BL23.

DATA SHEET 2 | Total file with differentially expressed genes after bacterial supplementations.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SS and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 April 2017
doi: 10.3389/fimmu.2017.00427

Gut Microbiota Species Can Provoke both Inflammatory and Tolerogenic Immune Responses in Human Dendritic Cells Mediated by Retinoic Acid Receptor Alpha Ligation

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Dendritic cells are considered as the main coordinators of both mucosal and systemic immune responses, thus playing a determining role in shaping the outcome of effector cell responses. However, it is still uncovered how primary human monocyte-derived DC (moDC) populations drive the polarization of helper T (Th) cells in the presence of commensal bacteria harboring unique immunomodulatory properties. Furthermore, the individual members of the gut microbiota have the potential to modulate the outcome of immune responses and shape the immunogenicity of differentiating moDCs *via* the activation of retinoic acid receptor alpha (RARα). Here, we report that moDCs are able to mediate robust Th1 and Th17 responses upon stimulation by *Escherichia coli Schaedler* or *Morganella morganii*, while the probiotic *Bacillus subtilis* strain limits this effect. Moreover, physiological concentrations of all-*trans* retinoic acid (ATRA) are able to re-program the differentiation of moDCs resulting in altered gene expression profiles of the master transcription factors RARα and interferon regulatory factor 4, and concomitantly regulate the cell surface expression levels of CD1 proteins and also the mucosa-associated CD103 integrin to different directions. It was also demonstrated that the ATRA-conditioned moDCs exhibited enhanced pro-inflammatory cytokine secretion while reduced their co-stimulatory and antigen-presenting capacity thus reducing Th1 and presenting undetectable Th17 type responses against the tested microbiota strains. Importantly, these regulatory circuits could be prevented by the selective inhibition of RARα functionality. These results altogether demonstrate that selected commensal bacterial strains are able to drive strong effector immune responses by moDCs, while in the presence of ATRA, they support the development of both tolerogenic and inflammatory moDC in a RARα-dependent manner.

Keywords: monocyte-derived dentritic cell, gut microbiota, all-*trans* retinoic acid, retinoic acid receptor alpha, interferon regulatory factor 4, T cell, CD1a, CD1d

OPEN ACCESS

Edited by:
Haruki Kitazawa, Tohoku University, Japan

Reviewed by:
Zoltan Simandi, Sanford-Burnham Institute for Medical Research, USA
Hervé M. Blottiere, INRA/Micalis Institute & INRA/MetaGenoPolis, France

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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *05 January 2017*
Accepted: *27 March 2017*
Published: *18 April 2017*

Citation:
Bene K, Varga Z, Petrov VO, Boyko N and Rajnavolgyi E (2017) Gut Microbiota Species Can Provoke both Inflammatory and Tolerogenic Immune Responses in Human Dendritic Cells Mediated by Retinoic Acid Receptor Alpha Ligation. Front. Immunol. 8:427. doi: 10.3389/fimmu.2017.00427

INTRODUCTION

The development and the metabolic activity of the human immune system critically depend on the amount and the diversity of the human microbiota acquired from the actual tissue microenvironment (1, 2). Upon birth, the human gastrointestinal tract becomes colonized by commensal microbes co-evolved with humans in a symbiotic or at least mutualistic manner together with the immune system

(3, 4). The local dendritic cell (DC) network involves a highly heterogeneous population of cells of myeloid and bone marrow origin (5), and in the course of this balancing regulation, moDCs also act as potent organizers of adaptive immunity leading to the maintenance of peripheral tolerance against the gut resident microbes. However, our knowledge about the interplay of molecular interactions during diet involving vitamin A supplementation, and the presence of gut microbiota species in the course of an ongoing human immune system is still limited in both health and diseases.

The uncontrolled disruption of the gut microbiota can be provoked by dysbiosis due to excessive hygiene conditions and/or the presence of antibiotics. This microbial perturbation may play role in the pathogenesis of chronic inflammatory and autoimmune diseases such as inflammatory bowel diseases (IBD), celiac disease, allergy, and metabolic and neurobehavioral diseases. For example, in Crohn's disease, the ratio of *Proteobacteria* could be increased (6), while the diversity and the fraction of *Firmicutes* in the gut microbiota are decreased (7). Colonization with commensal *Escherichia coli* 083 and *Lactobacillus rhamnosus* strains in early life is able to decrease the incidence of allergies and atopic dermatitis, respectively (8, 9). The various effects of probiotic gut bacteria also may prevent infection by pathogens such as the probiotic *E. coli Nissle* 1917 strain, which is able to inhibit the growth of enteropathogenic *E. coli*, which also may serve as a safe strain in IBD treatment (10–12).

Here, we focus to the underlying mechanisms involved in the recognition and processing of different species of gut commensal and beneficial bacteria and to their ability to polarize helper T (Th) lymphocytes. Considering that the human commensal microbiota is personalized (13) and exhibits high heterogeneity, it also contributes to the development of protective immune responses against pathogens *via* modulating the type and the composition of gut resident effector T cells (13–15). It is well established that pathogenic microbes or pathobionts, including fungal and bacterial species, are able to induce different types of immune responses (16, 17), which are modulated by external and internal signals. However, the means how non-pathogenic gut commensal species contribute to the coordination and fine tuning of immune responses by moDCs is not completely uncovered. In line with this, the primary goal of this study was to characterize a selected set of the normal gut microbiota including *Escherichia coli var. mutabilis* (*E. coli Schaedler*), *Morganella morganii* from *Proteobacteria*, and probiotic *Bacillus subtilis* 090 from *Firmicutes*, all with individual immunogenic and/or modulatory potential during moDC maturation and T-lymphocyte polarization. As it has previously been described, *E. coli Schaedler* and *M. morganii* exert unique stimulatory effects on the developing immune system and are also able to induce oral tolerance in mice (18), while *B. subtilis* is widely used in veterinary practice based on the active constituents of probiotic Monosporyn™ developed at the Uzhhorod National University. Upon interaction with the mucosal immune system, tolerogenic immune responses are raised against commensal and beneficial microbes. However, it is still poorly understood how the special but highly complex and dynamic intestinal milieu impacts the differentiation program of moDCs and the outcome of moDC-mediated immunological processes initiated by normal microbiota members and probiotic bacteria such as *B. subtilis* 090.

The differentiation program of monocytes during moDC generation is initiated by granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 and is regulated by the peroxisome proliferator-activated receptor gamma (PPARγ) (19). PPARγ is known to collaborate with retinoid receptors and acts as a master transcriptional regulator in human moDC differentiation and function (19). In addition, a set of genes encoding proteins related to metabolism, lipid antigen processing and presentation, invariant natural killer T (iNKT) cell activation, and RA synthesis are regulated by PPARγ and overlaps with those regulated by retinoic acid receptor alpha (RARα) (20–23), showing that RARα also serves as a master regulator of moDC functions. In humans, the vitamin A derivate all-*trans* retinoic acid (ATRA) is produced endogenously from retinol by DCs, macrophages, and epithelial and stromal cells (20, 24–27) and binds to RARα and retinoic X receptor alpha (RXRα) with different affinities (28) and enables to follow up the modulatory effects of the retinoid pathways in moDC-mediated immune responses. Besides targeting the highly conserved receptor RARα (29), ATRA also serves as a potential therapeutic drug in anticancer settings (30) and in combinations with other therapeutic agents such as GM-CSF (31) able to promote myelomonocytic differentiation.

We hypothesize that human monocytes migrating from the blood to the intestinal *lamina propria* have access to these special microenvironments, which are conditioned by growth factors and metabolites, including GM-CSF, exogenous and/or endogenous ATRA, and take part in the coordination of immune responses raised against the targeted gut commensal species. Intestinal mononuclear cells express mucosa-associated cell surface molecules such as CX_3CR1 and/or CD103 (32, 33). The main sources of human intestinal CX_3CR1^+ DCs are circulating monocytes, which lose this marker within 24 h (34). In contrast to this event, the CX_3CR1 chemokine receptor remains expressed on the cell surface of intestinal mononuclear phagocytes and acts directly as an inflammatory and migratory cell population with high phagocytic capacity (34–37), while mucosal $CD103^+$ DCs have been described as a dominant migratory population involved in triggering regulatory T cell responses raised against commensal bacteria *via* producing RA (38).

Based on this concept, *in vitro* conditions were designed to analyze the canonical pathways leading to the ATRA-modulated expression of the contributing master transcription factors including retinoid receptors, PPARγ and interferon regulatory factor 4 (IRF4) playing role in moDC differentiation in line with the impact of different, individual commensal bacteria exerted on moDC-mediated inflammation and effector T-lymphocyte priming. In this context, we will follow up the phenotypic changes and the functional activities of moDC populations by monitoring their phagocytic potential, inflammatory nature, and immunogenicity. Taken the unique intestinal microenvironment and the complex interplay of various exogenous effects, we sought to demonstrate how external and internal stimuli derived from the engulfed commensal *E. coli Schaedler*, *M. morganii*, and the probiotic *B. subtilis* bacteria may impact on the development of effector T-lymphocyte activation and polarization followed up by the production of interferon gamma (IFNγ) and IL-17 cytokines.

MATERIALS AND METHODS

Bacterial Strains and Reagents

The experiments were performed with the commensal bacteria as follows: *E. coli var. mutabilis (Schaedler)* (O83:K24:H31, member of the original Schaedler's flora), *M. morganii*, and *B. subtilis* 090. *M. morganii* was kindly provided by Michael Potter, National Institutes of Health, strain *E. coli Schaedler* was obtained from Russel Schaedler, USA, and *B. subtilis* 090 was provided by Nadiya Boyko, National University of Uzhhorod, Ukraine. Both commensal gut microbiota strains were received by the R&D Centre for Molecular Microbiology and mucosal immunology from Pennsylvania University in the framework of a research cooperation agreement. ATRA, the selective RARα antagonist BMS-195614 (BMS614), the vehicle dimethyl-sulfoxide (DMSO), and the anti-hβ-actin mAb were from Sigma-Aldrich, Schnelldorf, Germany. The anti-hIRF4 antibody was from Cell Signaling Technology, Inc. (Trask Lane, Danvers, MA, USA).

Human moDC Cultures

Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation and anti-CD14 microbeads, according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). After separation on a VarioMACS magnet, 96–99% of the cells were shown to be $CD14^+$ monocytes, as measured by flow cytometry. Isolated monocytes were cultured for 2 days in 12-well tissue culture plates at a density of 5.0×10^5 cells/ml in Gibco's serum-free AIM-V medium (Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium) and 100 ng/ml IL-4 (PeproTech EC, London, UK). The cells were differentiated in the presence or absence of 1 nM ATRA followed by a 75-min incubation period with or without 1 μM BMS614 specific RARα-antagonist at 37°C atmosphere containing 5% CO_2.

Bacterial Growth for moDC Activation

Selected gut commensal bacteria were grown in 2% lysogeny broth medium (Serva Electrophoresis GmbH, Heidelberg, Germany) for overnight with shaking at 37°C. Bacterial suspensions were washed with 25 ml sterile phosphate-buffered saline (PBS) three times and OD_{600nm} was measured by spectrophotometry and converted to cell/ml following $OD_{600nm} \times 2.5 \times 10^8$ CFU/ml. Human moDC cultures were activated with the specific toll-like receptor ligand bacterial lipopolysaccharide (LPS) (250 ng/ml ultrapure LPS, InvivoGen, San Diego, CA, USA) and with live commensal bacteria at a non-toxic ratio of 1:0.4 and were co-cultured for another 24 h.

Phagocytosis Assay

Live bacterial cells were centrifuged at $1{,}000 \times g$ for 5 min and washed three times in 25 ml PBS. Suspensions of bacterial cells were heat inactivated by heating at 65°C for 45 min and were re-suspended in 0.25 M carbonate–bicarbonate buffer (pH 9.0). The heat-killed bacterial cell suspensions (900 μl) were stained with 100 μl fluorescein-isothiocyanate (FITC) used at 5 mg/ml dissolved in DMSO and were rotated overnight at 4°C in dark. FITC-labeled bacteria were washed three times with cold PBS and were co-incubated for 3 h with moDCs at 37 or 4°C at a moDC:bacteria ratio of 1:20. moDCs positive for FITC-labeled bacteria were analyzed by flow cytometry using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA).

Flow Cytometry

Phenotyping of resting and activated moDCs was performed by flow cytometry using anti-human CD1d-phycoerythrin (PE), CD103-FITC, HLA-DQ-FITC, PD-L1-PE (BD Biosciences, Franklin Lakes, NJ, USA), CD1a-allophycocyanin (APC), CD40-FITC (BioLegend, San Diego, CA, USA), CX_3CR1-PE, CD80-FITC, CD83-FITC, CD86-PE, DC-SIGN-FITC, CCR7-PE, CD14-PE (R&D Systems, Minneapolis, MN, USA), B7RP1 (ICOSL)-PE (EBiosciences, Santa Clara, CA, USA), and isotype-matched control antibodies. The ratio of regulatory T-lymphocytes was measured by flow cytometry using anti-human CD25-PE (BD Pharmingen), CD4-FITC (BioLegend), FoxP3-APC (R&D Systems), and anti-IL-10-AlexaFluor488 (BioLegend). The viability of moDCs was determined with 2 μg/ml 7-amino-actinomycin D (LKT Laboratories Inc., St. Paul, MN, USA) dye followed by a 24-h activation period with live bacteria or LPS. Fluorescence intensities were measured by FACSCalibur (BD Biosciences), and data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA).

RNA Isolation, cDNA Synthesis, and Real-time Quantitative PCR

Briefly, total RNA was isolated by TriReagent (Molecular Research Centre, Inc., Cincinnati, OH, USA). Total RNA (1 μg) was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific). Gene-specific TaqMan assays (Thermo Fischer Scientific) were used to perform qPCR in a final volume of 12.5 μl in triplicates using DreamTaq DNA polymerase and ABI StepOnePlus real-time PCR instrument. Amplification of h36B4 was used as normalizing controls using specific primers and probe (Integrated DNA Technologies, Coralville, IA, USA). Cycle threshold values were determined using the StepOne Software, version 2.1 (Thermo Fischer Scientific). The sequences of the primers and probes are available upon request.

Measurement of Cytokine Concentration

Culture supernatants of moDCs were harvested 24 h after moDC activation, and the concentration of TNF-α, IL-1β, IL-6, IL-10, IL-12(p70), IL-23(p19) cytokines, and chemokine CXCL8 was measured using OptEIA kits (BD Biosciences) following the manufacturer's instructions.

Stimulation of moDCs to Measure T-Lymphocyte Polarization

To analyze the polarized effector T cells, immature and activated moDCs were washed and co-cultured with peripheral blood lymphocytes (PBLs) for 4 days in AIM-V medium at a moDC:T-cell

ratio of 1:20. The T cells were analyzed for IFNγ and IL-17 secretion by the avidin-horseradish peroxidase (HRP)-based enzyme-linked ImmunoSpot system (NatuTec GmbH, Frankfurt am Main, Germany). The co-cultures containing resting moDCs and T-cells as well as T-cells alone served as negative controls. To detect IL-17 secretion, the plates were coated with 0.5 μg/ml mouse anti-hCD3 antibody (BD Biosciences). The plates were analyzed by using the ImmunoScan plate reader (Cell Technology Limited, Shaker Heights, OH, USA). To detect regulatory T-lymphocytes, activated and resting moDCs were washed and co-cultured with PBL or naïve $CD4^+$ T-lymphocytes for 6 days in serum-free AIM-V medium at a moDC:T-cell ratio of 1:10. On day 6, cells were harvested, permeabilized, and fixed with Citofix/Cytoperm intracellular staining kit (BD Biosciences). The ratio of $CD4^+CD25^+FoxP3^+$ T cells was measured by flow cytometry. To detect the presence of intracellular IL-10, T cells were treated on day 6 with Golgi-Stop™ containing monensin (BD Biosciences) for 6 h followed by the surface CD25, CD4, and intracellular FoxP3 and IL-10 staining of cells. Naïve $CD4^+$ T-lymphocytes were isolated by the Naïve $CD4^+$ T Cell Isolation Kit II, human (Miltenyi Biotec).

Stimulation of moDCs to Measure iNKT Cell Expansion

Two-day moDCs were co-incubated with live bacteria, LPS, or 100 ng/ml α-galactosylceramide (α-GalCer, KRN7000, Funakoshi, Tokyo, Japan) for 24 h in AIM-V medium. Activated and resting moDCs were washed and co-cultured with PBL for 5 days in AIM-V medium at a moDC:T cell ratio of 1:10 in 24-well plates in AIM-V medium. On day 5, cells were labeled with anti-human CD3-PECy5, T cell receptor (TCR) Vα24-FITC, TCR Vβ11-PE monoclonal antibodies (Beckman Coulter, Brea, CA, USA), and the double-positive iNKT population was monitored by flow cytometry using FACSCalibur.

Western Blotting

Cells were lysed in Laemmli buffer, and the protein extracts were tested by antibody specific for IRF4 diluted to 1:1,000; secondary antibodies were used at 1:10,000. Anti-rabbit antibody, conjugated to HRP (GE Healthcare Life Sciences, Little Chalfont Buckinghamshire, UK), was used as a secondary antibody. The SuperSignal ECL system was used for probing target proteins (Thermo Fischer Scientific). After the membranes had been probed for the target protein, they were stripped and re-probed for β-actin.

Statistical Analysis

Student's unpaired two-tailed *t*-test or ANOVA followed by Bonferroni's multiple comparison tests were used as indicated in the relevant experiments. In case of significantly different variances ($P < 0.05$) between the two sets of samples, the Welch's correction was applied in the *t*-test. The results were expressed as mean + SD. All analyses were performed by using the GraphPad Prism software, version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered to be statistically significant at $P < 0.05$. Significance was indicated as $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; and $^{****}P < 0.0001$.

RESULTS

The Expression Profile of Master Transcription Factors and the Cell Surface Expression of CD1 Glycoprotein Receptors Differ in Human moDCs

We found that in the presence of 1 nM ATRA, monocytes generated in the presence of GM-CSF and IL-4 induced the differentiation of monocytes to moDCs within 2 days accompanied by the increasing expression levels of genes encoding the nuclear hormone receptor RXRα as well as its dimerization partners RARα and PPARγ in line with the aldehyde dehydrogenase-1 family member A2 (ALDH1A2)/retinaldehyde-dehydrogenase 2 (RALDH2) gene (**Figure 1A**) playing role in the regulation of retinoic acid production in moDCs. In the absence of ATRA, the CD1d gene was expressed in moDCs at low levels, but the CD1d gene transcripts and the cell surface expression of the translated protein was upregulated, while in ATRA-conditioned moDCs, the cell surface expression of CD1a decreased (**Figure 1B**). Moreover, on days 2 and 3, the differentiation of moDCs could be re-programmed to induce CD1d but inhibited CD1a expression, respectively (data not shown). Importantly, the cell surface expression of the DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) remained constant at these conditions (**Figure 1C**), while ATRA maintained the expression level of CD14 (**Figure 1D**) suggesting a decelerated differentiation phase of moDCs.

Dendritic cells can also be classified according to the expression levels of the transcription factors guiding both DC differentiation and re-programming (39, 40). Murine models suggested that $CD11b^+$ bone marrow-derived DCs cultured in the presence of GM-CSF and IL-4 express IRF4 and regulate the cell surface expression of the major histocompatibility gene complex II (MHC class II), while IRF4 increases the antigen-presenting capacity of moDCs resulting in potent T helper cell priming (41). In this human *in vitro* model system, we also found that moDCs express CD11b independent on the presence of ATRA (**Figure 1E**). Interestingly, ATRA was able to downmodulate the gene expression levels of IRF4 (**Figure 1F**) while upregulated the cell surface expression of CD103 (**Figures 3D,E**). Importantly, the relative mRNA level of interferon regulatory factor (IRF)8, responsible for regulating CD103 protein expression in DCs (41), remained unaffected by ATRA (**Figure 1G**). Collectively, these results demonstrate that nanomolar concentration of ATRA has the potential to modify the moDC differentiation program in a coordinated manner leading to increased mRNA levels of PPARγ, retinoid receptors, ALDH1A2, and CD1d, while the expression of CD1a and IRF4 remained inhibited. Based on this finding, we were able to identify two separate moDC subsets exhibiting distinct phenotypic characteristics based on the expression patterns of CD1 and CD103 proteins and transcription factors. The ATRA-primed $CD1a^-CD103^+CD1d^+$ cells are the $RARα^{hi}IRF4^{lo}$ subpopulation, and in contrast to this combination, the $CD1a^{+/-}$ $CD103^-CD1d^-$ cells are identified as a resting $RARα^{lo}IRF4^{hi}$ cell population.

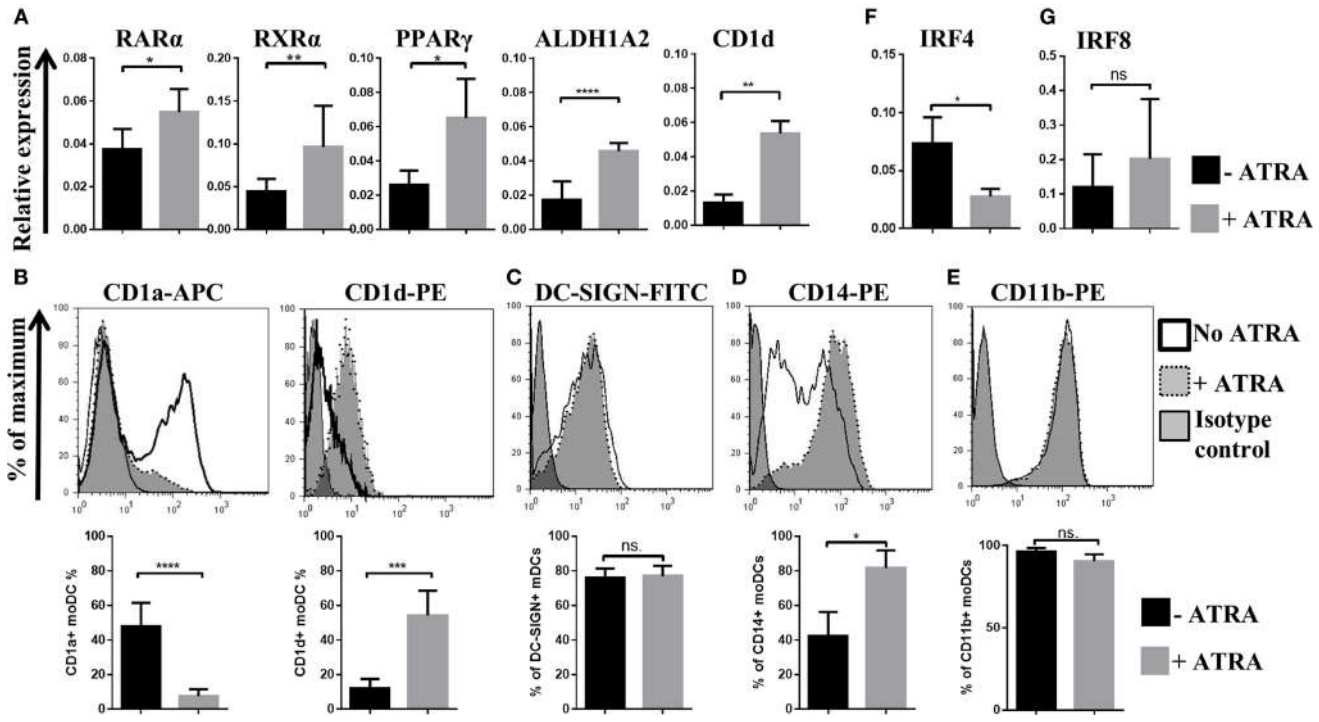

FIGURE 1 | The effects of all-*trans* retinoic acid (ATRA) on human monocyte-derived dentritic cell (moDC) differentiation. Two-day moDCs were differentiated in the absence or presence of 1 nM ATRA. The relative gene expression levels of retinoic acid receptor alpha (RARα), retinoic X receptor alpha (RXRα), peroxisome proliferator-activated receptor gamma (PPARγ), ALDH1A2, and CD1d **(A)**, interferon regulatory factor (IRF)4 **(F)**, and IRF8 **(G)** were measured by quantitative real-time PCR, and the cell surface expression level of CD1a, CD1d **(B)**, DC-SIGN **(C)**, CD14 **(D)**, and CD11b **(E)** was measured by flow cytometry. Mean values of relative mRNA levels and the ratio of moDCs positive for the measured cell surface proteins were calculated from five independent experiments +SD. Student's unpaired two-tailed *t*-test was used in the statistical analysis with significance defined as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Stimulation of RARα^{lo}IRF4^{hi} moDCs by Non-Pathogenic Commensal Bacteria Polarize Effector T-Lymphocytes Differently as Compared to RARα^{hi}IRF4^{lo} Cells

Besides the novel finding showing that the outcome of the inflammatory response of DCs to engulfed commensal bacteria is determined by the unique characteristics of the tested microbes (42), we were able to follow-up the immunomodulatory properties of a given microbe though monitoring the activation state and the direction of cell polarization of moDC-mediated autologous T-lymphocytes. In this experimental setting, moDCs were activated by live *E. coli Schaedler* or *M. morganii* both of them being capable to increase the number of IFNγ-producing T-lymphocytes (**Figure 2A**). By contrast, the Th17 response could be activated by all of the tested species (**Figure 2B**). In addition, ATRA-conditioned moDCs exhibited a completely different T-lymphocyte stimulatory potential as compared to moDCs manipulated in the absence of ATRA. In this case, the number of IFNγ-secreting T cells was decreased, while that of the Th17 cells remained undetectable in the moDC–T cell co-cultures. Taken the individual features of commensal bacteria, the RARα^{lo}IRF4^{hi} moDCs could be activated by both *E. coli Schaedler* and *M. morganii* leading to the differentiation of CD4^{+}CD25^{+}FoxP3^{+} regulatory T-lymphocytes, while the RARα^{hi}IRF4^{lo} reduced this effect (Figures S1A,B in Supplementary Material). To confirm this unexpected observation, we validated the existence of the regulatory T-cell population by detecting the level of the IL-10 cytokine derived from CD4^{+}CD25^{+}FoxP3^{+} T-lymphocytes co-cultured with moDCs upon the prior activation by commensal bacteria (Figure S1C in Supplementary Material). Based on these results, we were able to identify two moDC populations, which respond to gut commensal species differently, but in a strain- and ATRA-dependent manner. To get further insight how microbiota species guide immune responses of distinct characteristics, we sought to analyze the impact of selected bacterial strains driving the differentiation and functional activities of moDCs by using various experimental approaches.

The Commensal *E. coli Schaedler* and the Probiotic *B. subtilis* Modulate the Cell Surface Expression of CD1, CX_3CR1, and CD103 Proteins in an ATRA-Dependent Manner

To test how gut microbiota strains may act on human moDC differentiation at *in vitro* culture conditions mimicking the intestinal milieu, the cells were exposed to stimulatory signals such as LPS and selected live commensal bacteria. At this experimental

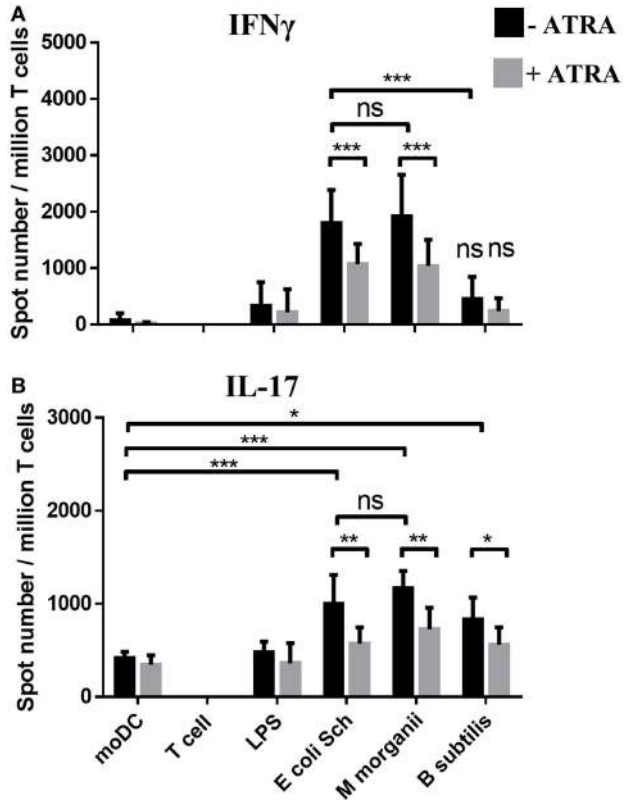

FIGURE 2 | Monitoring monocyte-derived dentritic cell (moDC)-mediated T-lymphocyte polarization induced by commensal stimuli. The T cell polarizing capacity of moDCs was monitored in moDC stimulated with *Escherichia coli Schaedler*, *Morganella Morganii*, and *Bacillus subtilis* or lipopolysaccharide (LPS) followed by co-culturing the cells with freshly isolated autologous T cells for 4 days. The number of cytokine producing T-lymphocytes induced by LPS or by moDCs exposed to commensal bacteria was measured by interferon gamma (IFNγ) **(A)** and interleukin (IL)-17 **(B)** enzyme-linked ImmunoSpot assays. T corresponds to T cells cultured without dendritic cells as negative control. The mean value of spot numbers was calculated from five independent experiments +SD. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

setting, exclusively *E. coli Schaedler* was capable to reduce the ratio of CD1a^{+} moDCs indicating the potential of this commensal bacterium to reduce CD1a expression selectively, but it had no effect on CD1d expression (**Figure 3A**), even though the viability of moDCs remained intact as compared to the immature cells (Figure S2 in Supplementary Material). Interestingly, *B. subtilis* exerted an opposing effect on the cell surface expression pattern of CD1 proteins, and LPS reduced the levels of both CD1d and CD1a in moDCs, while *M. morganii* had no effect on the cell surface expression level of CD1 proteins. These results indicated that lipid antigen presentation by moDCs *via* CD1a and CD1d proteins is regulated by both ATRA and the gut microbiota in a species-specific manner.

Using the *in vitro* system, we established the live commensal bacteria were able to upregulate the cell surface expression of CX_3CR1 within 24 h (**Figures 3B,C**) but had no effect on CD103 expression in the absence of ATRA (**Figures 3D,E**). Moreover, ATRA-conditioned moDCs downregulated the cell surface expression of CD103, but stimulation by commensal bacteria upregulated the CX_3CR1 receptor. These data altogether confirmed that in the presence of live commensal bacteria, ATRA drives the differentiation of moDCs leading to either synergistic or inhibitory directions, thus modulating the cell surface expression pattern of CD1 and that of the gut-tropic proteins.

The Phagocytic Capacity of moDCs Depends on the Individual Characteristics of the Tested Bacteria and on Actual Environmental Cues

The very first steps of moDC activation and the induction of antigen-induced immune responses are assisted by the phagocytic potential and the standby physiological activities of moDCs (42). These events can be further modulated by the unique characteristics of the internalized corpuscular antigens as well as by the cell surface receptor repertoire of the given cell. To assess the phagocytic potential of the previously identified moDC populations, we established an *in vitro* phagocytosis assay in which the FITC-labeled heat-inactivated bacteria were exposed to 37°C for 3 h, or were kept at 4°C as control (**Figures 3F,G**). As expected, the engulfment of commensal bacteria could be enhanced significantly and was found to be mediated by the RARα^{hi}IRF4^{lo} moDC population. When the moDCs were co-incubated with FITC-labeled bacteria at 4°C, background fluorescence intensities varied remarkably indicating differences in the individual functional characteristics of the tested commensal bacteria upon penetrating through the moDC membrane. These results altogether confirmed that in the presence of gut-derived microbial stimuli ATRA supports the differentiation of phagocytic CD1a^{-}CD1d^{+} moDCs, while the expression of the gut-tropic protein CD103 is partially downmodulated. It was also observed that in the absence of ATRA, the gated CD1a^{-} and CD1a^{+} moDC fractions engulfed the tested bacteria with similar activities as the CD1a^{+} cells (data not shown). Consequently, the median fluorescence intensity values within the gated moDC populations of the FITC-labeled bacteria remained similar demonstrating that the efficacy of moDC-mediated phagocytosis depends on both the unique features and the species of the engulfed bacteria, and this effector mechanism can be further enhanced by ATRA.

Activation of RARα^{hi}IRF4^{lo} moDCs by Commensal Bacteria Provokes Exacerbated Inflammation as Compared to RARα^{lo}IRF4^{hi} moDCs

Next, we continued to monitor the inflammatory potential of the selected commensals. Exposure of moDCs to live commensal bacteria such as *E. coli Schaedler* and *B. subtilis* or LPS for 24 h was found to increase the cell surface expression of CD83, while ATRA could downmodulate this response significantly (**Figure 4A**). The cell surface expression of the chemokine receptor CCR7, playing an essential role in driving DC migration to reach the secondary

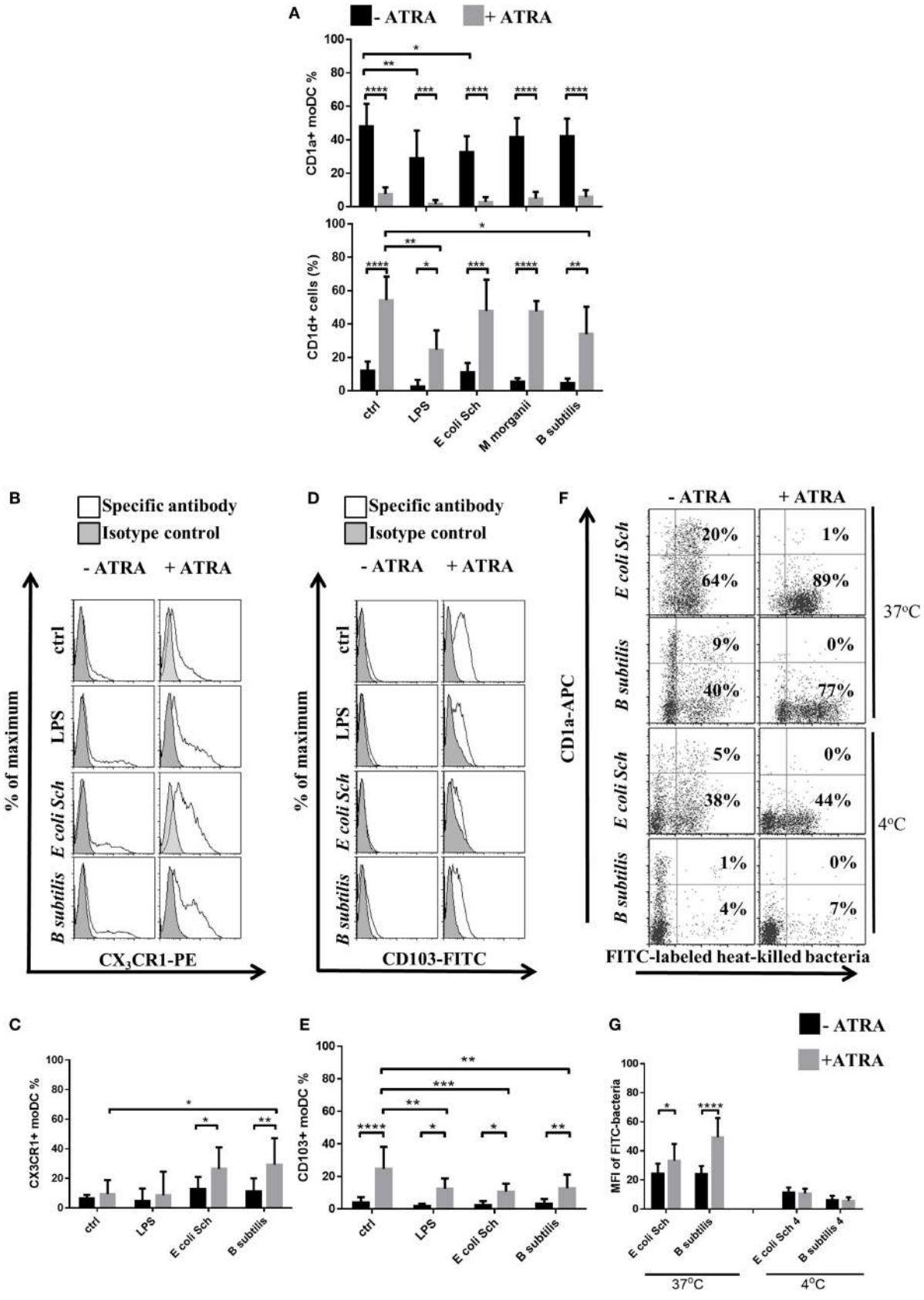

FIGURE 3 | Continued

FIGURE 3 | Continued

All-*trans* retinoic acid (ATRA) shifts the cell surface expression pattern of CD1, gut-related receptors, and the phagocytic capacity of monocyte-derived dentritic cells (moDCs) in an ATRA and commensal strain-dependent manner. Human moDCs were differentiated in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4 with or without 1 nM ATRA for 2 days. The surface expression level of CD1a and CD1d was measured on resting cells and moDCs activated with live commensal bacteria for 24 h **(A)** by flow cytometry. Histogram overlays show results derived from 1 representative donor of 10. The cell surface expression level of the mucosa-related CX_3CR1 **(B,C)** and CD103 **(D,E)** was measured by flow cytometry followed by a 24-h activation period with live commensal bacteria or lipopolysaccharide (LPS) served as a positive control. Mean values showing the ratio of moDCs positive for the measured surface protein were calculated from five independent experiments +SD. To monitor the phagocytic capacity of moDCs, on day 2, moDCs were co-cultured with heat-inactivated and fluorescein-isothiocyanate (FITC)-labeled bacteria at 37°C or at 4°C for 3 h at a moDC:bacteria ratio of 1:20. **(F,G)** Dot plots show one of four independent experiments. The ratio of moDC positive for heat-inactivated and FITC-labeled bacteria was measured by flow cytometry. The number of moDCs carrying FITC-labeled bacteria was calculated from four independent experiments +SD. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

lymphoid organs, could also be induced in the presence of LPS or *E. coli Schaedler*, but the expression level of CCR7 remained inhibited in ATRA-treated moDC (**Figure 4B**). In line with these results showing the potential of microbial components to generate mature moDCs, we detected the species-specific production of inflammatory cytokines including TNF-α, IL-1β, IL-6, and CXCL8 chemokine (**Figure 4C**). Furthermore, *B. subtilis* was found to induce negligible pro-inflammatory cytokine production as compared to Gram-negative *E. coli Schaedler*, but the effects of *B. subtilis* could be boosted significantly upon ATRA treatment confirmed by the increased secretion of TNF-α, IL-1β, and IL-6. We also observed that *M. morganii* induced the expression of a similar panel of moDC-derived inflammatory cytokines as compared to that of *E. coli Schaedler* (data not shown).

These results collectively indicate that *E. coli Schaedler* and *B. subtilis* harbor individual moDC-provoking potential, while ATRA can boost the production of pro-inflammatory mediators. In contrast to this finding, the expression level of CCR7 becomes downmodulated presumably associated with its decreased migratory potential guided by the $RAR\alpha^{hi}IRF4^{lo}$ moDC population. Based on these results, we conclude that *E. coli Schaedler* acts as a potent inducer of inflammatory responses in moDCs accompanied by the production of TNF-α, IL-1β, and IL-6, while *B. subtilis* is less efficient to trigger TNF-α and/or IL-1β secretion.

E. coli Schaedler and *B. subtilis* Increase the T-Lymphocyte Stimulatory and Polarizing Capacity of moDCs but ATRA Interferes with This Effect

The first signal for Th cell activation derives from the interaction of the TCR with MHC class II–peptide complexes presented by antigen-presenting proteins such as HLA-DQ and HLA-DR inducible by LPS or by the selected microbiota strains (**Figure 5A**). When moDCs were exposed to LPS or to commensal bacteria, the cell surface expression of the CD80 and CD86 co-stimulatory molecules was increased (**Figure 5B**). In such an experimental system, the secretion of the regulatory cytokine IL-10 was independent on ATRA in case of moDC activation by bacteria. More importantly, the secretion level of the Th1 polarizing cytokine IL-12 was decreased, while that of the IL-23 cytokine was enhanced significantly in the $RAR\alpha^{hi}IRF4^{lo}$ moDC population (**Figure 5C**). Interestingly, *B. subtilis* was unable to induce IL-23 secretion and the level of IL-12 also remained lower than the effect provoked by moDCs in the presence of the Gram-negative commensal bacterium *E. coli Schaedler*.

Considering that the differentiation of T-lymphocytes is regulated by both co-stimulatory and inhibitory signals, the cell surface expression of known co-stimulators of T-lymphocytes were also monitored. The results revealed that the cell surface expression of the co-stimulatory molecule CD40 could be induced by LPS and also by the two commensal strains, and this effect could be slightly enhanced in $PPAR\gamma^{hi}IRF4^{low}$ moDCs upon activation by *E. coli Schaedler* (**Figure 5D**). The induction of the effector T cell inhibitor PD-L1 could also be achieved if moDCs were stimulated by *E. coli Schaedler* (**Figure 5E**), in contrast to *B. subtilis* or LPS with no such effects. These data altogether suggest that both LPS and gut-associated commensal bacteria can induce the cell surface expression of T cell co-stimulatory and inhibitory molecules on the moDC cell surface in a strain-dependent manner, while ATRA-activated moDCs exhibit impaired cell surface expression of MHC class II, co-stimulatory, and inhibitory cell surface proteins.

Limited Commensal-Induced Effector Responses Mediated by $RAR\alpha^{hi}IRF4^{lo}$ moDCs Are Associated with Augmented Inflammation That Can Be Rescued by the Selective Inhibition of RARα

In a next step, we addressed the question how T-lymphocyte stimulation and maturation may modulate moDC responses in the presence of ATRA or commensal bacteria. Taken the fact that differentiation of moDCs can be modified in the presence of 1 nM ATRA, we also confirmed that the blockade of RARα signaling by a specific antagonist resulted in the prevention of CD1d and CD103 expression, while in the presence of ATRA, the cell surface expression of CD1a remained similar as control cells (**Figure 6A**). The chemical antagonist of RARα, i.e., BMS614 was unable to increase the cell surface expression level of CD1a on the cell surface showing that a minimal concentration of endogenous ATRA is presented by moDCs.

In a further step, we also demonstrated that the enhanced secretion of the pro-inflammatory cytokines (**Figure 6B**) and IL-23 (**Figure 6C**) induced by commensal bacteria could be ameliorated by the prior blockade of RARα. Moreover, the reduced antigen-presenting capacity of the ATRA-conditioned moDCs could be restored by the inhibition of RARα (**Figure 6D**).

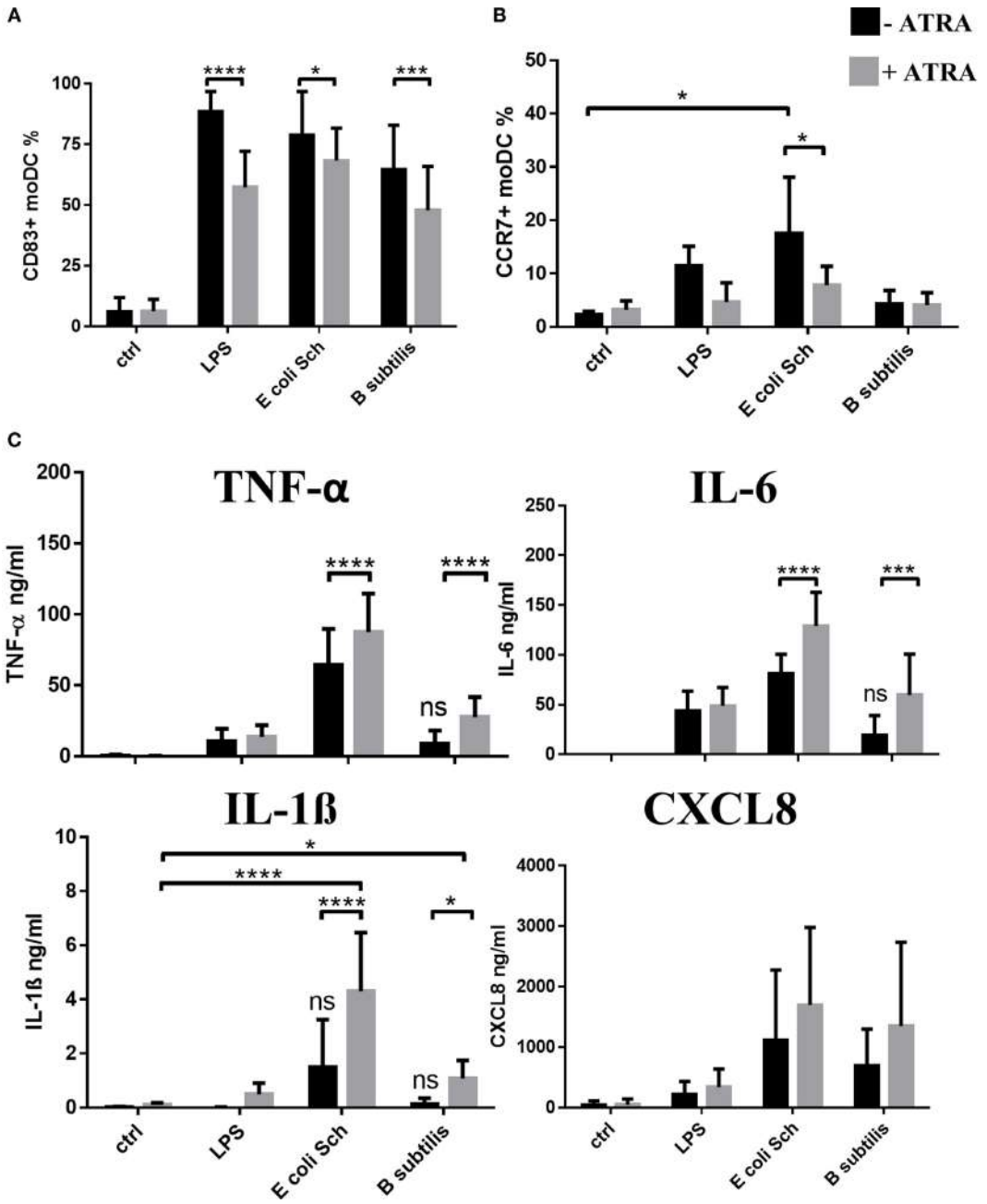

FIGURE 4 | Characteristics of the inflammatory and migratory potential of monocyte-derived dentritic cell (moDC) populations induced by commensal bacteria. Two-day moDCs were co-incubated with live commensal strains or with 250 ng/ml lipopolysaccharide (LPS) used as control for 24 h. Expression of the moDC-associated activation marker CD83 **(A)** and CCR7 **(B)** was measured by flow cytometry. Mean values were calculated from five to seven independent experiments +SD. The concentration of TNF-α, interleukin (IL)-1β, IL-6 pro-inflammatory cytokines, and the chemokine CXCL8 **(C)** was measured by ELISA followed by a 24-h activation of moDC in five independent experiments. Mean values +SD are shown. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Considering that the IRF4 transcription factor plays a pivotal role in setting the degree of DC-mediated antigen presentation (41), in a final experimental setting, we described for the first time in human moDCs that the protein level of IRF4 could be upregulated by live commensal bacteria and this effect could be decreased in a RARα-dependent manner (**Figure 6E**). As we expected, the decreased effector T-lymphocyte polarizing capacity of moDCs could be recovered by the selective blockade of RARα leading to strong Th1 (**Figure 6F**) and Th17 (**Figure 6G**) responses against the selected microbiota strains.

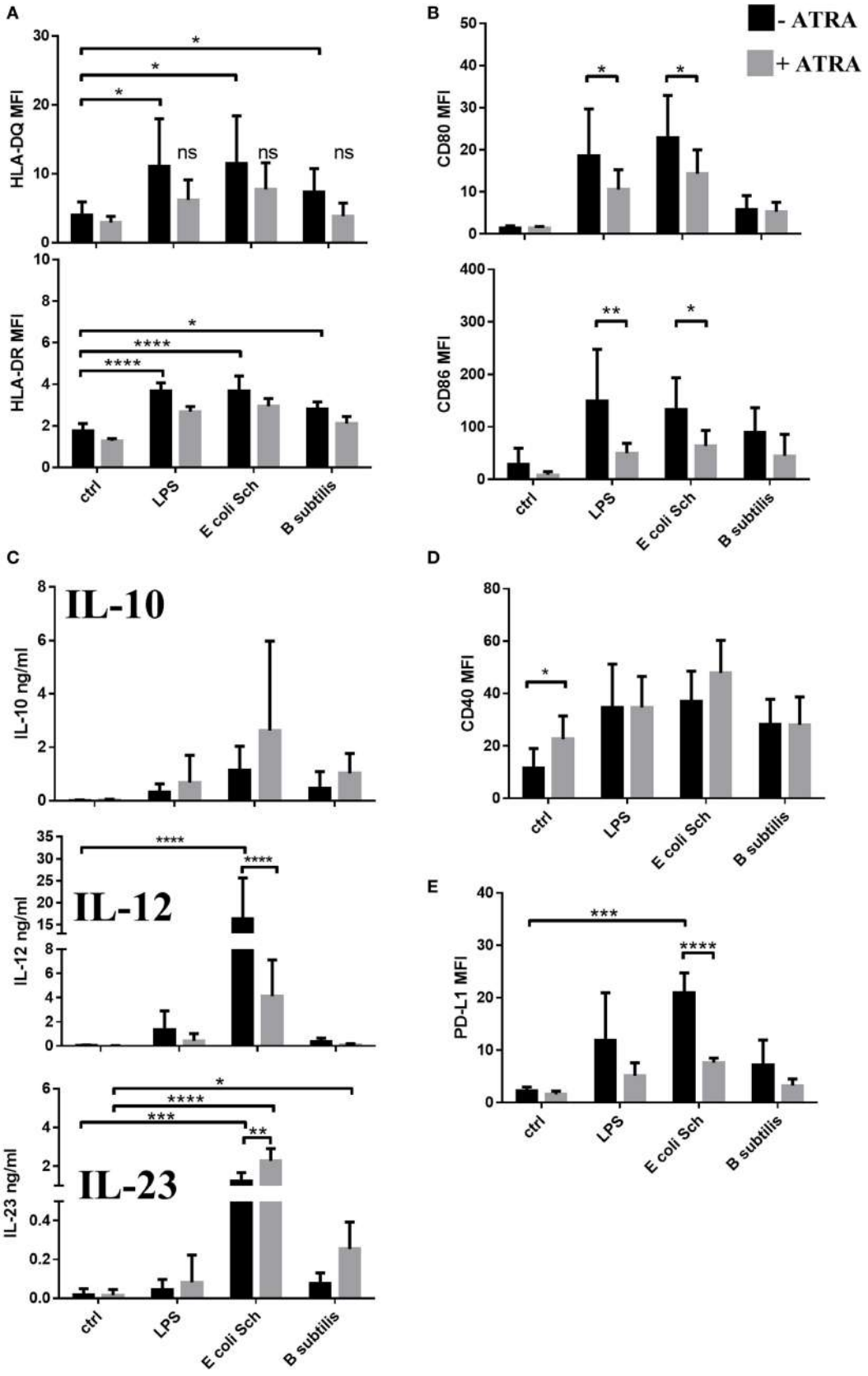

FIGURE 5 | **Continued**

FIGURE 5 | **Continued**

The T-lymphocyte activating and polarizing capacity of monocyte-derived dentritic cells (moDCs) activated by selected commensal bacteria. Two-day moDCs were co-incubated with live commensal strains or with 250 ng/ml lipopolysaccharide (LPS) used as control for 24 h. The expression levels of HLA-DQ and HLA-DR **(A)**, the co-stimulatory proteins CD80 and CD86 **(B)**, CD40 **(D)**, and the inhibitory molecule PD-L1 **(E)** was measured by flow cytometry. Mean values of median fluorescence intensities (MFIs) were calculated from five to seven independent experiments +SD. The concentration of interleukin (IL)-12, IL-23, and IL-10 cytokines was measured by ELISA followed by a 24-h activation of moDC and was tested in seven independent experiments **(C)**. Mean values +SD are shown. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Based on these results, we propose that the differentiation program of moDC initiated by GM-CSF and IL-4 can readily be modulated by ATRA, and this effect is associated specifically to the RARα nuclear receptor. In line with the results showing that ATRA is able to downmodulate the gene expression of IRF4 in both resting and ATRA-conditioned activated moDCs in the presence of commensal bacteria, the cell surface expression of antigen-presenting HLA-DQ molecules is decreased in a RARα-dependent manner.

DISCUSSION

This study focuses to the interplay of moDCs differentiated *in vitro* and designed to accommodate to various microenvironments having the potential to guide autologous effector T-lymphocyte functional activities. In this context, the polarization and the actual expression patterns of the cell surface molecules exhibiting co-stimulatory and/or inhibitory potential were monitored in the presence and absence of selected members of the gut microbiota exemplified by *E. coli Schaedler*, *M. morganii*, and *B. subtilis*. Based on our concept, the outcome of moDC differentiation is able to accommodate to unique cellular microenvironments (21, 44) and remains remarkably plastic until the terminal differentiation of the moDCs ensues. In line with this, we also demonstrated that during the very early phase of moDC differentiation, the cells remain programmable at physiologically relevant doses of environmental cues such as in the presence of nanomolar ATRA (45). Importantly, these events can be prevented by the selective ligation of RARα acting through its natural antagonist resulting in a moDC phenotype similar to that of the "gold standard" of moDCs (43) differentiated by GM-CSF and IL-4.

In a retinoid-rich milieu, moDCs shift the cell surface expression pattern of CD1 proteins, and in resting moDCs, the expression level of CD103 remains inducible supporting the development of a mucosa-related phenotype (46, 47). This observation allowed us to distinguish the characteristics of the expressed cell surface molecules such as CD1 and CD103 on various moDC types. These proteins can be expressed by the $CD1a^{+/-}CD1d^{-}CD103^{-}$ and the $CD1a^{-}CD1d^{+}CD103^{+}$ cell populations, respectively (**Figure 7A**).

We first characterized and compared the expression levels of the contributing transcription factors, including IRF4, PPARγ, and RARα in moDCs. DCs expressing IRF4 were shown to be the less potent inducers of cytotoxic T-lymphocytes as compared to cells expressing IRF8, a DC subset localized to the gut mucosa (39, 48). The results revealed that $IRF4^{hi}$ moDCs can be characterized as immunogenic cells provoking commensal-induced Th1 and Th17 immune responses, but this pattern could be reduced in case the T cells were primed with microbiota-stimulated $RARα^{hi}PPARγ^{hi}IRF4^{lo}$ moDCs supporting the notion that these cells remain highly inflammatory, lose their potential to activate autologous effector T helper cells, and also lack molecular interactions, which may play role in preventing effector T cell responses induced by commensal bacteria (**Figure 7B**). This observation is further supported by previous studies showing that the increased expression level and activity of PPARγ is associated with CD1d expression and the development of tolerogenic moDCs (20). Ligation of the CD40 cell surface molecule enhances the inflammatory potential of DCs (49) and the resting moDCs concomitantly conditioned with ATRA upregulate the cell surface expression of CD40, which can be further increased by *E. coli Schaedler* as compared to moDCs differentiated in the absence of ATRA. This observation is also confirmed by the concept that resting DCs express high levels of CD40 on the cell surface representing a semi-activated DC population with tolerogenic features (50, 51).

It has also been demonstrated that in the presence of heat-killed *E. coli Schaedler* and *B. subtilis* bacteria, the phagocytic capacity of moDCs could be facilitated by ATRA, similar to a previous work showing increased PPARγ activity in moDCs upon internalizing corpuscular antigens more efficiently than moDCs with low PPARγ activity (52). In addition to these findings, we also demonstrated that the stimulation of moDC with selected commensal bacteria resulted in moDCs expressing CX_3CR1 supported by ATRA and showing a phenotype similar to that of the $CD11b^{+}CX_3CR1^{+}CD103^{-}$ mononuclear mucosal phagocytes of myeloid origin. Moreover, in the presence of selected bacterial strains, the ATRA-primed moDCs induced the secretion of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-23 at high levels. Considering that these inflammatory cytokines play central role in the maintenance and/or disruption of mucosal integrity, exemplified by secreted IL-23 of both DC and macrophage origin. These regulatory circuits may serve as double-edged swords in the maintenance of balance in health and disease. The increased level of secreted IL-23 could directly be associated with several chronic inflammatory diseases including IBD (53). However, the presence of microbiota provide signals for both CX_3CR1^{+} inflammatory cells and $CD11b^{+}CD103^{+}$ DCs in the *lamina propria* to produce IL-23 and induce IL-22 secretion by innate lymphoid cells, thus playing a critical role in promoting mucosal healing in colitis (37, 54). Pro-inflammatory *lamina propria*-derived TNF-α can also exacerbate colitis through CX_3CR1^{+} DCs indicating that this DC subset also plays role in the maintenance of balanced inflammatory and/or standby conditions upon gut homeostasis (32).

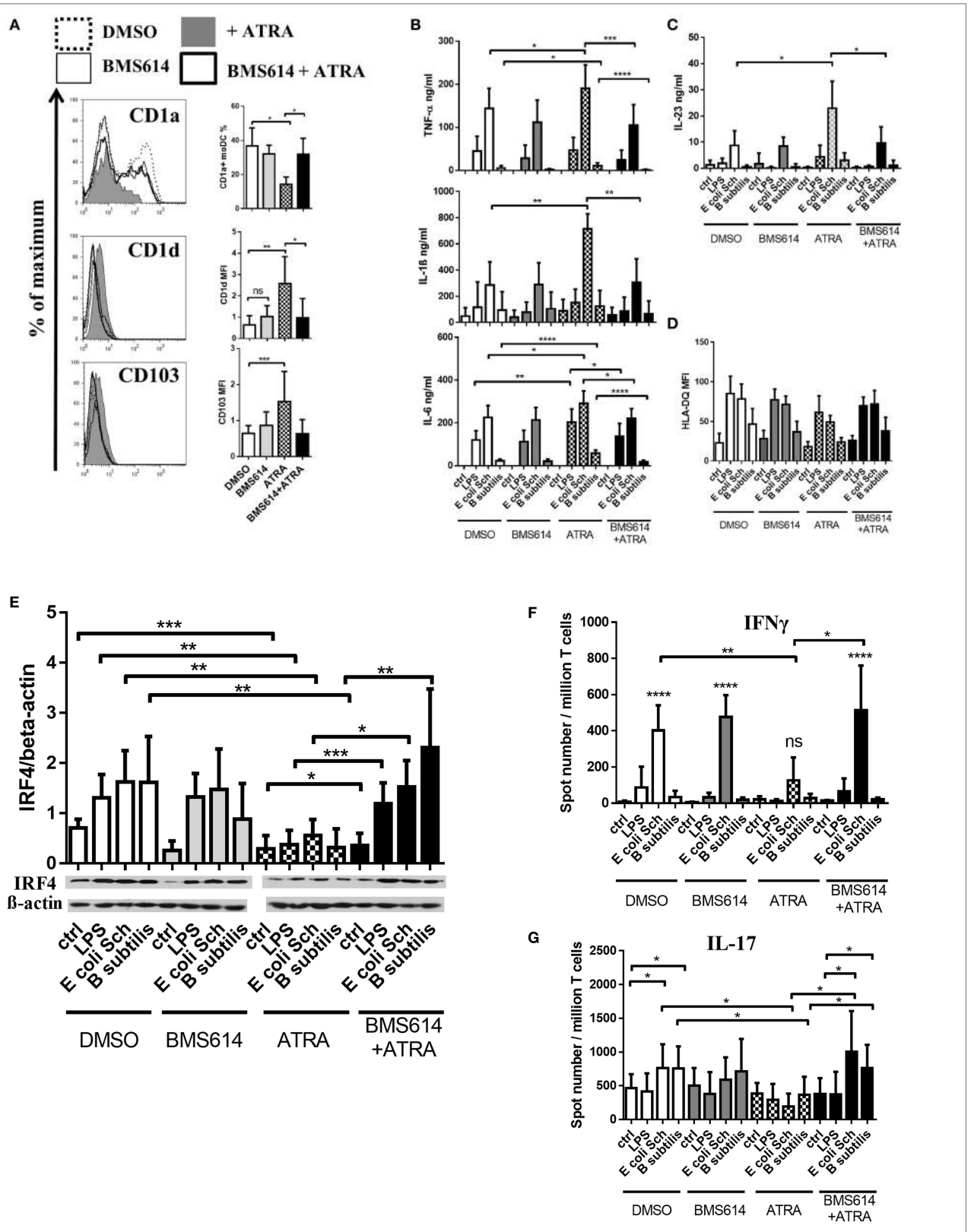

FIGURE 6 | **Continued**

FIGURE 6 | Continued

The selective inhibition of retinoic acid receptor alpha (RARα) prevents the all-*trans* retinoic acid (ATRA)-induced signature of microbiota-generated immune responses mediated by monocyte-derived dentritic cells (moDCs). To analyze how ATRA acts on the moDC-mediated immune response against microbiota species, the cells were treated with the RARα antagonist BMS614 prior to treating the cell culture medium with ATRA. The cell surface expression level of CD1a, CD1d, and CD103 was measured by flow cytometry in 2-day moDCs **(A)**. The concentration of TNF-α, interleukin (IL)-6, and IL-1β **(B)** and IL-23 **(C)** was measured by ELISA followed by a 24-h activation of moDC performed in seven independent experiments. Mean values +SD are shown. The cell surface expression level of HLA-DQ was measured by flow cytometry followed by a 24-h incubation period with live commensal bacteria. **(D)** Mean values of cells positive for the measured cell surface molecules were calculated from the results of seven independent donors +SD. Analysis of interferon regulatory factor 4 (IRF4) expression in moDCs. **(E)** Two-day moDCs were activated by live commensal bacteria for 24 h, and the relative expression levels of IRF4 protein was measured by Western blotting. Bar graphs show IRF4/β-actin ratios measured after 24 h of stimulation. Mean values of protein densities were calculated from five independent experiments +SD. The T cell polarizing capacity of moDCs was monitored in moDCs activated with the selected commensal strains or with lipopolysaccharide (LPS) followed by co-culturing the cells with autologous T cells. Freshly isolated peripheral blood lymphocytes were co-cultured with autologous moDCs for 4 days. The number of cytokine producing T-lymphocytes, induced by LPS or moDCs exposed to *Escherichia coli Schaedler* and *Bacillus Subtilis*, was measured by interferon gamma (IFNγ) **(F)** and IL-17 **(G)** enzyme-linked ImmunoSpot assays. The mean value of spot numbers was calculated from five independent experiments +SD. Statistical analysis was performed by the Student's unpaired two-tailed *t*-test with significance defined as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

In the presence of live bacteria, ATRA boosts the secretion of Th17 polarizing cytokines; however, the polarizing capacity of these moDCs is reduced. This observation is also supported by our previous study showing that moDCs "educated" by the supernatant of ATRA-primed colonic epithelial cells were able to reduce CCR7-dependent cell migration as well as their Th17 polarizing capacity as compared to control moDCs (44). Interestingly, in a murine model, Th17 differentiation was found to be dependent on IRF4 and IL-6 secreted by $CD11b^{+}CD103^{+}$ DCs derived from the mesenteric lymph nodes (55). The same group also showed that the human equivalent of these DCs could be identified as the intestinal IRF4 protein expressing $CD103^{+}SIRP\alpha^{hi}$ DCs.

Based on the known regulatory functions of DCs, this study demonstrates that the selected commensal bacteria also secrete IL-10, an inhibitory cytokine acting independently on the bacterial species. At our experimental conditions, the cell surface expression of PD-L1 protein became upregulated in a bacterial strain-dependent manner, which could be demonstrated also in the ATRA-primed moDCs, even though its expression level was significantly lower as compared to the respective ATRA free moDC counterpart. In addition to these results, the secretion of IL-12 cytokine with known inflammatory properties was downmodulated by ATRA as shown before by others (56). In contrast to these findings, we demonstrated that ATRA had no effect on IL-10 secretion in moDCs. Collectively, these data indicate that the decreased levels of IL-12, the reduced co-stimulatory and antigen-presenting capacity of $RAR\alpha^{hi}IRF4^{lo}$ moDCs, together with the production inhibitory IL-10 create a local milieu, which is inefficient to induce potent effector T helper cell responses upon targeting the selected gut microbiota species.

Our results clearly demonstrated that in resting moDCs, ATRA is able to upregulate the relative mRNA levels of RARα, previously confirmed also by others (56). In addition, we can exclude the effects of other RAR isoforms such as RARβ, as it is not expressed and the expression of RARβ could not be induced in moDCs in the presence of ATRA. It has also been shown that the effects of ATRA on the differentiation and the microbiota-induced stimulation of moDCs could be prevented by the selective inhibition of RARα, a transcription factor playing critical role in regulating moDC differentiation and guiding mucosal immune responses. It has also been found that the gut microbiota has an impact on retinoid signaling-mediated immune homeostasis transmitted by microbial metabolites such as short-chain fatty acids (57). Furthermore, retinoid supplementation through diet also acts on the composition of the gut microbiota and on energy metabolism of the host (58). For example, vitamin A deficiency causes perturbations in the gut microbiota by reducing the ratio of *Firmicutes* and *Proteobacteria* on a Myd88- and TRIF-dependent manner (59). It has previously been demonstrated that RA is associated to inflammatory macrophages, as patients with Crohn's disease exhibit an increased capacity to generate RALDH-derived RA, which is associated with $CD14^{+}$ macrophages derived from the intestinal mucosa, thus maintaining an inflammatory phenotype mediated by RARα (26). This group also showed that clinical samples derived from Crohn's disease patients involve both $CD103^{+}$ and $CD103^{-}$ DCs with elevated expression levels of the ALDH1A2 gene, which is undetectable in RA-producing macrophages. Retinoids involving ATRA also improves the antitumor immunity in microbiota-induced colorectal cancer, as it increases the efficacy of tumor-specific cytotoxic T-lymphocytes by increasing RARα-mediated MHCI expression in tumor cells (60).

Human moDCs not only provoke antigen-specific immune responses but also induce the activation and expansion of innate lymphoid cells; among them, iNKT cells (20, 61) and also present lipid antigens *via* cell surface CD1 glycolipid receptors. Remarkably, the level of CD1a and CD1d expression can be modified by commensal bacteria to different extents supporting the notion that this effect is not even related to the local lipid/retinoid environment, the activity of PPARγ (21), or the presence of pathogenic microbes (62), but their activities may resemble some microbiota species such as *E. coli Schaedler* and *B. subtilis*. moDCs with increased PPARγ activity also induce the expansion of IFNγ-secreting iNKT cell at high levels as compared to moDCs with low PPARγ activity (52). Surprisingly, we were unable to detect changes in the number of iNKT cells in moDCs stimulated by commensal bacteria, when the activated moDC–T cell cultures were tested. Instead, moDCs generated processed lipid antigens derived from commensal bacteria indicating that these lipids are unable to provide ligands for CD1a or CD1d proteins (Figure S3 in Supplementary Material). However, it was previously reported that bacterial colonization of the murine colon

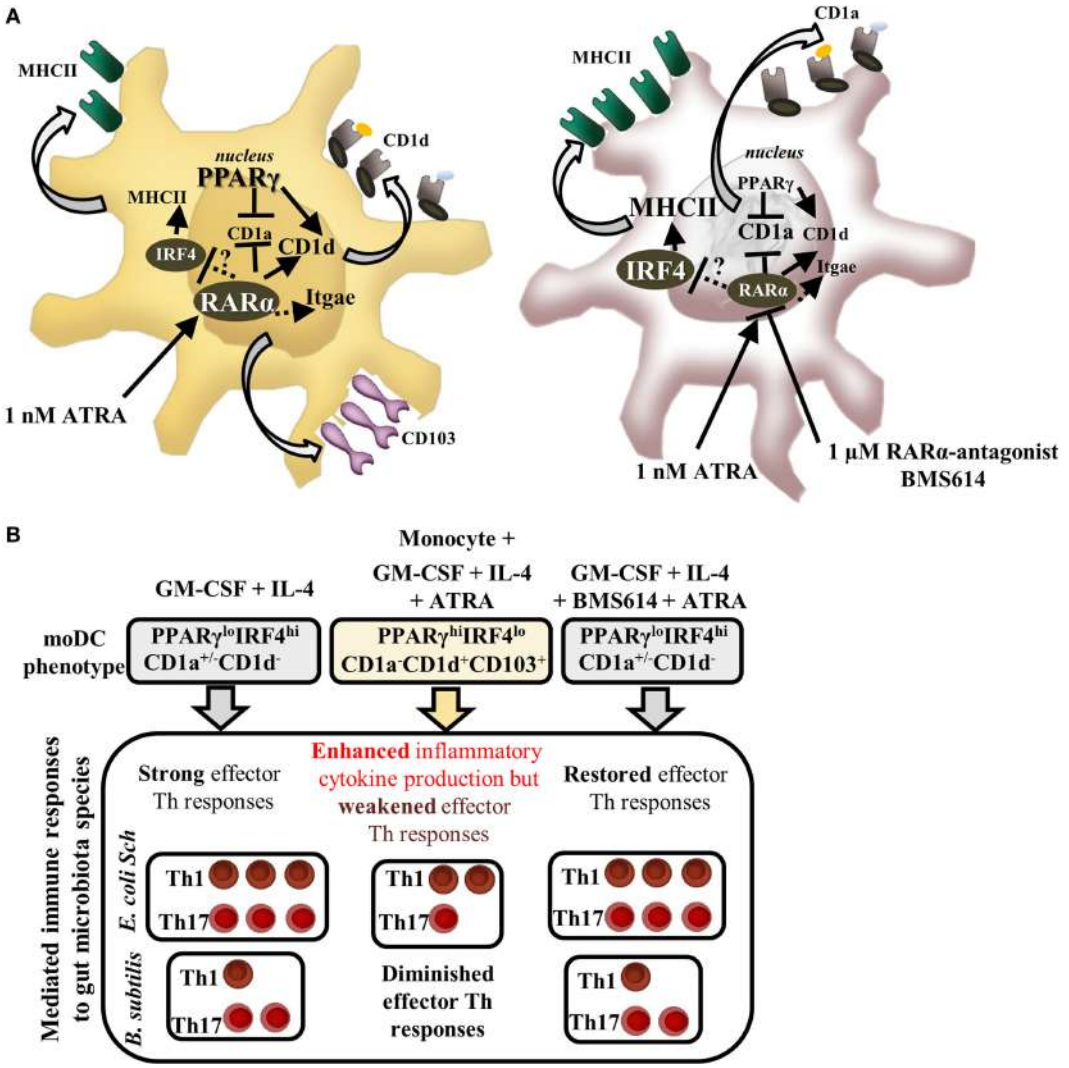

FIGURE 7 | The role of retinoic acid receptor alpha (RARα) in guiding monocyte-derived dentritic cell (moDC) development and microbiota-induced immune responses. All-*trans* retinoic acid (ATRA) modifies the differentiation of moDCs that could be prevented by the selective inhibition of RARα. **(A)** In the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, monocytes differentiate to $CD1d^{-}CD1a^{+}$ DCs (43). Peroxisome proliferator-activated receptor gamma (PPARγ) and RARα regulate the gene expression of CD1d and ALDH1A2 both directly and indirectly in human moDCs (20). Interferon regulatory factor 4 (IRF4) mediates the differentiation and antigen-presenting capacity of human moDCs, which is downregulated by the ligation of RARα resulting in decreased mRNA and protein levels of IRF4 together with CD1a. Selected microbiota species provoke different types of immune responses mediated by moDCs. **(B)** *Escherichia coli Schaedler* induces full maturation in moDCs leading to strong inflammatory and microbiota-induced effector helper T (Th) responses, while *Bacillus subtilis* induces inflammation in the absence of IL-12 and IL-23 and provokes decreased effector Th1/Th17 immune responses. ATRA downmodulates the immunogenicity of moDCs resulting in diminished Th1 and undetectable Th17 responses, which effect can be restored in moDCs by the prior inhibition of RARα in moDCs. Solid lines represent known mechanisms; dotted lines indicate unknown molecular interactions.

with *E. coli Schaedler* stimulates intestinal epithelial cells and intraepithelial innate lymphoid cells (63) independently, and this effect may play role in the pathogenesis of colitis as demonstrated in adoptive transfer models using SCID mice, which may also operate in patients with IBD.

Collectively, we offer a sensitive *in vitro* assay system appropriate for the comparative analysis of selected individual microbes in the course of collaboration with human phagocytic cells such as primary moDCs, playing essential roles in orchestrating the outcome of immune responses. We also confirmed that the vitamin A derivative ATRA has the potential to drive the differentiation program of moDCs in a RARα-dependent manner and thus confers suppressive signals during gut commensal bacteria-induced effector T-lymphocyte responses in line with enhancing their local inflammatory potential.

The interactions of diet, gut microbiota and the host build up a highly complex network of regulatory circuits to drive the development of both mucosal and systemic immune responses. Preferentially in early childhood, imbalances in food supplementation together with the acquired perturbance of the gut

microbiota increase the risk of chronic immune and metabolic disorders; however, how the environmental and genetic factors determine the outcome of such immune failures requires further analysis.

ETHICS STATEMENT

Leukocyte-enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service of the University of Debrecen, Faculty of Medicine (Hungary) and from the Regional and Institutional Research Ethical Committee of the University of Debrecen (DEOEC RKEB/IKEB 3855-2013). Written, informed consent was obtained from the blood donors prior blood donation, their data were processed and stored according to the directives of the European Union.

AUTHOR CONTRIBUTIONS

KB designed and performed the experiments, analyzed the results, organized the data, and wrote the manuscript. ZV contributed to protein-based experiments. VP contributed to the isolation and cultivation of commensal microbes. NB provided initial experimental idea and revised the manuscript. ER designed the concept, developed the interpretation, and revised the manuscript.

ACKNOWLEDGMENTS

This research was supported by the "Host/bacterial interaction—mechanisms of the regulation of mucosal immune response by the commensal bacteria," State Found of Fundamental Research (SFFR in Ukraine with the International Participation), 20/451-2007, 0107U008933, the Ukrainian-Hungarian Intergovernmental S&T Project: "Nosocomial Infections of population in Hungarian-Ukrainian Transborders Regions: current state and effective ways for their prevention," M/220-20070107U007971 and "Immunomodulatory role of the commensal microflora: new mechanisms, potential target cells and the prosperity of their usage," M/27-2009. This research was also supported by the TORNADO FP7-KBBE-2007-2A "Molecular targets open for regulation by the gut flora: *New avenues for improved diet to optimize European health*," a project supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TAMOP 4.2.4.A/2-11-1-2012-0001 and TAMOP 4.2.2.A-11/1/KONV-2012-0023. The authors thank Istvan Szatmari for revising the manuscript, Peter Gogolak for his valuable experimental advices, and Zsuzsanna Biro-Debreceni and Erzsebet Nagyne Kovacs for their excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00427/full#supplementary-material.

FIGURE S1 | **Regulatory T-lymphocyte polarizing capacity of monocyte-derived dentritic cell (moDC) populations stimulated by *Escherichia coli Schaedler*, *Morganella morganii*, and *Bacillus subtilis***. To detect the number of regulatory T-lymphocytes, resting and stimulated moDCs were co-cultured with peripheral blood lymphocyte for 6 days. The ratio of $CD25^{+}FoxP3^{+}$ Treg cells **(A,B)** and the interleukin (IL)-10-producing Treg cells **(C)** were analyzed by flow cytometry, respectively. Dot plots show one out of five independent experiments. The mean value of Treg cell numbers was calculated from five independent experiments +SD. In the statistical analysis, ANOVA followed by Bonferroni's multiple comparison tests were used with significance defined as $^{*}P < 0.05$.

FIGURE S2 | **Monitoring the viability of monocyte-derived dentritic cells (moDCs) exposed to live commensal bacteria in the absence or presence of all-*trans* retinoic acid (ATRA)**. moDCs were differentiated with or without ATRA for 2 days in serum-free culture medium. On day 2, moDCs were co-incubated with live commensal bacteria for 24 h followed by labeling the cells with 7-amino-actinomycin D (7-AAD) dye. Mean values of moDCs positive for 7-AAD staining were calculated from five independent experiments +SD.

FIGURE S3 | **The invariant natural killer T (iNKT) cell inducing capacity of monocyte-derived dentritic cell (moDC) populations stimulated by *Escherichia coli Schaedler* and *Bacillus subtilis***. To detect the number of iNKT cells, moDCs were stimulated with live bacteria or with lipopolysaccharide (LPS) followed by co-incubation with autologous peripheral blood lymphocyte for 5 days, and the moDC cultures were incubated with the CD1d ligand α-GalCer served as a positive control. The ratio of $CD3^{+}$ cells expressing Vα24Vβ11 T cell receptors was analyzed by flow cytometry. The mean values of iNKT cell numbers were calculated from three independent experiments +SD.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 27 December 2016
doi: 10.3389/fimmu.2016.00651

Local Treatment with Lactate Prevents Intestinal Inflammation in the TNBS-Induced Colitis Model

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Lactate has long been considered as a metabolic by-product of cells. Recently, this view has been changed by the observation that lactate can act as a signaling molecule and regulates critical functions of the immune system. We previously identified lactate as the component responsible for the modulation of innate immune epithelial response of fermented milk supernatants *in vitro*. We have also shown that lactate downregulates proinflammatory responses of macrophages and dendritic cells. So far, *in vivo* effects of lactate on intestinal inflammation have not been reported. We evaluated the effect of intrarectal administration of lactate in a murine model of colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). The increase in lactate concentration in colon promoted protective effects against TNBS-induced colitis preventing histopathological damage, as well as bacterial translocation and rise of IL-6 levels in serum. Using intestinal epithelial reporter cells, we found that flagellin treatment induced reporter gene expression, which was abrogated by lactate treatment as well as by glycolysis inhibitors. Furthermore, lactate treatment modulated glucose uptake, indicating that high levels of extracellular lactate can impair metabolic reprograming induced by proinflammatory activation. These results suggest that lactate could be a potential beneficial microbiota metabolite and may constitute an overlooked effector with modulatory properties.

Keywords: innate immunity, lactate, TNBS-induced colitis, flagellin, immunomodulation

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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *06 October 2016*
Accepted: *14 December 2016*
Published: *27 December 2016*

Citation:
Iraporda C, Romanin DE, Bengoa AA, Errea AJ, Cayet D, Foligné B, Sirard J-C, Garrote GL, Abraham AG and Rumbo M (2016) Local Treatment with Lactate Prevents Intestinal Inflammation in the TNBS-Induced Colitis Model. Front. Immunol. 7:651. doi: 10.3389/fimmu.2016.00651

INTRODUCTION

Inflammatory bowel disease (IBD) involves a group of chronic, inflammatory disorders of the gastrointestinal tract, including Crohn's disease and ulcerative colitis, affecting people of all ages including the pediatric population. The etiology of IBD is still unknown but is thought to be due to a combination of genetic, microbial, immunological, and environmental factors that result in an abnormal and excessive immune response against commensal microbiota (1). The intestinal microbiota profoundly regulates the host immune function under physiological conditions and is likely the most important environmental factor in IBD as the target of the inflammatory response (2).

Dysbiosis or a lack of specific bacteria with anti-inflammatory properties may be responsible for gut inflammation (3–6). Although the molecular mechanisms of host–microbiota interactions are still not fully elucidated, manipulation of microbiota by probiotics or prebiotics is becoming increasingly recognized as a therapeutic option, for the treatment of the dysfunction or inflammation of the intestinal tract (7). The metabolic output of the modification of gut microbiota is the production of different profiles of short chain fatty acids (SCFA) such as butyrate, propionate, and acetate, and these metabolites are of relevance in the modulation of key signaling pathways involved in the inflammation of the gastrointestinal mucosa (7–9).

The impact of probiotic bacteria on intestinal health with the aim to prevent IBD or improve its treatment has been studied (10–12), as well as it has been shown that metabolites present in the supernatants of fermented dairy products can exert a protective effect *ex vivo* on intestinal mucosa exposed to inflammatory insults (13).

Lactate is the main metabolite of many fermented products and can also be generated *in situ* on the intestinal mucosa. Although lactate has been known to biochemists for over 200 years, it has been considered as a mere intermediate of carbon metabolite with specific organoleptic/antimicrobial properties rather than a bioactive molecule. Recently, lactate has been rediscovered as an active signaling metabolite in multiple fields of biology and medicine (14). Lactate mediates signaling pathways on several cell types, including production of pro- and anti-inflammatory mediators by T cells and macrophages and migratory changes and metabolic adaptation in T cells, endothelial cells, and neurons. Intracellular lactate can directly bind to proteins, influence the redox state *via* the lactate dehydrogenase reaction, stabilize hypoxia inducible factor-1, induce reactive oxygen species, and act as an inhibitor of glucose breakdown (15). The occurrence of these effects might depend on the cell type. Hoque et al. (16) demonstrated that administration of lactate reduced inflammation and organ injury in mice with immune hepatitis (16). Moreover, besides immunomodulation, Okada et al. (17) showed that luminal lactate-stimulated enterocyte proliferation in a murine model of hunger feedback, contributing to maintain intestinal barrier function (17). We have recently shown that lactate abrogates TLR and IL-1β dependent NF-κB activation of intestinal epithelial cells (18) and can regulate critical functions of several key players of the immune system such as macrophages and dendritic cells (19). In order to determine if the immunomodulatory capacity of lactate operates *in vivo*, the present work evaluated the effect of lactate in innate-driven murine model of colitis.

MATERIALS AND METHODS

Chemicals and Reagents

Different chemical reagents used 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2 deoxyglucose (2DO), sodium oxamate, sodium 3-bromopyruvate (3BrPA) were purchased to Sigma Chemicals. DL-lactic acid (J. T. Baker) was employed. Flagellin was purified from *Salmonella*, detoxified, and controlled as previously described (20). Other proinflammatory stimulators, such as human interleukin-1β (IL-1β) and tumor necrosis factor (TNF), were purchased from R&D Systems (Minneapolis, MN, USA).

Animals

Male BALB/c AnN, 6 weeks old mice with weight over 20 g were purchased from Faculty of Science Veterinary from National University of La Plata, Argentina. The animals kept in polypropylene cages were maintained under standard conditions. The experimental protocols were approved by the Animal Ethics Committee of Faculty of Exact Sciences, National University of La Plata, Argentina (Approval No 011-01-15). Before conducting experiments, animals were acclimatized to animal facility conditions for 7 days.

Treatment and Induction of Experimental Colitis Using TNBS

Procedure was performed as previously described (21). Briefly, mice randomly divided into four groups were instilled with PBS (200 μL) (two groups) and with lactate solution in PBS 200 mM (200 μL) (two groups) by intrarectal route. Two hours post-administration, experimental colitis was induced by intrarectal instillation of 0.5 mg TNBS (SIGMA-Aldrich, USA) in ethanol 50% (v/v). Control animals were instilled with ethanol 50% (v/v) in distilled water. Enemas were gently instilled through a polyurethane catheter (18 G) inserted into the colon 4 cm proximally to the anal verge, and mice were held thereafter in a head-down position for 30 s. The weight of each mouse was determined and blood sampled at the beginning of the experiment and at 24 and 48 h. After 48 h, animals were sacrificed by cervical dislocation; colon tissues were collected for histological analysis (hematoxylin and eosin staining); and livers were aseptically taken to determine microbial translocation.

Serum IL-6 Determination

Blood was collected by submandibular bleeding and serum was isolated. Serum IL-6 determination was performed using BD Bioscience OptEIATM Mouse IL-6 ELISA Kit (Franklin Lakes, NJ, USA), according to manufacturer instructions.

Assessment of Colonic Epithelial Damage and Inflammation

Histopathological damage was determined following the criteria described previously (21). This system records two separate scores evaluating epithelial damage and infiltration. Briefly, the epithelial damage was scored as 0 for none, 1 for a minimal loss of goblet cells, 2 for extensive loss of goblet cells, 3 for a minimal loss of crypts and extensive loss of goblet cells, and 4 points for extensive loss of crypts; the infiltration was scored as 0 for none, 1 for an infiltrate around crypts bases, 2 for an infiltrate in *muscularis mucosa*, 3 for extensive infiltrate in *muscularis mucosa* with edema, and 4 points for the infiltration of submucosa. Preparations were assessed double blind, and the histopathological activity index was calculated as the sum of the epithelial damage and the infiltration score, ranging between 0 and 8 points from unaffected to severe colitis.

Microbial Translocation

Portions of liver were aseptically collected and placed in a sterile tube with a volume of BHI broth (Oxoid, England) in order to obtain 1 g organ/10 mL. These suspensions were homogenized, enriched in total viable bacteria by incubation 24 h at 37°C and used to inoculate BHI agar plates. Translocation of bacteria was defined by growth of microorganism on plates after 48–72 h of incubation at 37°C.

Cell Culture and CCL20:LUC Reporter Assay

Caco-2 cells stably transfected with a luciferase reporter construction under the control of the chemokine-ligand-20 (CCL20) promoter (Caco-2-*CCL20:LUC*) have been previously described (20). The cells were routinely grown in Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM, GIBCO BRL Life Technologies, Rockville, MD, USA); supplemented with 15% (v/v) heat-inactivated (30 min, 60°C) fetal-bovine serum (FBS, PAA, GE Healthcare Bio-Sciences Corp., USA), 1% (v/v) non-essential amino acids (GIBCO BRL Life Technologies, Rockville, MD, USA) and the following antibiotics (Parafarm, Saporiti SACIFIA, Buenos Aires, Argentina): penicillin (12 IU/mL), streptomycin (12 μg/mL), and gentamicin (50 μg/mL). Caco-2-*CCL20:LUC* cells were used at 24 h post-confluence after 8 days of culture at subculture passages between 12 and 22 from the original stocks. All experiments were performed in serum-free medium.

Confluent Caco-2-*CCL20:LUC* cells cultured in 48-well plates were treated for 30 min with different concentrations of lactate pH 7.4 or different solutions of glycolysis inhibitors. The cells were then exposed to stimulation by flagellin (1 μg/mL), Il-1β (10 ng/mL), or TNF-α (100 ng/mL), during 6 h at 37°C in an atmosphere of 5% CO_2—95% air. A basal condition without any treatment was included as a control lacking stimulation; while flagellin, TNF-α, or IL1-β was added to cell that did not receive any treatment as control of 100% of induction of the proinflammatory response. The cells were next lysed with lysis Buffer (Promega, Madison, WI, USA), and luciferase activity was evaluated using the Luciferase Assay Kit (Promega, Madison, WI, USA) following manufacturer's instructions and measured in a luminometer (Luminoskan TL Plus). Luminescence was normalized to the stimulated control cells and expressed as a percentage of the normalized average luminescence (% normalized luciferase activity) ± SD from at least three independent experiments.

Cytotoxicity Assay

As a method of assessing treatment-induced cytotoxicity, mitochondrial activity was evaluated employing commercial kit CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following manufacturer's instruction.

Glucose Consumption by Epithelial Cells against TLR5 Agonist Stimulation

Confluent Caco-2/TC-7 epithelial cells cultured in 48-well plates were incubated at 37°C, in controlled atmosphere 5% CO_2 in DMEM containing initially 2 g/L glucose. Glucose uptake was determined in the culture medium employing a commercial enzymatic kit (Wiener lab, Rosario, Argentina). Samples were taken after 3, 6, 15, and 24 h of incubation either in basal condition, stimulated with flagellin with and without lactate 100 mM in the culture medium.

Statistical Analysis

The results are expressed as mean ± SD. Data analysis was performed using Graph Pad Prism version 5.01 for Windows (GraphPad Software, CA, USA). Analyses of variance followed by Dunnet Test or Bonferroni Test were applied. A p-value <0.05 indicated a significant difference.

RESULTS

Lactate Treatment Prevents Tissue Inflammation, Early IL-6 Production, and Bacterial Translocation in a TNBS-Induced Colitis Model

To address the *in vivo* immunomodulatory capacity of lactate, we evaluated the capacity to protect mice from colitis induced by intrarectal administration of TNBS. During the experiment, we compared the development of TNBS-induced colitis in mice that received intrarectal administration of lactate 200 mM or PBS as control. Such administration guaranteed lactate contact with intestinal cells exposed to the TNBS. The intrarectal administration of PBS or lactate followed by vehicle administration did not induce any significant changes of animal weight. In contrast, the rectal administration of TNBS causes progressive weight loss reaching up to 15% of the initial weight at 48 h. In both TNBS-treated groups (PBS/TNBS and Lactate/TNBS), a significant weight loss was observed (Figure S1 in Supplementary Material). Although the differences were not significant, the weight loss was lower in lactate/TNBS than in PBS/TNBS (10 versus 15%). Histological features of colitis were observed in the PBS/TNBS group as determined by epithelial damage, loss of goblet cells, edema, and infiltration of immune cells, leading to a pathology index of 4.67 ± 2.33 (**Figure 1**). In contrast, the group of mice pretreated with lactate (lactate/TNBS) showed significant protection from TNBS-induced inflammation, with lack of epithelial damage and minimal edema. Indeed, the histological sections were similar to the control groups that did not receive TNBS (PBS/vehicle and lactate/vehicle). The histopathology index of 1.40 ± 0.54 was significantly different from that of PBS/TNBS group ($p = 0.039$) (**Figures 1A,B**). This was in concordance with a clear better behavior of lactate-treated animals, indicating that lactate treatment prevents intestinal inflammation in the TNBS colitis model.

Inflammation is associated with the production of various inflammatory mediators, primarily cytokines that are key players in the innate and adaptive immune responses. Levels of circulating IL-6 were determined in the different experimental groups before and 24 or 48 h after instillation (**Figure 2**). IL-6 levels were significantly increased 24 h after treatment with TNBS in control group but decreased to the baseline at 48 h. In contrast, lactate treatment abolished the production of circulating IL-6 at

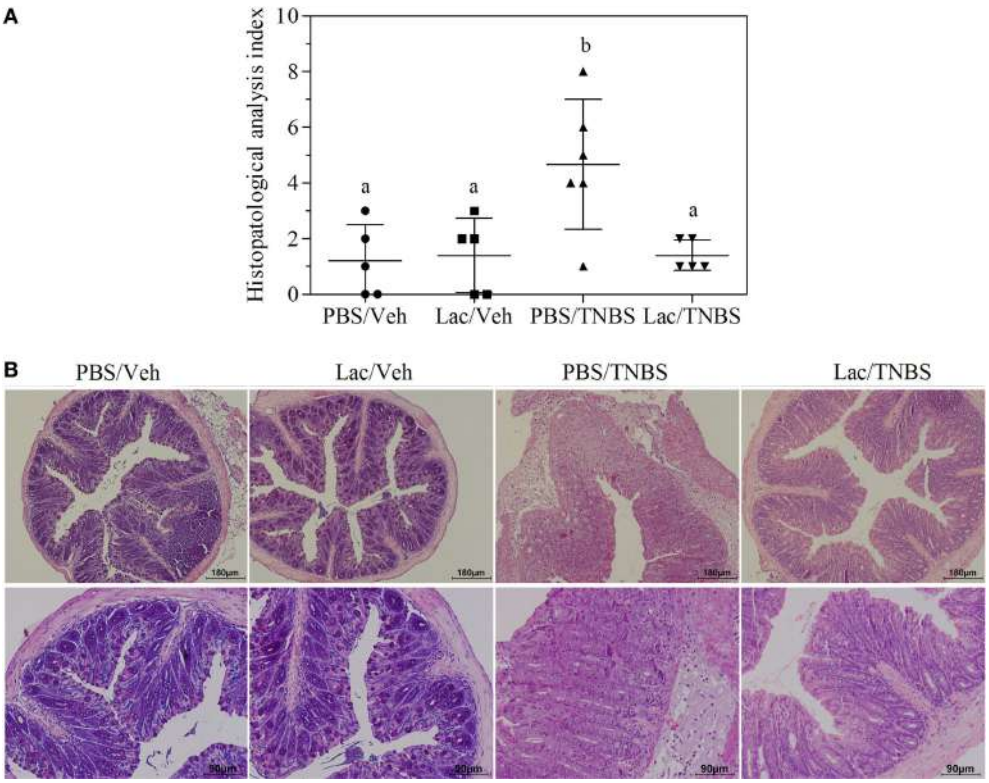

FIGURE 1 | **Rectal administration of lactate protects animals against damage in TNBS acute colitis model**. Mice were treated with lactate or PBS (i.r.) 2 h before TNBS or vehicle instillation and 48 h afterward tissue was collected for histopathological analysis. In all cases, groups of at least five mice were used. Results from a representative experiment out of five are shown. **(A)** Histopathological activity index assigned to different experimental groups. Different letter indicates significant differences with $p < 0.05$. **(B)** Photomicrograph of H&E-stained cross section (×100 top line and ×200 bottom line) of distal colon of a representative mouse of each experimental groups.

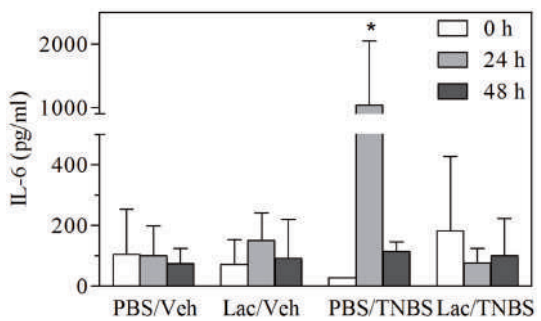

FIGURE 2 | **Rectal administration of lactate protects animals against early IL-6 production**. Serum was obtained from animals treated as in **Figure 1,** and levels of IL-6 were measured by ELISA. Serum levels of IL-6 (pg/ml) (□) before (▒) 24 h (■) 48 h, after TNBS or vehicle administration. Results from a representative experiment out of five are shown, expressed as the mean ± SD, *indicates significant difference with $p < 0.05$ respect to its corresponding control.

24 h, resulting in levels similar to control group. These results indicate that lactate pretreatment also prevents systemic alteration induced by TNBS treatment.

2,4,6-trinitrobenzenesulfonic acid is known to disrupt the mucosal barrier function by interacting with surface-active phospholipids of the colonic mucosa, a process that is evidenced by microbial translocation. We assessed the disruption by measuring the presence of bacteria into the liver. Our data demonstrated bacterial translocation four out of six animals in the PBS/TNBS group (**Table 1**). On the contrary, we did not find any bacteria in liver of animals pretreated with lactate and exposed to TNBS. These results were similar to the groups of mice that received vehicle (PBS/vehicle and lactate/vehicle) and did not experimented disruption of the mucosal barrier. Overall, our results show that luminal lactate could prevent bacterial translocation and reduce tissue inflammation induced by TNBS.

Lactate Downregulates Proinflammatory Response in Intestinal Epithelial Cells and Induces Metabolic Changes

In order to unravel the mechanisms of the anti-inflammatory effect of lactate in mice, an *in vitro* assay in the intestinal epithelial cell line Caco-2-*CCL20:LUC* that enables the monitoring of proinflammatory activation was used. In concordance to previous reports, pretreatment of Caco-2-*CCL20:LUC* cells with

lactate produced a significant decrease of luciferase activity induced by various proinflammatory stimuli, i.e., flagellin (the TLR5 agonist), the cytokines IL1-β, and TNF (**Figure 3A**). In all stimulation conditions, a similar pattern of downregulation of the proinflammatory signaling was observed. For instance, exposure to concentrations of lactate of 5 mM or higher elicited a significant decrease of IL1-β-induced activation. These inhibitory effects of lactate were increased in a dose-dependent manner.

We have previously observed that lactate treatment abrogates enhanced glycolysis in TLR-stimulated macrophages, which correlates with its activity as modulator of innate response (22), Caco-2-*CCL20:LUC* reporter cell line was utilized to evaluate if the effects of lactate on epithelial cells could be related to metabolic changes.

TABLE 1 | Microbial translocation to liver observed 48 h after 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis.

Treatment	Animals with positive translocation/total animals in the group
PBS/VEH	0/5
LAC/VEH	0/5
PBS/TNBS	4/6
LAC/TNBS	0/5

Results from a representative experiment out of five are shown.

Treatment of cells during 6 h with glycolysis inhibitors such as 2DO or 3BrPA (competitive inhibitors of hexokinase) and oxamate (inhibitor of lactate dehydrogenase) in different concentrations did not affect luciferase activity in non-stimulated condition. Luciferase activity induced by flagellin was significantly lower in cells pretreated with glycolysis inhibitors, and this effect was dose-dependent (**Figures 3B–D**). Cell viability was not affected by 6 h incubation with glycolysis inhibitors, showing an enzymatic activity on MTT reduction over 85% in all cases (not shown).

We observed that Caco-2 intestinal epithelial cells decreased their rate of glucose consumption in the presence of lactate either in basal as well as with flagellin conditions. This could be associated with an inhibition of glycolysis in presence of lactate (**Figure 4**). Overall, these results indicate that lactate modulation of epithelial response, correlates with its capacity to alter glycolytic activity, which alters the capacity to trigger the effectors of innate response activation.

DISCUSSION

Although the etiology of IBD is still unknown, increasing evidence shows that IBD may involve in genetically susceptible individuals a dysregulation of their immune response to resident microbiota (23). It is now widely accepted that a misbalanced gut ecosystem also plays an important role other pathologies of the gastrointestinal tract (24).

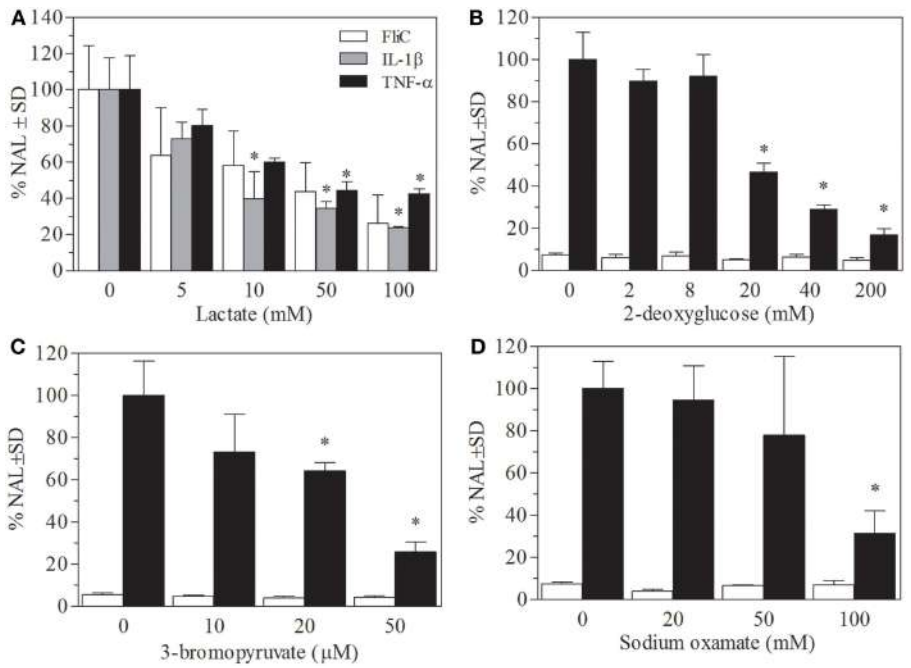

FIGURE 3 | Lactate pretreatment as well as glycolysis inhibition leads to downregulation of inflammatory response in Caco-2-*CCL20:LUC* cells. Reporter cells were stimulated with IL1-β (10 ng/mL), tumor necrosis factor (TNF)-α (100 ng/mL), or flagellin (1 μg/mL), after pretreatment with different concentrations of lactate. **(A)** Results are expressed as normalized luciferase activity, using the levels of stimulated cells in absence of lactate as 100% of activation. The Caco-2-*CCL20:LUC* cells pretreated with solutions of glycolysis inhibitors **(B)** 2DG (mM) **(C)** 3-bromopyruvate (μm) **(D)** Oxamate (mM). Results shown are the mean and SEM from independent triplicates. Results from a typical experiment out of at least three are depicted. □ Non-stimulated and ■ stimulated Flic. *Indicates a significant difference from the cells without treatment and stimulated with flagellin, IL-1β, and TNF-α, respectively, with $p < 0.05$.

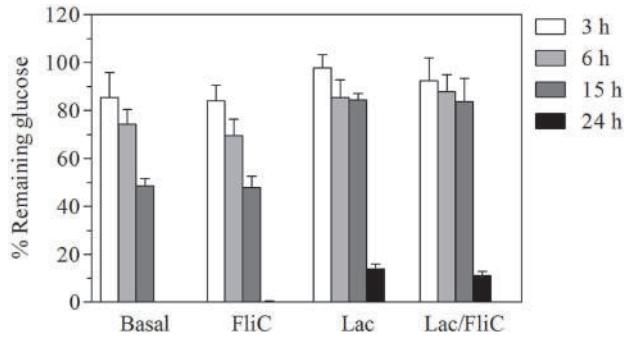

FIGURE 4 | Lactate treatment decreases glucose uptake of Caco-2/TC-7 cells either in basal as well as stimulated conditions. Percentage of glucose remaining in culture medium of Caco-2/TC-7 cells either with lactate 100 mM or not, non-stimulated and stimulated with flagellin (1 μg/mL), incubated for (□) 3, (▒) 6, (■) 15, and (■) 24 h.

Several therapeutic strategies proposed to reduce the symptoms of IBD are based on the use of anti-inflammatory drugs (such as corticoids, 5-aminosalicylic acid, and anti-TNF-α antibodies), all having marked long-term side effects. Other proposed treatments are based on microbiota-based dietary interventions, either by the use of probiotics or prebiotics (25). Therapeutic administration of probiotic species such as *Bifidobacterium* spp., *Lactobacillus* spp., or *Propionibacterium* has been shown to have protective effects on IBD models through the production of anti-inflammatory metabolites (26, 27). Almost two decades ago, proof of concept in clinical studies demonstrated the efficacy of SCFAs-based treatments in IBD, specifically in ulcerative colitis, treating the inflamed region using a mixture of SCFAs (acetate 80 mM, butyrate 40 mM and propionate 30 mM) enemas (28–30). In the recent years, therapeutic strategies related to intestinal SCFAs to manage IBDs have renewed interest based on studies from either animal models or intestinal metabolomic/microbiota analysis on patients. Interventions in animal models resulting in increased exposure of intestinal tissue to specific SCFAs have shown protective effects in intestinal mucosa (31, 32) and new combined interventions with pharmaceuticals and oral SCFAs formulated to be released in large intestine have shown efficacy in patient management (33).

We have previously shown that lactate can downregulate the proinflammatory responses of immune cells such as macrophages and dendritic cells, as well as those of mucosal structural cells like intestinal epithelial cells (18, 19). The diverse effects of lactic acid on various immune cells suggest that lactic acid or lactate may influence widely used signaling pathways. Indeed, both molecules have been demonstrated to influence several MAP kinases, NFkB signaling, or the PI3K/AKT pathway (15, 34). Aiming to analyze effects of lactate on inflammation *in vivo*, in a proof of concept experimental design, we found that pretreatment with lactate 200 mM modulates the epithelial damage and infiltration induced by TNBS. This effect was not observed when lactate 200 mM was administered in drinking water, on account of low lactate levels measured in distal colon (not shown). This reduced content could be due to either intestinal absorption by enterocytes, lactate consumption by microbiota, or both. To have protective effect, lactate luminal levels should be high, such as those reached by intrarectal administration. TNBS i.r. administration has been used as model for innate-driven intestinal inflammation due to epithelial damage and increased access of microbial-derived molecules to the immune cells in the lamina propria compartment (35). The use of the TNBS model that produce an acute local activation of inflammatory response allowed us to evaluate the local effect of lactate after a short term exposure, upon i.r intervention. Due to experimental design, contribution of microbiota, other fermentation metabolites, or other microbial products to the anti-inflammatory effect is expected to be low, indicating that is lactate the main responsible for the modulation observed.

Using different strategies, several authors showed that prevention of inflammation in the TNBS model is usually correlated with lower bacterial translocation from the gut to mesenteric lymph nodes and systemic compartment (36–40). In coincidence with these results, we have shown that lactate administration protects against microbial translocation to the liver in animals treated with TNBS and the increase in lactate concentration in colon alleviates TNBS-induced colitis. Furthermore, lactate treatment also prevented serum rise of levels of IL6 (**Figure 2**), in coincidence with our previous observations that lactate pretreatment abrogates NFkB activation and proinflammatory gene expression such as IL12, IL1β, or IL6 (18, 19). Some proinflammatory cytokines that may be modulated in this way, such as IL1b and IL18, have also the capacity to trigger epithelial renewal and reinforce barrier function (41). Nevertheless, in our system, the overall effect of lactate is to promote tissue protection as appreciated by hystopathological analysis (**Figure 1**).

There are several possible non-mutually exclusive mechanisms that may explain the capacity of lactate to prevent inflammation in our model. We have recently shown that lactate, as other SCFAs, may prevent TLR-mediated activation of macrophage and dendritic cells (19). Several reports indicate that the blockage of macrophage activation can modulate colonic inflammation in different acute models; Du et al. (42) have shown that targeting intestinal macrophages with gadolinium chloride block colitis in a TNBS model (42). Furthermore, several treatments targeting intestinal macrophage activation, using miRNAs or modulation of specific GPCRs, can also modulate colitis in TNBS model (43, 44). Besides, previous studies have shown that lactate can modulate innate activation of intestinal epithelial cells (18, 19). Since epithelial cells can also contribute to the amplification of inflammation in the TNBS model, this could be another possible cellular target that explains the bioactive properties of lactate. In line with this possibility, Cheng et al. have shown that targeting intestinal epithelial cells may reduce colitis in IBD models (45).

Beyond the cellular target of lactate, there are also several mechanisms that may account for its activity. We have recently shown that lactate impairs macrophage metabolic reprograming after LPS activation in a GPR81-independent manner (22), which has also been associated with blunting the proinflammatory cytokine response (46). In accordance with these results, Selleri et al. (47) have shown that local increase of lactate in the environment of mesenchymal stromal cells shifts macrophage M1

activation toward the less inflammatory M2 (47). Colegio et al. (48) have shown also the blunting of M1 macrophage activation in the solid tumor environment due to high lactate production of Warburg metabolism of tumor cells (48). Kreutz and colleagues have shown that increase in extracellular lactate of macrophages impair proinflammatory activation by altering its capacity to rise its glycolitic flux, effect that is enhanced at low pH (34, 49). Inhibition of lactate efflux from macrophages blocks LPS-driven activation by a mechanism also associated to impairment of glycolytic reprograming of macrophage upon activation (50).

Metabolic reprograming upon proinflammatory activation of myeloid cells implicates enhanced glycolysis with low respiratory rate (51). In the case of macrophages, this implies high rate of urea cycle intermediates for the production of NO from arginine (46) and production of lipid metabolites from citrate. In the case of dendritic cells, metabolic reprograming supplies carbon from glycolysis to lipid metabolites, mainly to allow expansion of endoplasmic reticulum in order to facilitate antigen presentation (52). Although it is not clear that the extent of metabolic reprograming takes place in epithelial cells upon TLR activation, there are some reports that show similarities on macrophage and epithelial cell response to mediators of metabolic reprograming (53). Our results indicate that blocking of glycolysis impairs flagellin-induced CCL20 transcriptional activation observed in our reporter system (**Figure 3**). Furthermore, we observed less consumption of glucose in the presence of extracellular lactate (**Figure 4**). These results are consistent with the necessity of enhancement of glycolysis rate in epithelial cells for a full functional TLR response, as is the case of macrophages.

Although our experimental design was first aimed to confirm the bioactive properties of lactate observed *in vitro* in a preclinical model, it opens the possibility of using lactate in local treatments to modulate inflammation. Furthermore, it can be considered that local production of lactate by probiotic microorganisms that attach to the intestinal epithelium may also contribute to their protective capacity in inflammatory situations (14), providing alternative cues for selection of microorganisms to be used as complement in the management of IBDs.

CONCLUSION

Results shown here were conclusive in relation to the effect of lactate at local level in a model of acute intestinal inflammation, contributing to a decrease in epithelial damage, signs of inflammation, and the secretion of proinflammatory cytokine IL-6, presenting a first approximation *in vivo* about the role of lactate in preventing intestinal inflammation.

Although several possibilities remain to be considered to explain the cellular and molecular mechanisms responsible for the observed effect, a correlation between impairment of glycolysis and proinflammatory activation of epithelial cells was observed, in coincidence with previous works in macrophages.

These results suggest that lactate could be a potential beneficial microbiota metabolite and may contribute to health-promoting properties on the intestinal mucosa.

AUTHOR CONTRIBUTIONS

CI performed experimental work, participated in the study design and conception and manuscript writing. AB, DR, AE, and DC performed experimental work, participated in study design. BF performed experimental work, participated in study design and manuscript writing. J-CS participated in study design, funding, and manuscript writing. GG, AA, and MR participated in study design and conception, funding, and manuscript writing.

ACKNOWLEDGMENTS

CI and AB are fellows of Argentina National Research Council (CONICET), DR, AE, AA, GG, and MR are members of Scientific Career of CONICET. DC, J-CS, and BF are supported by the Institut Pasteur de Lille, the University of Lille, CNRS and Inserm. Work was supported by grants from CONICET, Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) and ECOS A12B03 grant of the National Ministry of Science, Technology and Innovation (MINCYT-Argentina).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00651/full#supplementary-material.

FIGURE S1 | Intrarectal administration of lactate protects animals against weight loss in 2,4,6-trinitrobenzenesulfonic acid (TNBS) acute colitis model. Weight variation after 24 and 48 h of TNBS-induced colitis (% of initial weight). In all cases, groups of at least five mice were used. Results from a representative experiment out of five are shown. Different letter indicates significant differences with $p < 0.05$.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with several of the authors and states that the process nevertheless met the standards of a fair and objective review.

frontiers in Immunology

ORIGINAL RESEARCH
published: 23 January 2017
doi: 10.3389/fimmu.2017.00023

Intestinal Dysbiosis Is Associated with Altered Short-Chain Fatty Acids and Serum-Free Fatty Acids in Systemic Lupus Erythematosus

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OPEN ACCESS

Edited by:
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Reviewed by:
Martin Rumbo, National University of La Plata (CONICET)
Paola Gauggin Cano, Centro de Referencia Para Lactobacilos (CONICET)

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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *04 October 2016*
Accepted: *05 January 2017*
Published: *23 January 2017*

Citation:
Rodríguez-Carrio J, López P, Sánchez B, González S, Gueimonde M, Margolles A, de los Reyes-Gavilán CG and Suárez A (2017) Intestinal Dysbiosis Is Associated with Altered Short-Chain Fatty Acids and Serum-Free Fatty Acids in Systemic Lupus Erythematosus. Front. Immunol. 8:23. doi: 10.3389/fimmu.2017.00023

Metabolic impairments are a frequent hallmark of systemic lupus erythematosus (SLE). Increased serum levels of free fatty acids (FFA) are commonly found in these patients, although the underlying causes remain elusive. Recently, it has been suggested that factors other than inflammation or clinical features may be involved. The gut microbiota is known to influence the host metabolism, the production of short-chain fatty acids (SCFA) playing a potential role. Taking into account that lupus patients exhibit an intestinal dysbiosis, we wondered whether altered FFA levels may be associated with the intestinal microbial composition in lupus patients. To this aim, total and specific serum FFA levels, fecal SCFA levels, and gut microbiota composition were determined in 21 SLE patients and 25 healthy individuals. The *Firmicutes* to *Bacteroidetes* (F/B) ratio was strongly associated with serum FFA levels in healthy controls (HC), even after controlling for confounders. However, this association was not found in lupus patients, where a decreased F/B ratio and increased FFA serum levels were noted. An altered production of SCFA was related to the intestinal dysbiosis in lupus, while SCFA levels paralleled those of serum FFA in HC. Although a different serum FFA profile was not found in SLE, specific FFA showed distinct patterns on a principal component analysis. Immunomodulatory omega-3 FFA were positively correlated to the F/B ratio in HC, but not in SLE. Furthermore, divergent associations were observed for pro- and anti-inflammatory FFA with endothelial activation biomarkers in lupus patients. Overall, these findings support a link between the gut microbial ecology and the host metabolism in the pathological framework of SLE. A potential link between intestinal dysbiosis and surrogate markers of endothelial activation in lupus patients is supported, FFA species having a pivotal role.

Keywords: free fatty acids, systemic lupus erythematosus, dysbiosis, microbiota, short-chain fatty acids

INTRODUCTION

Epidemiological studies have consistently shown an increase in the prevalence and severity of a number of metabolic disorders in patients with systemic lupus erythematosus (SLE) compared to the general population (1–3). Among them, metabolic syndrome, disturbed glucose metabolism, or altered lipid metabolism are the most relevant. These disorders are related to an increased risk of

cardiovascular disease (CVD) development, the most important cause of mortality in SLE (4, 5), thus highlighting the clinical relevance of the metabolic alterations in SLE.

Immune dysregulation and chronic inflammation are known to promote endothelial dysfunction in SLE (6, 7). Increased levels of pro-inflammatory cytokines [such as tumor necrosis factor alpha (TNFα), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1)], adipokines, and autoantibodies are associated with the progression of endothelial dysfunction toward atherosclerosis development (6). At the local level, these mediators can impair the balance between endothelial repair and damage, whereas a number of systemic effects can be also triggered, including a shift to a pro-oxidant status (8) and altered lipid metabolism. In this scenario, although the underlying mechanisms are not totally understood, the relationship between systemic inflammation, metabolic disorders, and CVD may be explained, at least in part, by the free fatty acids (FFA) (9). FFA are fatty acid molecules released from adipocytes and several cell types upon lipolysis (10). Increased FFA levels in serum have been described in several metabolic disorders. Moreover, elevated serum FFA have also been found in immune-mediated diseases, such as SLE or rheumatoid arthritis, although striking differences were noted between both conditions (11). Rather than inflammatory or clinical parameters, the body mass index (BMI) was found to be the main predictor of FFA serum levels in lupus (11). However, these clinical studies did not allow the elucidation of the exact mediators and mechanisms involved.

Obesity is the result of an imbalance between energy intake and expenditure, which results in an excess of fat accumulation. However, several epidemiological studies have identified people with low BMI exhibiting markers of metabolic dysfunction (12, 13). Similarly, healthy metabolic profiles are found in a subset of obese subjects (14, 15), hence suggesting that metabolic dysfunction (that is, impaired fatty acid mobilization) rather than adiposity should be considered as the underlying cause. Therefore, it is feasible that factors related to energy intake and expenditure may underlie altered FFA levels and thus, metabolic disorders.

A mounting body of evidence shows that the gut microbiota can influence the host metabolism as well as the energy harvest and storage (16–18). Actually, the gut microbiota is seen by some authors as a separate endocrine organ involved, through a molecular cross talk with the host, in the maintenance of energy homeostasis and fat deposition (19). Currently, extensive research efforts have been focused on deciphering the basis of the cross talk between the microbiota and the host metabolism in the development and progression of host diseases and have revealed the relevance of the intestinal microbiota–host metabolism axis mediated by different bacterial and host metabolites (20, 21). Thus, it may be speculated that changes in the intestinal microbial ecology could disrupt this homeostatic cross talk and precipitate the development of pathological outcomes in the host.

Recently, we have reported that SLE patients exhibit an altered intestinal composition compared to healthy subjects, mainly characterized by a decreased abundance of members of the *Firmicutes* phylum and an overrepresentation of those of *Bacteroidetes* (22). However, the clinical impact of this SLE-associated intestinal dysbiosis remains to be elucidated. Taking into account the former assumptions, we hypothesized that altered gut microbiota composition in SLE may underlie increased FFA serum levels. Accordingly, the main aims of the present report were (i) to analyze the potential association between the microbiota composition and FFA serum levels, (ii) to elucidate whether microbial metabolites can have a role in this interaction, (iii) to evaluate whether a different profile of FFA can be found in lupus patients, and (iv) to study the associations of these parameters with clinically relevant serum biomarkers.

MATERIALS AND METHODS

Ethical Approval

Ethical approval for this study was obtained from the Institutional Review Board (Comité de Ética de Investigación Clínica del Principado de Asturias) in compliance with the Declaration of Helsinki. All participants were informed and gave a signed informed consent prior their inclusion in the study.

Patients and Controls

Our study involved 21 SLE patients, all fulfilling classification criteria for SLE. According to the 1982 revised criteria from the American College of Rheumatology, a definitive SLE diagnosis can be established when a patient exhibit at least 4 out of the 11 SLE criteria (malar rash, discoid lesions, photosensitivity, oral ulcers, arthritis, serositis, renal disorders, neurological disorder, cytopenia, raised anti-DNA titers, and positivity to antinuclear antibodies) (23). A complete clinical examination, including Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) calculation and anti-dsDNA autoantibodies assessment, was performed at the time of sampling. All patients were in remission (SLEDAI <8) at the sampling time. Information on clinical features along the disease course as well as the therapies received during the last 6 months was obtained from their clinical records. A group of 25 age- [median 43.50 (range 23.00–63.00) years] and gender-matched (23 females) healthy individuals recruited from the general population was included as the healthy controls (HC). Patients and controls did not differ in age ($p = 0.293$) and gender distribution ($p = 0.495$). Exclusion criteria were the history of recent infections, diagnosis of metabolic diseases, or the use of antibiotics, glucocorticoids, or monoclonal antibodies in the previous 6 months.

Upon acceptance of the individuals to participate in the study, a strict overnight fast (more than 8 h) blood sample was obtained in tubes without anticoagulant. Serum was collected, divided into aliquots, and samples were stored at −80°C until experimental procedures were performed. Additionally, basic serum blood lipid analyses were carried out on fresh samples at the time of sampling, by standardized procedures.

Quantification of Total FFA Serum Levels

Total FFA serum levels were analyzed by a colorimetric enzymatic assay using a commercial kit (NEFA kit half-microtest, Roche Life

Sciences, Penzberg, Germany) following the instructions from the manufacturer. Final absorbance was measured at 546 nm, and the detection limit was 0.2 mM.

Assessment of Serum FFA Profiles

Individual FFA were analyzed in serum samples following a methyl-*tert*-butylether-based extraction protocol (MTBE) as previously described (24), with minor modifications. Briefly, serum samples (100 µl) were spiked with 5 µl of internal standard (600 ppm heptadecanoic acid). Proteins were precipitated by the addition of 200 µl methanol chromasolv grade (Sigma Aldrich, MO, USA). Organic phases were obtained by the addition of 1,200 µl MTBE chromasolv grade (Sigma) followed by an incubation in an ultrasound water bath at 15°C for 30 min. Finally, organic phases were isolated by centrifugation at 5,000 rpm (7 min, 15°C) after the addition of 200 µl milliQ water. The extraction protocol was repeated once with 100 µl MetOH, 500 µl MTBE, and 100 µl milliQ H_2O. Lipid extracts were dried in a miVac centrifugal evaporator (Genevac Ltd., UK) and redissolved in 100 µl of water:acetonitrile (38:62).

The analyses of fatty acids in the samples were performed in a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Bremen, Germany) equipped with a column Zorbax Eclipse Plus C18 (50 mm × 2.1 mm, 1.8 µm). Mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% of formic acid. Fatty acids were separated in an injection volume of 2 µl by a gradient program as follows: 62% B (held for 4.5 min) followed by a linear increase up to 100% B in 10 min (held for 1 min). The column temperature was set at 45°C. Mass detection was carried out in a Bruker Impact II q-ToF mass spectrometer with electrospray ionization, operating in the negative mode. The settings of the mass spectrometer were as follows: spray voltage 4.5 kV; drying gas flow 12 l/min; drying gas temperature 250°C; and nebulizer pressure 44 psi.

For quantification, calibration curves for each compound were prepared by dissolution of the pure standards in methanol to adequately encompass the expected concentration of the analytes in the samples. The calibration ranges were as follows: 0.4–12.5 µg/ml for EPA and γ-linolenic; 1.2–37.5 µg/ml for DHA and linolenic; 2.3–75 µg/ml for AA and palmitoleic; 3.9–125 µg/ml for linoleic; and 7.8–250 µg/ml for oleic, palmitic, and stearic acids. A good linearity was observed in all cases ($r^2 > 0.994$). Heptadecanoic acid was used as internal standard to account for potential biases during the extraction protocol.

Analysis of Fecal Microbiota

Fecal sample collection and metagenomic analyses of fecal microbiota were performed as previously reported (22). Briefly, fresh fecal material was processed within 3 h from collection and immediately homogenized and stored at −80°C. Fecal DNA was extracted with a QIAampDNA stool minikit (Qiagen, Strasse, Germany). Then, 16S rRNA gene sequences were amplified, and 16S rRNA and gene-based amplicons were sequenced by an Ion Torrent PGM sequencing platform as described elsewhere (22).

Analysis of Short-Chain Fatty Acids (SCFA) in Fecal Samples

Analysis of SCFA (acetate, propionate, and butyrate) was performed by gas chromatography. Briefly, 1 g of fecal samples was diluted 1:10 in sterile PBS and homogenized in a LabBlender 400 stomacher (Seward Medical, London, UK) at full speed for 4 min. Then, supernatants were obtained by centrifugation (10.000 × *g*, 30 min, 4°C), filtered through 0.2-µm filters, mixed with 1:10 of ethyl butyric acid (2 mg/ml) as an internal standard, and stored at −80°C until analysis.

A gas chromatograph 6890N (Agilent Technologies Inc., Palo Alto, CA, USA) connected to a mass spectrometry (MS) 5973N detector (Agilent Technologies) and to a flame ionization detector was used for identification and quantification of SCFA. Data were collected using the Enhanced ChemStation G1701DA software (Agilent). Samples (1 µl) were injected into the gas chromatograph equipped with an HP-Innowax capillary column (60-m length by 0.25-mm internal diameter, with a 0.25-µm film thickness; Agilent) using He as a gas carrier (flow rate of 1.5 ml/min). The temperature of the injector was kept at 220°C, and the split ratio was 50:1. Chromatographic conditions were as follows: initial oven temperature of 120°C, 5°C/min up to 180°C, 1 min at 180°C, and a ramp of 20°C/min up to 220°C to clean the column. In the MS detector, the electron impact energy was set at 70 eV. The data collected were in the range of 25 to 250 atomic mass units (at 3.25 scans/s).

SCFA were identified by comparison of their mass spectra with those held in the HP-Wiley 138 library (Agilent) and by comparison of their retention times with those of the corresponding standards (Sigma Aldrich, St. Louis, MO, USA). The peaks were quantified as relative abundances with respect to the internal standard. The concentration (in millimolar) of each SCFA was calculated using the linear regression equations ($R^2 \geq 0.99$) from the corresponding standard curves.

Analysis of Serum Biomarkers

Soluble Biomarkers

Serum levels of vascular endothelial growth factor (VEGF), granulocyte monocyte colony-stimulating factor (GM-CSF), and IL-8 were analyzed by Cytometric Bead Arrays (BD Biosciences, NJ, USA) using a BD FACS Canto II and FACS Diva software. Detection limits were 4.5, 0.2, and 1.2 pg/ml, respectively.

Epidermal growth factor (EGF), TNFα, MCP-1, interferon gamma-inducible protein-10 (IP-10), and leptin serum levels were assessed by plate immunoassays using commercial kits by Peprotech (NJ, USA), following manufacturer's instructions. Detection limits were 3.9, 3.9, 8, 3.9, and 24 pg/ml, respectively.

Malondialdehyde (MDA)

Malondialdehyde serum levels were determined by means of a colorimetric method using a commercial kit (LPO-596, Byoxytech, Oxis International, France). Final absorbance was read at 586 nm.

Anthropometric Measures

Height was measured using a stadiometer with an accuracy of ±1 mm (Año-Sayol, Barcelona, Spain). The subjects stood barefoot, in an upright position and with the head positioned in the Frankfort horizontal plane. Weight was measured on a scale with an accuracy of ±100 g (Seca, Hamburg, Germany).

Nutritional Assessments

Dietary intakes were assessed by means of an annual semiquantitative validated food frequency questionnaire including 160 items (25). During an interview by trained dietitians, subjects were asked, item by item, whether they usually ate each food and, if so, how much they usually ate. For this purpose, three different serving sizes of each cooked food were presented in pictures to the participants so that they could choose from up to seven serving sizes (from "less than the small one" to "more than the large one"). For some of the foods consumed, amounts were recorded in household units, by volume, or by measuring with a ruler. Information on the cooking practices, number and amount of ingredients used in each recipe, and other relevant information for the study was collected. Methodological issues concerning dietary assessment have been detailed elsewhere (25). The consumption of foods was converted into energy intake (kilocalories per day), macronutrients (carbohydrates, lipids, and proteins, grams per day), and total fiber (grams per day) using the nutrient food composition tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID) (26). CESNID, a foundation, involves different institutions, universities, and companies related to the food and nutrition area, and its food composition databases are supported by the Spanish Association of Nutrition and Dietetics.

Statistical Analyses

Continuous variables were expressed as median (interquartile range) or mean ± SD. Mann–Whitney *U*, Student's *t* or Kruskal–Wallis tests were performed to assess statistical differences. Correlations were analyzed by Spearman's rank or Pearson tests, depending on the distribution of the data. Categorical variables were summarized as *n* (%), and differences were analyzed by χ^2 tests. A principal component analysis (PCA) was performed to avoid potential bias due to collinearity. The adequacy of the data was studied by Kaiser–Meyer–Olkin test and Bartlett test of sphericity. The number of components retained was based on eigenvalues (>1), and loadings greater than 0.5 were used to identify the variables comprising each component. Unsupervised cluster analysis was performed based on squared Euclidean distances, and Ward's Minimum Variance method was used to identify the clusters. Heatmaps were generated under R package *heatmap.2*. SPSS 21.0, R 3.0.3, and GraphPad Prism 5.0 for Windows were used.

RESULTS

Total FFA Serum Levels in SLE Patients: Association with Intestinal Dysbiosis

The concentration of total FFA was measured in serum samples from 21 SLE patients and 25 matched HC (**Table 1**). SLE patients exhibited higher FFA serum levels (**Table 1**). Differences between groups in the levels of FFA remained significant after adjusting for age and gender ($p = 0.024$). Moreover, no associations were found with demographical parameters, cholesterol and triglycerides levels, and dietary intakes (**Table 2**). Furthermore, FFA were neither related to clinical manifestations (**Table 3**) (all $p > 0.050$) nor disease activity ($r = -0.349$, $p = 0.169$), duration ($r = -0.005$,

TABLE 1 | Serum-free fatty acids (FFA) levels, nutritional parameters and blood lipid profiles of the healthy controls (HC) and systemic lupus erythematosus (SLE) patients recruited in this study.

	HC (*n* = 25)	SLE (*n* = 21)	*p*
FFA assessment			
Total FFA (mM)	0.27 (0.17)	0.41 (0.26)	0.045
Blood lipid analyses			
Total cholesterol (mg/dl)	191.50 (49.00)	200.00 (61.25)	0.732
HDL-cholesterol (mg/dl)	62.00 (14.75)	62.00 (24.00)	0.740
LDL-cholesterol (mg/dl)	114.50 (50.00)	111.50 (57.50)	0.530
Triglycerides (mg/dl)	68.50 (54.25)	71.55 (45.25)	0.715
Nutritional parameters			
Total energy (kcal/day)	1,888.88 (226.53)	2,186.11 (208.16)	0.069
Carbohydrates (g/day)	202.50 (51.60)	205.46 (102.95)	0.944
Lipids (g/day)	78.13 (29.32)	79.42 (63.57)	0.789
Proteins (g/day)	96.98 (15.81)	102.60 (34.30)	0.782
Fiber (g/day)	24.68 (6.17)	26.47 (6.57)	0.609
Body mass index (kg/m^2)	24.96 (4.47)	24.58 (7.78)	0.715

Variables are represented as median (interquartile range) or n (%) unless otherwise stated. Differences in demographical and blood lipid variables were assessed by Mann–Withney U tests, whereas differences in daily intakes were analyzed by multivariate analyses adjusted for confounders. Energy was adjusted by gender and age, whereas the rest of the nutrients were adjusted by gender, age, and energy.

TABLE 2 | Analysis of the correlation between serum FFA levels and demographical and nutritional features in healthy controls (HC) and systemic lupus erythematous (SLE) patients.

	HC	SLE
Age	$r = -0.260$	$r = -0.179$
	$p = 0.231$	$p = 0.451$
Total cholesterol	$r = -0.108$	$r = -0.203$
	$p = 0.625$	$p = 0.390$
HDL-cholesterol	$r = 0.220$	$r = 0.114$
	$p = 0.313$	$p = 0.633$
LDL-cholesterol	$r = -0.028$	$r = -0.248$
	$p = 0.898$	$p = 0.291$
Triglycerides	$r = -0.224$	$r = -0.078$
	$p = 0.305$	$p = 0.743$
BMI	$r = 0.247$	$r = 0.214$
	$p = 0.268$	$p = 0.366$
Total energy	$r = 0.082$	$r = -0.212$
	$p = 0.710$	$p = 0.369$
Carbohydrates	$r = -0.107$	$r = -0.311$
	$p = 0.628$	$p = 0.182$
Lipids	$r = -0.048$	$r = -0.220$
	$p = 0.826$	$p = 0.352$
Proteins	$r = 0.010$	$r = -0.005$
	$p = 0.964$	$p = 0.985$
Fiber	$r = 0.125$	$r = -0.394$
	$p = 0.568$	$p = 0.086$

Correlations were assessed by Spearman ranks tests (r coefficient and p-value is indicated for each parameter).

TABLE 3 | Demographical and clinical parameters of the systemic lupus erythematosus (SLE) patients.

	SLE ($n = 21$)
Age, (years), mean (range)	48.35 (27.00–70.00)
Gender, (f/m)	21/0
Age at diagnosis, (years)	33.00 (14.50)
Disease duration, (years), median (range)	7.00 (2.00–24.00)
SLEDAI score	4.00 (3.25)
Clinical manifestations, *n*(%)	
Malar rash	12 (57.1)
Photosensitivity	16 (76.2)
Discoid lesions	6 (28.6)
Arthritis	10 (47.6)
Oral ulcers	10 (47.6)
Serositis	4 (19.0)
Renal disorder	3 (14.3)
Neurological disorder	0 (0.0)
Cytopenia	11 (52.4)
Autoantibodies, *n*(%)	
ANAs	21 (100)
Anti-dsDNA titer, (U/ml), mean ± SD	25.30 ± 33.89
Anti-SSa	11 (52.4)
Anti-SSb	2 (9.5)
Anti-Sm	2 (9.5)
Anti-RNP	1 (4.8)
Treatments, *n* (%)	
None or NSAIDs	3 (14.2)
Antimalarials	18 (85.7)

Variables are represented as median (interquartile range) or n(%), unless otherwise stated.

TABLE 4 | Association between serum-free fatty acids (FFA) levels and gut microbiota composition in healthy controls (HC) and systemic lupus erythematosus patients (SLE).

	HC	SLE)
Actinobacteria	$r = 0.075$ $p = 0.733$	$r = -0.415$ $p = 0.069$
Bacteroidetes	**$r = 0.721$** **$p < 0.0001$**	$r = 0.311$ $p = 0.182$
Firmicutes	**$r = -0.574$** **$p = 0.007$**	$r = -0.117$ $p = 0.622$
Cyanobacteria	$r = -0.120$ $p = 0.539$	$r = -0.401$ $p = 0.080$
Euryarchaeota	$r = -0.213$ $p = 0.328$	$r = -0.063$ $p = -0.792$
Fusobacteria	$r = -0.052$ $p = 0.812$	$r = 0.139$ $p = 0.560$
Lentisphaerae	$r = 0.272$ $p = 0.210$	$r = 0.019$ $p = 0.937$
Proteobacteria	$r = -0.158$ $p = 0.471$	$r = 0.230$ $p = 0.329$
Tenericutes	$r = -0.105$ $p = 0.634$	$r = -0.220$ $p = 0.227$
TM7	$r = 0.015$ $p = 0.945$	$r = 0.018$ $p = 0.939$
Verrucomicrobia	$r = -0.325$ $p = 0.130$	$r = 0.209$ $p = 0.376$
Synergistetes	$r = -0.211$ $p = 0.334$	$r = -0.104$ $p = 0.661$

The associations between serum FFA levels and the abundance of microbial groups at the level of phyla in HC and SLE patients were analyzed by Spearman ranks tests (r coefficient and p-value is indicated for each parameter). Statistical analyses with a p-value below 0.050 are highlighted in bold.

$p = 0.982$), or anti-dsDNA levels ($r = -0.350$, $p = 0.130$) in SLE. Therefore, parameters other than those indicated may explain the altered serum FFA levels registered in SLE.

Then, we wondered whether gut microbial composition may account for the increased FFA serum levels in SLE. To this aim, the associations between FFA levels and the main intestinal microbial groups analyzed by a metagenomic approach as already described (22) were assessed. As previously reported, diminished *Firmicutes* to *Bacteroidetes* (F/B) ratio characterized the intestinal dysbiosis found in SLE compared to healthy subjects [1.94 (1.51) vs 4.27 (5.93), $p < 0.001$] (22). Among the phyla analyzed (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Cyanobacteria*, *Euryarchaeota*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria*, *Tenericutes*, TM7, *Verrucomicrobia*, and *Synergistetes*), FFA levels displayed opposite correlations with the *Firmicutes* and *Bacteroidetes* groups in HC, but not in lupus patients (**Table 4**). Consequently, a negative association with the F/B ratio was observed in HC, but not in SLE (**Figure 1**). Moreover, this association remained significant after adjusting for potential confounders (**Table 5**). Therefore, F/B ratio was found to be the main predictor of FFA serum levels in HC, whereas this effect was not seen in lupus patients, hallmarked by a decreased F/B ratio and elevated FFA levels.

Since some heterogeneity within groups in the FFA levels was observed and in order to gain more insight into the connections between gut microbiota and serum FFA, further analyses were performed. Focusing on the main microbial groups at the level of phyla, individuals were classified into groups by means of a cluster analysis. Interestingly, two main clusters were identified (thereafter referred to as clusters I and II) (**Figure 2A**), mainly differing in the F/B ratio (8.84 ± 5.53 vs 1.70 ± 0.76, respectively; $p < 0.0001$). Notably, a distinct distribution of individuals was observed, as HC were mainly found within the cluster I (14/25), whereas SLE patients were marginally present in this group (3/21, $p = 0.004$). On the one hand, this result highlights a shift in the microbiota composition in SLE patients compared to HC, hence supporting an association between a biased distribution of the intestinal microbial groups and elevated FFA serum levels. More importantly, when FFA levels were compared among HC and SLE subjects stratified by microbial clusters, it was noted that HC grouping within the cluster II exhibited similar FFA serum levels as SLE patients (**Figure 2B**), thus reinforcing the relevance of the microbiota composition on the FFA serum levels.

Overall, our findings disclose a strong association between FFA levels in serum and specific groups of the gut microbiota in healthy individuals, but not in lupus patients where a profound intestinal dysbiosis was registered.

SCFA and FFA Levels

Our results point to a relationship between the gut microbiota and the host metabolism at the systemic level, but the actual mediators are unclear. Since SCFA may affect the human metabolism

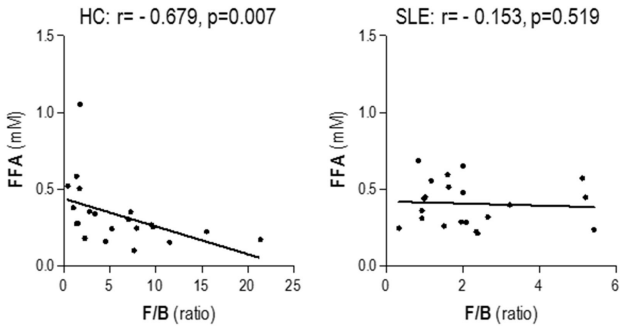

FIGURE 1 | Serum-free fatty acids (FFA) levels and *Firmicutes* to *Bacteroidetes* (F/B) ratio. The association between serum FFA levels and the F/B ratio was analyzed by Spearman's ranks correlation tests.

TABLE 5 | *Firmicutes*/*Bacteroidetes* (F/B) ratio is the main predictor of FFA levels in healthy controls (HC) but not in systemic lupus erythematosus (SLE) patients.

		β	*B* [95% CI]	*p*
HC	F/B ratio	−0.636	−0.334 [−0.557, −0.111]	**0.007**
	Age	−0.354	−0.652 [−1.436, 0.132]	0.093
	Gender	0.150	0.114 [−0.179, 0.407]	0.405
	BMI	−0.039	−0.002 [−0.022, 0.018]	0.832
	CRP	0.191	0.133 [−0.170, 0.436]	0.352
	Total energy	−0.010	0.001 [−0.044, 0.042]	0.961
	Lipids	0.276	0.003 [−0.004, 0.010]	0.315
	Carbohydrates	−0.372	−0.002 [−0.004, 0.001]	0.073
	Proteins	0.092	0.001 [−0.005, 0.007]	0.690
	Fiber	−0.223	−0.007 [−0.020, 0.005]	0.239
SLE	F/B ratio	−0.025	−0.014 [−0.332, 0.303]	0.923
	Age	−0.229	−0.336 [−1.315, 0.643]	0.466
	BMI	0.415	0.012 [−0.008, 0.032]	0.205
	CRP	0.186	0.072 [−0.191, 0.335]	0.558
	Total energy	−0.072	−0.007 [−0.045, 0.031]	0.687
	Lipids	−0.193	−0.001 [−0.005, 0.003]	0.698
	Carbohydrates	−0.265	−0.001 [−0.003, 0.002]	0.649
	Proteins	0.369	0.002 [−0.002, 0.006]	0.308
	Fiber	−0.351	−0.006 [−0.019, 0.008]	0.365

The association between F/B ratio and FFA serum levels in HC was studied by multiple lineal regression analysis including demographical parameters and nutritional intakes as potential confounders. HC: R^2 (model) = 0.762; SLE: R^2 (model) = 0.426. Statistical analyses with a p-value below 0.050 are highlighted in bold.

and an altered gut microbiota composition leads to a dysregulated SCFA production, the associations between fecal SCFA levels and those of serum FFA were analyzed.

On the one hand, higher levels of all SCFA studied were observed in lupus patients compared to HC (**Table 6**). However, no differences were found between SLE and HC when relative proportions were compared (all $p > 0.050$). On the other hand, all SCFA exhibited a positive correlation with FFA serum levels in HC (**Table 6**).

Importantly, the F/B ratio was negatively correlated to the fecal levels of propionate and butyrate in HC but not in SLE patients (**Figure 3**). Notably, stronger associations were found in HC when only *Bacteroidetes* abundance was considered: propionate ($r = 0.653$, $p < 0.001$) and butyrate ($r = 0.623$, $p = 0.002$).

In order to gain further insight into the relevance of the intestinal microbiota and the SCFA production and serum FFA levels, we performed additional analyses by stratifying subjects according to the clusters obtained from the microbiota analysis (**Figure 2**) instead of their clinical condition. Interestingly, a negative association between F/B ratio and fecal SCFA was found in cluster I (propionate: $r = -0.621$, $p = 0.024$ and butyrate: $r = -0.654$, $p = 0.015$), but not in those grouped in cluster II (propionate: $r = -0.015$, $p = 0.940$ and butyrate: $r = 0.220$, $p = 0.271$). Overall, this picture mirrors that of found for the HC vs SLE populations according to our previous findings and confirms a pivotal role of the intestinal microbiota in this scenario. However, SCFA and FFA levels did not remain associated after stratifying the whole population by the clusters, hence suggesting the involvement of additional factors, such as the clinical condition, to explain the connection between gut microbiota composition, SCFA, and serum FFA levels.

All these results highlight a role for the gut microbiota in the maintenance of serum FFA levels, SCFA having a potential role orchestrating this interaction. Additional factors, such as disease status, may also influence the outcome of the associations between gut microbiota composition and the interaction SCFA–FFA. Indeed, altered gut microbiota composition found in SLE patients was linked to an altered SCFA production and increased FFA levels in serum, thus supporting this hypothesis.

FFA Profiling in SLE Patients

Although elevated FFA levels were found in SLE, whether a global increase in all FFA species underlies this finding, or if some specific FFA were altered was not clear. To address this issue, a number of FFA species were measured, and the differences between SLE and HC were studied.

Overall, no striking differences were observed between patients and controls (**Table 7**). However, since some collinearity among FFA species existed, a PCA was carried out on the FFA species analyzed to avoid potential biases. PCA demonstrated a good adequacy of the data (KMO statistic = 0.781, Barlett sphericity test $p = 10^{-44}$), and three components were identified (eigenvalues >1) explaining 77.53% of the total variance. Based on their loadings, PC1 (53.32% variance explained) retained γ-linolenic, palmitoleic, palmitic, oleic, linolenic, linoleic, and arachidonic acids, and PC2 (13.95% variance explained) mainly retained EPA and DHA. Finally, PC3 (10.26% variance explained) only retained stearic acid (**Figure 4**).

On the one hand, these results underlie the outstanding heterogeneity of FFA species, a clearly distinct pattern of grouping depending on their chemical structure (chain length or double-bond position) not being found. Globally, saturated, monounsaturated, and w6 fatty acids were grouped together within the PC1, whereas the most important anti-inflammatory w3 FFA did in the PC2. Stearic acid, of controversial immunological and metabolic role, was grouped in the third component. Hence, these findings support a functional, rather than structural, relationship of FFA.

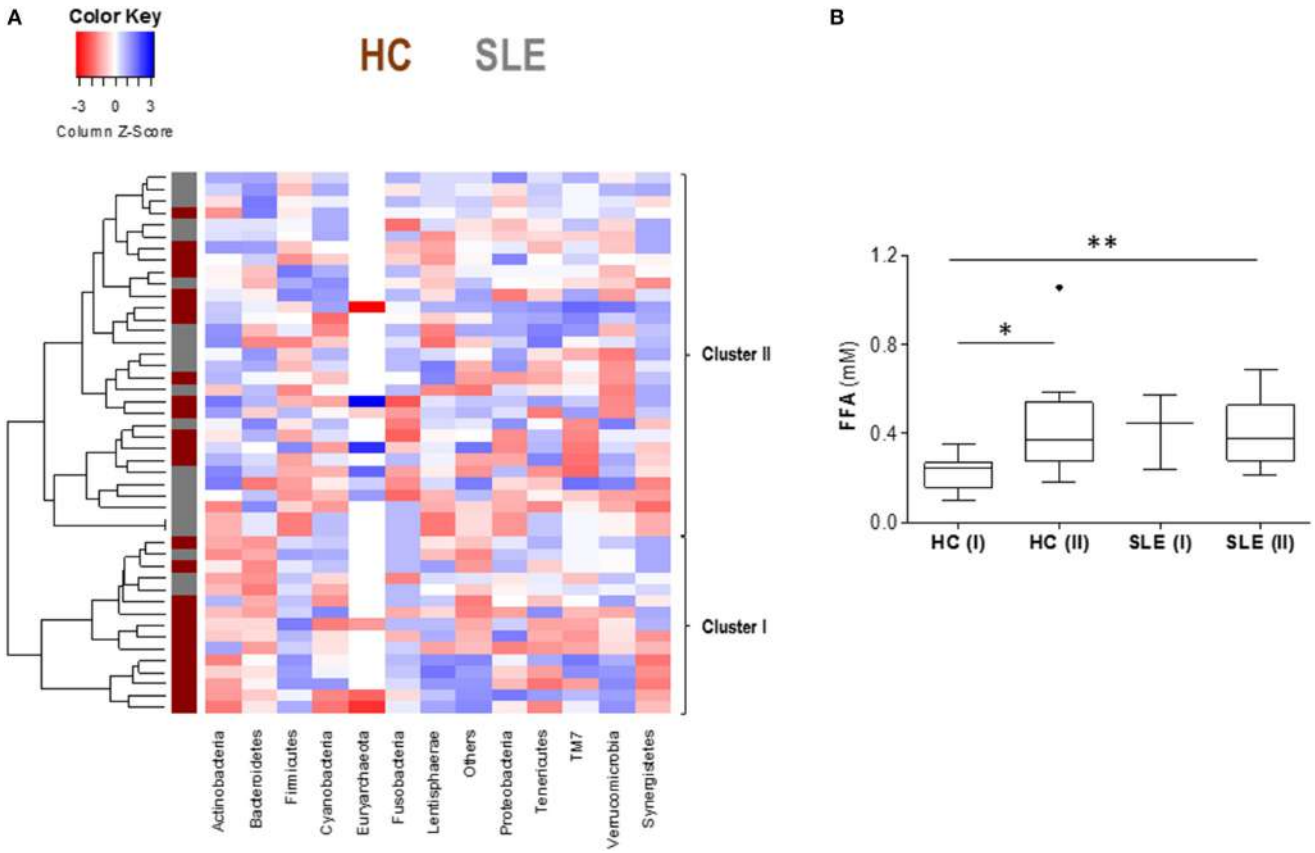

FIGURE 2 | Cluster analysis revealed a link between gut microbiota composition and total free fatty acids (FFA) serum levels. **(A)** Heatmap plotting gut microbiota composition, based on the main phyla, in healthy controls (HC) and systemic lupus erythematosus (SLE) patients. Tiles are colored based on the abundance of each group, red and blue colors indicating low or high levels, respectively. Each row represents an individual, and the vertical colored bar at the left represents HC (dark red) or SLE patient (gray). Two clusters were identified and are highlighted at the right (clusters I and II). **(B)** Comparison of total FFA serum levels among groups after stratifying by disease status (HC or SLE) and microbiota clusters (I or II). Boxes represent median and interquartile range, whereas whiskers represent minimum and maximum values. Differences were assessed by Kruskal–Wallis test and Dunn–Bonferroni *post hoc* correction for multiple comparisons tests. *$p < 0.050$, **$p < 0.010$.

TABLE 6 | Analysis of fecal short-chain fatty acids (SCFA) levels and their correlation with serum-free fatty acids (FFA) levels in healthy controls (HC) and systemic lupus erythematosus (SLE) patients.

	HC	SLE	*p*
SCFA levels (mM)			
Acetate	41.14 (12.30)	57.63 (19.63)	**0.005**
Propionate	11.96 (8.72)	20.61 (9.80)	**0.003**
Butyrate	7.78 (3.97)	10.50 (7.14)	0.075
SCFA–FFA correlations			
Acetate	***r* = 0.770** ***p* < 0.001**	*r* = 0.067 *p* = 0.793	
Propionate	***r* = 0.790** ***p* < 0.001**	*r* = 0.230 *p* = 0.359	
Butyrate	***r* = 0.764** ***p* < 0.001**	*r* = −0.066 *p* = 0.795	

The differences between fecal SCFA levels found in lupus patients and those in HC were analyzed by Mann–Withney U tests; whereas the correlation analyses were performed by Spearman rank's tests. Variables are summarized as median (interquartile range). Statistical analyses with a p-value below 0.050 are highlighted in bold.

On the other hand, no differences in the PCA scores were registered between SLE and HC groups (PC1: $p = 0.169$, PC2: $p = 0.378$, and PC3: $p = 0.916$), thus suggesting that SLE patients did not exhibit a different FFA profile compared to HC.

Finally, whether PCA scores could be related to gut microbiota composition was analyzed. Notably, F/B ratio was positively correlated with the PC2 score (**Table 8**) in HC but not in SLE, thereby suggesting a beneficial effect of gut microbiota composition on the serum FFA pool in healthy individuals. Again, when the phyla were independently studied, *Bacteroidetes* exhibited a stronger correlation with PC2 score ($r = -0.433$, $p = 0.039$) than that of *Firmicutes* ($r = 0.411$, $p = 0.052$) in HC individuals. Thus, gut microbiota seems to quantitatively and qualitatively impact the FFA serum pool.

FFA and Serum Biomarkers in SLE Patients

Since some associations between gut microbiota and specific FFA PCA scores were observed, we aimed to evaluate whether these

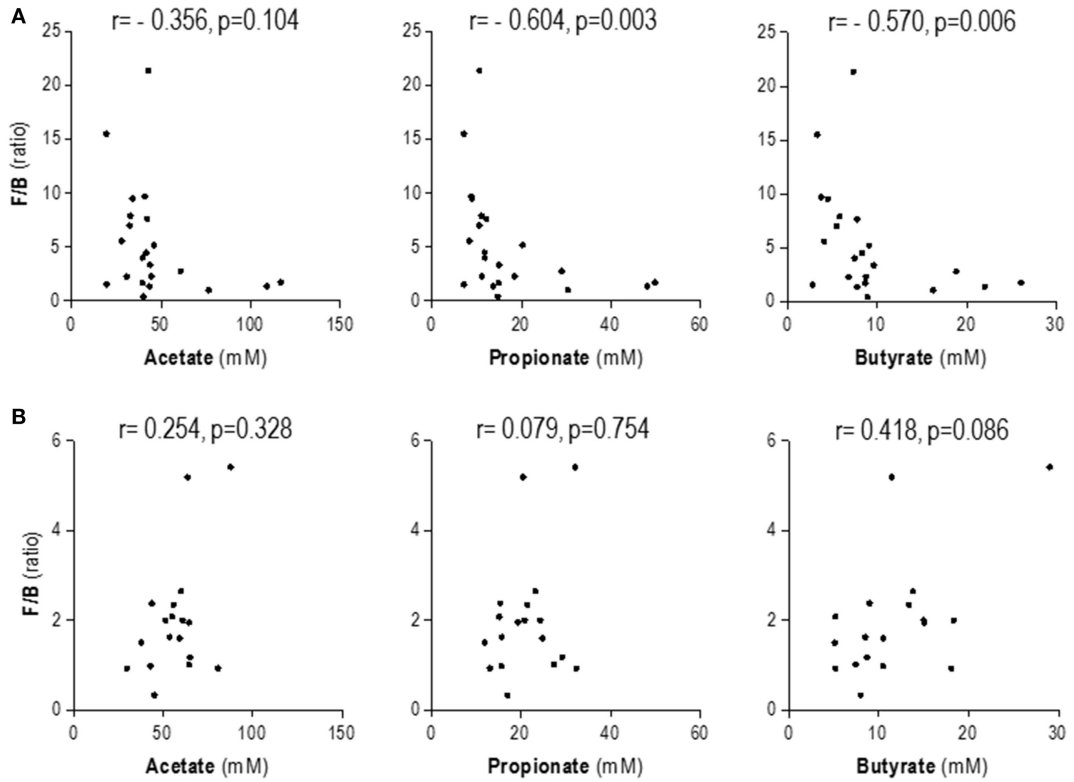

FIGURE 3 | Association between fecal short-chain fatty acids (SCFA) levels and *Firmicutes* to *Bacteroidetes* ratio. Correlation analyses by Spearman's ranks tests were performed in order to analyze the association between fecal SCFA levels and those of serum-free fatty acids in healthy controls **(A)** and systemic lupus erythematosus patients **(B)**.

TABLE 7 | Specific free fatty acids (FFA) in healthy controls (HC) and systemic lupus erythematosus (SLE) patients.

FFA (μg/ml)	HC	SLE	*p*
Palmitic (16:0)	32.76 (15.59)	30.35 (8.37)	0.982
Stearic (18:0)	28.51 (12.95)	29.12 (6.19)	0.873
Palmitoleic (16:1ω7)	1.93 (1.62)	2.67 (0.99)	0.351
Oleic (18:1ω9)	27.76 (20.61)	34.39 (17.77)	0.467
Linoleic (18:2ω6)	6.99 (6.47)	10.26 (7.35)	0.246
γ-Linoleic (18:3ω6)	0.08 (0.08)	0.10 (0.06)	0.785
AA (20:4ω6)	1.96 (1.68)	2.74 (1.74)	**0.045**
Linolenic (18:3ω3)	0.18 (0.17)	0.20 (0.16)	0.539
EPA (20:5ω3)	0.07 (0.15)	0.15 (0.17)	0.363
DHA (22:6ω3)	1.47 (1.46)	1.65 (1.76)	0.209

The differences in specific FFA serum levels between control and patients were assessed by Mann–Withney U tests. Variables are summarized as median (interquartile range). Statistical analyses with a p-value below 0.050 are highlighted in bold.

parameters could be related to some relevant serum biomarkers in lupus. To this end, a panel of serum biomarkers of endothelial activation (VEGF, GM-CSF, EGF, IL-8, TNFα, MCP-1, IP-10, and leptin) and oxidative stress (MDA) were measured (**Table 9**).

First, the associations between these biomarkers and the gut microbiota composition were analyzed. Interestingly, the F/B ratio was negatively associated to leptin ($r = -0.369$, $p = 0.006$) and MCP-1 serum levels ($r = -0.304$, $p = 0.025$) in the whole group.

On the other hand, divergent associations were noted among FFA PCA scores and these biomarkers in SLE patients. Whereas PC1 was positively correlated with biomarkers of endothelial activation [VEGF ($r = 0.444$, $p = 0.044$), IL-8 ($r = 0.522$, 0.015), and EGF ($r = 0.400$, $p = 0.070$)], negative associations were observed for PC2 [EGF ($r = -0.425$, $p = 0.055$), MCP-1 ($r = -0.640$, $p = 0.002$), IP-10 ($r = -0.397$, $p = 0.075$), TNFα ($r = -0.410$, $p = 0.065$), and MDA ($r = -0.375$, $p = 0.057$)]. Interestingly, negative associations were also found for PC3 [VEGF ($r = -0.534$, $p = 0.013$) and IL-8 ($r = -0.459$, $p = 0.036$)]. No associations were observed in the HC.

All these findings seem to point to a link between gut microbiota, FFA serum pool, and biomarkers of endothelial activation in lupus, thus emphasizing the systemic effect of the gut microbiota in this condition. Additionally, differences among FFA PCA are in line with their proposed functional diversity.

DISCUSSION

Over the last decade, several studies have revealed a number of interactions between the gut microbiota and the host in homeostatic conditions. Accordingly, dysbiosis has been consistently related to different pathological situations, from immune-mediated to metabolic diseases (27–29). In this sense, we have recently reported the existence of an intestinal dysbiosis in SLE

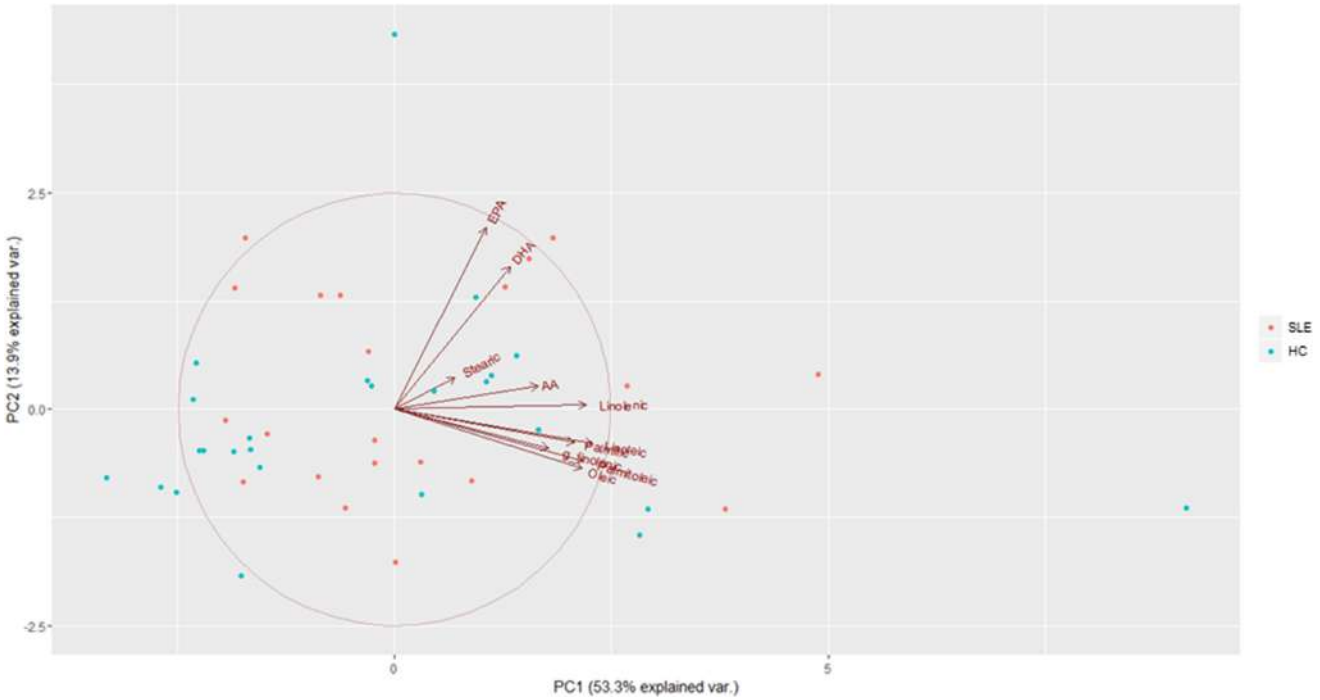

FIGURE 4 | Principal component analysis (PCA) of specific free fatty acids (FFA) profiling. Biplot obtained in the PCA of specific FFA serum levels. PC1 and PC2 are represented in the horizontal and vertical axes, respectively. Arrows represent the vectors showing the associations among the raw variables entered in the analysis. Individuals are represented in colored dots: healthy controls (HC) (turquoise) and systemic lupus erythematosus (SLE) (red).

TABLE 8 | Associations between *Firmicutes* to *Bacteroidetes* ratio and free fatty acids-principal component analysis scores in healthy controls (HC) and systemic lupus erythematosus (SLE) patients.

	HC	SLE
PC1	$r = -0.246$ $p = 0.257$	$r = 0.078$ $p = 0.736$
PC2	**$r = 0.437$** **$p = 0.037$**	$r = 0.110$ $p = 0.635$
PC3	$r = -0.075$ $p = 0.734$	$r = 0.089$ $p = 0.700$

Correlations between these parameters were assessed by Pearson correlation tests. Statistical analyses with a p-value below 0.050 are highlighted in bold.

TABLE 9 | Levels of serum biomarkers analyzed in healthy controls (HC) and systemic lupus erythematosus (SLE) patients.

	HC	SLE	*p*
Vascular endothelial growth factor [VEGF] (pg/ml)	83.38 (49.49)	70.73 (98.87)	0.326
Granulocyte monocyte colonystimulating factor [GM-CSF] (pg/ml)	0.35 (0.92)	0.35 (2.77)	0.424
Epidermal growth factor [EGF] (pg/ml)	114.29 (81.35)	65.57 (78.61)	**0.019**
Interleukin-8 [IL-8] (pg/ml)	14.69 (22.51)	27.85 (21.92)	**0.016**
Tumor necrosis factor alpha [TNFα] (pg/ml)	174.40 (309.4)	188.14 (292.92)	0.789
Monocyte chemoattractant protein-1 [MCP-1] (pg/ml)	444.55 (481.45)	616.13 (402.07)	**0.011**
Interferon gamma-inducible protein-10 [IP-10] (pg/ml)	98.82 (148.81)	167.67 (152.76)	**0.019**
Leptin (ng/ml)	7.73 (7.14)	14.16 (20.20)	**<0.001**
Malondialdehyde [MDA] (μM)	2.78 (0.71)	2.90 (0.44)	0.658

The differences in serum levels between control and patients were assessed by Mann–Withney U tests. Variables are summarized as median (interquartile range). Statistical analyses with a p-value below 0.050 are highlighted in bold.

(22). Additional studies from our group allowed us to associate the dysbiotic state with the dysregulated Treg/Th17 responses found in lupus patients (30). In the present report, we go a step further by addressing the study of the potential connections between the intestinal dysbiosis and the metabolic impairment in SLE, focusing on the role of FFA. Thus, gut microbiota may not only be related to disease pathogenesis itself but also to some comorbidities frequently present in lupus patients. Since the origin of such alterations is ill-defined, these new findings allow us to gain some insight into this complex situation and may help to delineate new therapeutic targets. Actually, the experimental modulation of the gut microbiota has yielded promising results in other scenarios (17). In fact, the lupus-like immune over-activation was partially reestablished *in vitro* by the supplementation with specific bacterial strains (30). Thus, our study warrants future research to assess whether this therapy may be also advisable to counteract the metabolic alterations in SLE patients.

Metabolic disorders, including metabolic syndrome, are common hallmarks of SLE and other related diseases (31–33).

However, the underlying causes of these traits are not well defined. The findings herein presented suggest that gut microbiota may play a role in this condition. This notion can explain why a wide range of diseases, with striking differences in their clinical presentations, are associated with similar comorbidities (such as metabolic alterations). Thus, it is feasible to think that similar patterns of intestinal dysbiosis may underlie this situation. This idea is supported by the altered F/B ratios that were reported in other diseases exhibiting increased serum FFA levels (34–36). The fact that this ratio is a continuum may explain the differences in prevalence and severity of metabolic complications among different conditions. However, it must be remarked that studies on the alterations in the F/B ratio have yielded contradictory results in different contexts, such as obesity (37–39). Schwiertz and colleagues have recently published a reduced F/B ratio in obese and overweighed individuals in a large study population (40). Similarly, the enrichment of *Firmicutes* in the intestinal ecosystem has been related to improved lipid absorption and homeostasis in animal models (41). It must be taken into account that differences in sequencing techniques, data analysis, and characteristics of the recruited populations may be an important source of discrepancy in this field. Thus, the associations of the microbiota with metabolic traits in different scenarios must be interpreted with caution, since direct comparison is not always possible. Additional studies focusing on the F/B ratio in non-obese healthy population are warranted.

The association between the F/B ratio and the serum levels of FFA emphasizes the ability of the gut microbiota to promote systemic responses in the host. Moreover, it supports that gut microbiota can modulate the energy metabolism of the host (42). However, the identification of the actual mediators involved and the potential impact of this interaction in pathological conditions is currently lacking in the literature. Our findings in the present work point to the SCFA as potential orchestrators of the cross talk between gut microbiota and the host metabolism. Although these compounds are thought to be of key relevance in the interactions between intestinal microbial populations and the host (16), their link with the lipid metabolism remains controversial. Our results suggest that the SCFA production paralleled the FFA serum levels in healthy individuals. However, this association was absent in the pathological framework of lupus, where an increased SCFA production, together with elevated FFA serum levels, was noted. These results are in line with those from other metabolic conditions [reviewed in Ref. (43)]. Additionally, our analyses revealed that this aberrant SCFA production can be linked to an altered microbial gut composition, as a reduced F/B ratio was related to increased SCFA fecal levels. In this scenario, the potential involvement of the propionate deserves a special mention. Propionate is mainly produced by *Bacteroidetes* species (44), thereby supporting its negative association with the F/B ratio observed. It is important to note that increased propionate fecal levels have been reported in obese individuals exhibiting a decreased F/B ratio (40). Furthermore, exposure of human intestinal organoids to propionate led to an upregulation of genes belonging to lipolytic pathways (45). Moreover, experimental evidence from animal models and *ex vivo* experiments with human material have identified a mechanistic link between exposure to propionate and increased lipolysis mediated by the increased expression of the enzyme lipoprotein lipase (46–48). Moreover, a homeostatic role for propionate on glucose metabolism and regulation of energy intake has also been proposed. However, which is the actual role for propionate in human diseases required further elucidation. Taken together, our finding may provide a possible explanation for the elevated FFA serum levels in SLE, altered SCFA production, and overrepresentation of *Bacteroidetes* in the intestinal microbiota playing a pivotal role. The stronger associations of *Bacteroidetes* alone compared with those of the F/B ratio are in line with this point.

Another important result of our study was the association between FFA and some biomarkers of endothelial activation. Because of their nature, FFA are considered as common mediators between immunity, inflammation, and metabolism. Although some authors have previously proposed a role for the gut microbiota in the etiology of metabolic alterations and CVD (49), the actual players are far from being clear. Interestingly, experimental studies revealed a mechanistic link between propionate and leptin expression by human adipose and omental tissues (50), which is in line with our results. Nevertheless, the exact significance of this finding *in vivo* is not known. Taking into account the effects of FFA on inflammation, oxidative stress, and expression of adhesion molecules (51–53), our results may support a role for FFA as a link between the (altered) gut microbiota, host metabolism, and disease status. It is interesting to note that differences among FFA in their ability to promote endothelial activation *in vivo* or *in vitro* have been demonstrated (54–56), which is in line with the associations found in our study. Importantly, these biomarkers are considered as early preclinical indicators of CVD development in the long term (57–60). Taking this into account, these associations may point to a very early role of the altered gut microbiota in the etiology of these complications. This is reinforced by the negative association between the intestinal dysbiosis in lupus and the levels of the protective IgM antibodies against phosphorylcholine (30). These antibodies are known to enhance apoptotic-cell clearance and induce anti-inflammatory pathways, explaining its negative association with markers of subclinical atherosclerosis (61) and CVD development (62) in lupus.

Finally, our approach did not identify a different FFA serum profile in SLE patients in comparison to HC. Although a similar pattern of grouping of FFA species in the PCA was observed in rheumatoid arthritis patients by our group (24), differences among FFA species between patients and controls were not observed in the case of lupus. Interestingly, no differences in plasma FFA profiling were observed in a previous study with lupus patients (63), although slight alterations in the polyunsaturated fatty acids were reported in those with a previous history of CVD. Our results are, at least in part, in line with these findings, although differences in the experimental procedures between both studies are important. It is worthy to note that our group of patients was characterized by a low disease activity, even in the absence of glucocorticoid and immunosuppressive drugs. Thus, a larger study involving SLE patients with a higher degree of disease activity is warranted. However, despite no differences being found in the specific FFA levels, the results from the PCA emphasize the heterogeneity among FFA classes and suggest that FFA, despite being mostly

unaltered, can develop different roles under different milieu. This hypothesis is in line with current evidence in this field (64, 65) and stresses the underestimated significance of FFA as key mediators for the human health.

Due to the lack of direct mechanistic data in our approach, these results pose the question on whether the microbiota composition is responsible of the altered FFA levels or if, alternatively, increased FFA levels may lead to changes in the gut ecology. Based on the literature currently available, several research studies seem to align with the former idea. The fact that no changes in the gut microbiota were related to disease duration (in spite of the wide range of disease duration studied in the present report) is also in line with this idea, probably suggesting that intestinal dysbiosis could be present at the preclinical stages of the disease. Similarly, experiments of fecal transplantation in obese and lean mice also support the causative role of the microbiota in shaping the host metabolism (66). However, due to the role of FFA on inflammation, it is tempting to speculate that these molecules can prompt a shift toward a systemic pro-inflammatory state, which can, in turn, disrupt the intestinal microbiota. Experimental studies with mice have revealed that diet-induced obesity is accompanied by changes in the gut microbiota and damaged intestinal barrier (67, 68). Interestingly, a diet with high ω6 content resulted in intestinal dysbiosis in mice, the inflammatory pathways playing a crucial role (69). Moreover, ω3 fatty acids seem to counteract the effects of diet- or antibiotics-induced dysbiosis through different mechanisms (70, 71). Additionally, an antibacterial effect was observed for some FFA (72). Therefore, it is feasible that a bidirectional cross talk between the gut microbiota and host metabolism is established, with immune circuits participating in this interaction.

In summary, our data indicate that increased serum FFA levels in SLE patients may be associated with changes in the gut ecosystem in the framework of lupus dysbiosis. The association between serum FFA and SCFA supports this notion. Additionally, different associations between FFA species and serum biomarkers of endothelial activation were found, hence not only underscoring the heterogeneity among FFA compounds but also shedding new light on the gut–metabolism–CVD axis. Although the reduced sample size and the lack of a mechanistic data are the limitations of our study, to the best of our knowledge this is the first report supporting a connection between gut microbiota, FFA, and biomarkers of endothelial activation. Moreover, we have provided a proof of concept evidence on the involvement of the intestinal dysbiosis in the metabolic alterations in lupus.

AUTHOR CONTRIBUTIONS

All the authors listed made substantial contributions to the design of the work, analysis, or interpretation of the results obtained; involved in drafting the manuscript, revising it critically for intellectual content, and approving the final version; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical assistance of Ana M. Hernández-Barranco (IPLA) with the analysis of SCFA in fecal samples as well as of the staff of the scientific core facilities from the University of Oviedo (Unidad de Espectrometría y Espectrofotometría, Servicios Científico-Técnicos, Universidad de Oviedo) with the FFA analysis. The authors also show our deepest gratitude to all the study volunteers.

FUNDING

This work was funded through the grants GRUPIN14-043 "Microbiota Humana, Alimentación y Salud" from the "Plan Regional de Investigación del Principado de Asturias" and cofounded from European Union FEDER funds, AGL2010-14952 from the Spanish Ministry of Science and Innovation, and PI012/00523 from the "Fondo de Investigaciones Sanitarias, Instituto de Investigación Carlos III." JR-C is supported by a contract from the grant GRUPIN14-043.

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Conflict of Interest Statement: The authors declared no financial conflicts of interest. The funders have no role in study design, data analysis, or decision to publish.

frontiers in Immunology

DATA REPORT
published: 07 April 2017
doi: 10.3389/fimmu.2017.00413

Complete Genome Sequence of *Lactobacillus casei* LC5, a Potential Probiotics for Atopic Dermatitis

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Keywords: atopic dermatitis, probiotics, *Lactobacillus casei*, genome sequence, PacBio

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *30 October 2016*
Accepted: *23 March 2017*
Published: *07 April 2017*

Citation:
Kang J, Chung W-H, Lim T-J, Whon TW, Lim S and Nam Y-D (2017) Complete Genome Sequence of Lactobacillus casei LC5, a Potential Probiotics for Atopic Dermatitis. Front. Immunol. 8:413. doi: 10.3389/fimmu.2017.00413

BACKGROUND

Probiotics are living microorganisms providing health beneficial effect to the host (1). Probiotics have been used for the treatment or prevention of various diseases related to diarrhea (2), cholesterol (3) immune function (4), and inflammatory bowel disease (5). In addition, recent study also presents that probiotic bacteria in the *Bifidobacterium* and *Lactobacillus* genera are able to have therapeutic effects in the patients of psychological disorders, such as depression, anxiety, and memory (6).

Lactobacillus casei is a Gram-positive bacterium that naturally inhabits the human and animal gastrointestinal and mouth organs (7). As its name implies, this heterofermentative microorganism is the dominant species present in ripening cheddar cheese (8). In probiotic aspects, *L. casei* showed beneficial roles in the activation of the gut mucosal immune system (9), treatment of diabetics (10), and chronic constipation (11). In the previous study, we isolated *L. casei* LC5 strain from fermented dairy products, which showed immune regulatory functions, especially, therapeutic effect on atopic dermatitis as a member of complex probiotics (12–14).

In order to gain better insight of the probiotic effect on atopic dermatitis, we analyzed the genome sequence of *L. casei* LC5. According to the report of NCBI Genome,[1] more than two hundreds of *Lactobacillus* organisms are sequenced and their beneficial properties derived from genomic information are used in the food industry. However, the available genomes of *L. casei* strains as members of health promoting probiotics are still insufficient. Furthermore, *L. casei* strains are frequently confused with the closely related strains such as *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. Therefore, comparative study in a whole genome scale is required to clarify taxonomic association of *L. casei* LC5 as well as its functional characteristics. The availability of the genomic information of *L. casei* LC5 will aid as a basis for further in-depth analysis of the probiotic function of *L. casei* strains.

MATERIALS AND METHODS

Bacterial Strains and DNA Preparation

Lactobacillus casei LC5 was isolated from fermented dairy products and commercially used as probiotics in Korea (15). *L. casei* LC5 was cultured aerobically in MRS medium (Difco, USA) at 37°C for 18 h. Genomic DNA from *L. casei* LC5 was extracted and purified using a QIAamp DNA

[1] https://www.ncbi.nlm.nih.gov/genome/?term=Lactobacillus.

Mini Kit (Qiagen, Germany). The concentration of genomic DNA was qualified with NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, USA) and Qubit 2.0 fluorometer (Life Technology, USA).

Genome Sequencing, Assembly, and Annotation

Whole genome sequencing of *L. casei* LC5 was carried out by using PacBio RS II platform. A 20 kb DNA library was constructed according to the manufacturer's instruction and sequenced using single molecule real-time (SMRT) sequencing technology with the P6 DNA polymerase and C4 chemistry. A total of 138,180 subreads (1.04 Gb) were obtained with 400-fold coverage. The average length of subreads was 7,550 bp and N50 was 10,940 bp. Genome assembly was performed using HGAP 3.0 (16) with default options. The annotation was carried out with NCBI Prokaryotic Genome Annotation Pipeline (17) through NCBI Genome submission portal (GenomeSubmit at http://ncbi.nlm.nih.gov). The chromosome topology was drawn using DNAPlotter (18). Clusters of orthologous groups (COG) categories were assigned to the coding genes using BLASTP (e-value: 1e−3) against COG database (19).

Phylogenetic Analysis and Comparative Genomic Analysis

For phylogenetic and comparative study, we downloaded 19 genome sequences of *L. casei* group (10 of *L. casei*, 8 of *L. paracasei*, 1 of *Lactobacillus zeae*, and 1 of *L. rhamnosus*) from NCBI genome database.[2] A list of the reference genomes are as follows: *L. casei* Zhang (NC_014334), *L. casei* BL23 (NC_010999), *L. casei* BD-II (NC_017474), *L. casei* LC2W (NC_017473), *L. casei* 12A (NZ_CP006690), *L. casei* W56 (NC_018641), *L. casei* LcY (NZ_CM001848), *L. casei* LcA (NZ_CM001861), *L. casei* LOCK919 (NC_021721), *L. casei* ATCC 393 (NZ_AP012544), *L. paracasei* ATCC 334 (NC_008526), *L. paracasei* 362.5013889 (NC_022112), *L. paracasei* N1115 (NZ_CP007122), *L. paracasei* JCM (NZ_AP012541), *L. paracasei* CAUH35 (NZ_CP012187), *L. paracasei* L9 (NZ_CP012148), *L. paracasei* KL1 (NZ_CP013921), *L. zeae* DSM 20178 (NZ_AZCT01000001), and *L. rhamnosus* GG (NC_013198). The assembly levels of all genomes are "complete genome" or chromosome except *L. zeae* DSM 20178 (includes 55 scaffolds). Because we failed to fetch full-length 16S rRNA gene from the genome of *L. zeae* DSM 20178, we alternatively used a 16S rRNA gene of *L. zeae* RIA 482 (NR_037122), the closest sequence of DSM 20178 (sequence identity = 99.9%), in the phylogenetic analysis.

The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model (20). All positions containing gaps and missing data were eliminated. There were a total of 1521 positions in the final dataset. Those phylogenetic analyses were conducted in MEGA6 (21). To compute genomic distance, we first computed orthologous average nucleotide identity (OrthoANI) values using orthologous average nucleotide identity tool (22). The OrthoANI values were converted to distance values by following formula: distance = 1 − (OrthoANI/100). The evolutionary distance was computed using the neighbor-joining method of MEGA6 (21). The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The resulting phylogenetic tree was produced using MEGA6. Pan-genomic study using Panseq (23) was performed to investigate the genomic conservation and finding novel region in the sequenced genome.

[2]http://www.ncbi.nlm.nih.gov/genome/.

RESULTS

Genome Characteristics of *L. casei* LC5

We obtained a complete genome sequence of *L. casei* LC5 using SMRT sequencing. This genome has a chromosome and no organelle sequences. The total size of the genome is 3,132,867 bp and its GC content is 47.9%. A total of 2,925 genes were detected from the genome sequence. The number of coding CDS is 2,817 and pseudogenes is 31. Seventy seven RNAs (15 rRNAs, 59 tRNAs, and 3 non-coding RNAs) were also identified. Repeating region or CRISPR array was not identified. Genomic features of *L. casei* LC5 are shown in **Figure 1A**.

Although *L. casei* LC5 was identified as a strain of *L. casei*, it showed different genomic features compared to the other published *L. casei* strains; According to the summary of 37 *L. casei* genomes deposited in NCBI Assembly, the median length is 3.01993 Mb, the median of coding genes is 2,712, and the median of GC contents is 46.4%. An interesting point is that those genomes can be split into two groups by the difference of GC contents, high-GC group (47.7–47.9%) and low-GC group (46.2–46.6%). Five genomes (ATCC 393, N87, 867_LCAS, Lbs2, JCM 1134) and *L. casei* LC5 belong to the high-GC group and the other genomes belong to the low-GC group (**Table 1**).

Comparative Study of *L. casei* Group

Comparative study of both 16S rRNA genes and whole genome sequences revealed that the closest genome of *L. casei* LC5 was *L. casei* ATCC 393 and second closest one was *L. zeae* DSM 20178. The three genomes which showed distinguishable differences on the comparative study, LC5, ATCC 393, and *L. zeae* DSM 20178, belong to the high-GC group as described in the above section. In contrast to the phylogenetic distances based on 16S rRNA gene among the high-GC group (below 0.001), the distances between the high-GC group and the low-GC group were above 0.003 (**Figure 1C**). It was also supported by the estimation result of the whole genomic comparison. Average nucleotide identity (ANI) values among the high-GC group were above 94% whereas ANI values between two groups were below 80% (**Figure 1D**). All the *L. casei* strains and *L. paracasei* strains belonging to the low-GC group showed the high genomic similarity of 98% or higher.

Functional Classification

Functional classification based on COG assigned the 2,334 CDSs into the 1,309 COG numbers. From the comparison of functional

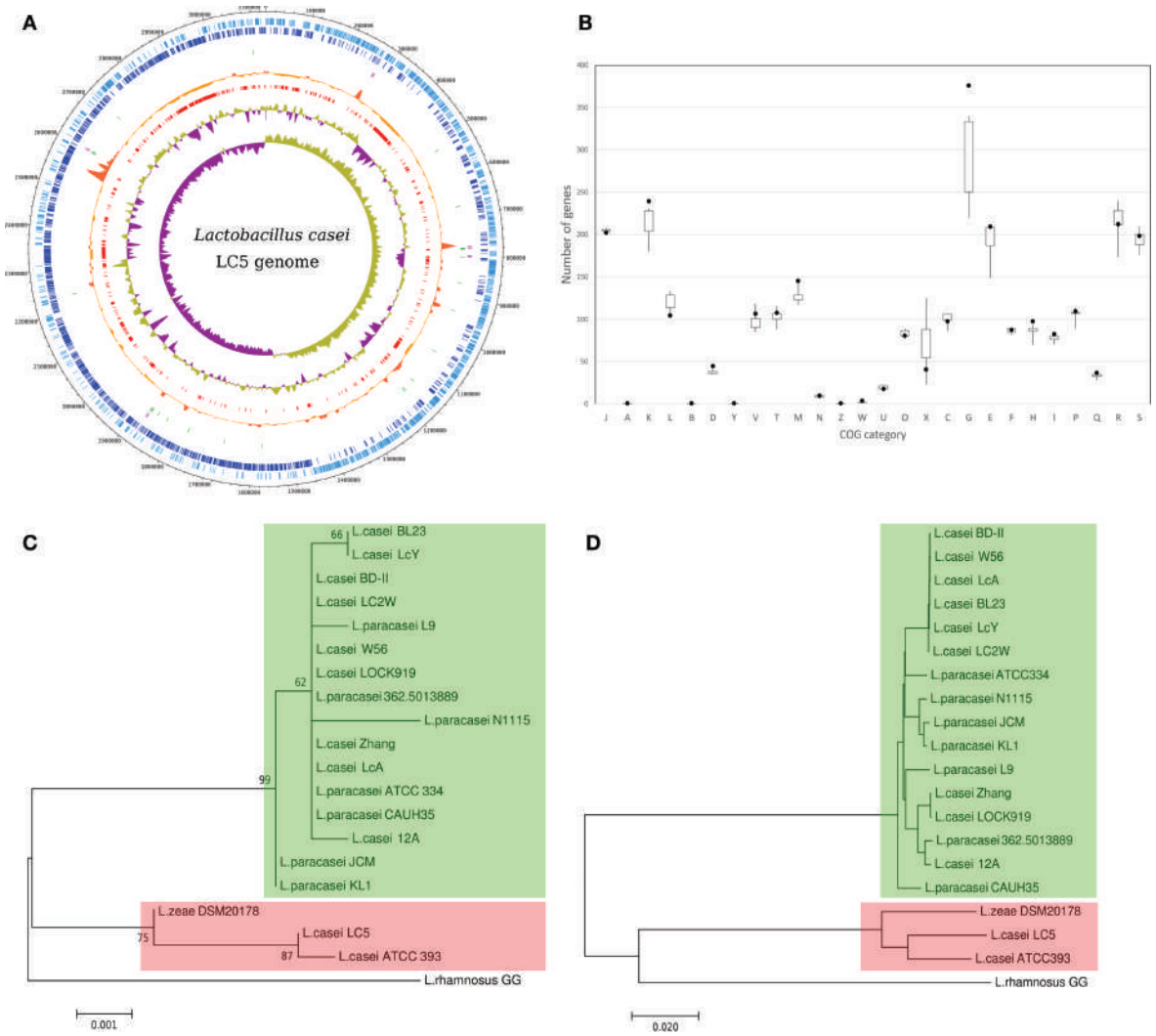

FIGURE 1 | **Genome characteristics of *Lactobacillus casei* LC5 genome**. **(A)** Circular map of genomic features; eight tracks were plotted in the map. Track 1 (light blue; outermost), forward-stranded coding CDS; Track 2 (blue), reverse-stranded coding CDS; Track 3 (light purple), rRNA including 5S, 16S, and 23S; Track 4 (green), Trna; Track 5 (orange), peak of pan-genomic conservation; Track 6 (red), novel regions (below 85% similarities with the other genomes); Track 7 (light green and purple), GC content; and Track 8 (light green and purple), GC skew. **(B)** Abundance of clusters of orthologous groups (COG) categories; black point indicates the abundance of LC5 for each category. A box and whisker plot indicates a statistical distribution of the COG categories of 19 *L. casei* genomes. **(C)** Phylogenetic tree of *L. casei* group based on 16S rRNA genes and **(D)** phylogenetic tree of *L. casei* group based on average nucleotide identity. The value 0.02 of the ruler in **(D)** indicates 2% of genomic dissimilarity. Red boxes on the **(C,D)** indicate the genomes associated to the high-GC group and green boxes indicate the genomes associated to the low-GC group.

categories against the 19 *L. casei* group genomes, we found that *L. casei* LC5 contains the high number of proteins which associate with "carbohydrate transport and metabolism (G)" (376 proteins) and "transcription (K)" (239 proteins) excluding two unknown categories, "general function prediction only (R)" and "function unknown (S)" as shown in **Figure 1B**. *L. casei* LC5 has at least 36 more proteins than the other genomes on the category G and has at least 8 more proteins than the other genomes on the category K. The gene expansion of those two functional categories in the LC5 genome is not found on the other members of high-GC group. Although the genomes belonging to high-GC group showed high similarities to each other and the genomes belonging to the high-GC group do not have excessive proteins on the categories, G and K, when compared to those belonging to the low-GC group. Moreover, *L. casei* ATCC 393 which is the most similar genome of LC5 has fewer proteins than the average number of those categories, 223 proteins for the category G and 192 proteins for the category K.

In the previous study, probiotic LC5 strain isolated from Korean fermented dairy product showed great therapeutic effect on atopic dermatitis. Here, we report a genomic overview and distinguishing gene features of LC5 by comparative genomic analysis of 20 related strains. The genomic data presented in this report will broaden our knowledge about roles and mechanisms

TABLE 1 | Genome summary of *Lactobacillus casei* group.

Organism/name	Strain	Clade	Assembly level	Size (Mb)	GC%	GC group
L. casei LC5	LC5	*L. casei*	Complete genome	3.13	47.9	High
L. casei str. Zhang	Zhang	*L. casei*	Complete genome	2.90	46.4	Low
L. casei BL23	BL23	*L. casei*	Complete genome	3.08	46.3	Low
L. casei BD-II	BD-II	*L. casei*	complete genome	3.13	46.3	Low
L. casei LC2W	LC2W	*L. casei*	Complete genome	3.08	46.4	Low
L. casei 12A	12A	*L. casei*	Complete genome	2.91	46.4	Low
L. casei W56	W56	*L. casei*	Complete genome	3.13	46.3	Low
L. casei LOCK919	LOCK919	*L. casei*	Complete genome	3.14	46.2	Low
L. casei subsp. casei ATCC 393	ATCC 393	*L. casei*	Complete genome	2.95	47.9	High
L. casei LcY	LcY	*L. casei*	Chromosome	3.10	46.3	Low
L. casei LcA	LcA	*L. casei*	Chromosome	3.13	46.3	Low
L. casei A2-362	A2-362	*L. casei*	Scaffold	3.19	46.2	Low
L. casei	KL1-Liu	*L. casei*	Scaffold	2.85	46.6	Low
L. casei DSM 20011 = JCM 1134	DSM 20011	*L. casei*	Scaffold	2.82	46.5	Low
L. casei 21/1	21/1	*L. casei*	Contig	3.22	46.2	Low
L. casei 32G	32G	*L. casei*	Contig	3.01	46.4	Low
L. casei A2-362	A2-362	*L. casei*	Contig	3.36	46.1	Low
L. casei CRF28	CRF28	*L. casei*	Contig	3.04	46.3	Low
L. casei M36	M36	*L. casei*	Contig	3.15	46.3	Low
L. casei T71499	T71499	*L. casei*	Contig	3.00	46.2	Low
L. casei UCD174	UCD174	*L. casei*	Contig	3.07	46.4	Low
L. casei UW1	UW1	*L. casei*	Contig	2.87	46.4	Low
L. casei UW4	UW4	*L. casei*	Contig	2.76	46.4	Low
L. casei Lc-10	Lc-10	*L. casei*	Contig	2.95	46.4	Low
L. casei Lpc-37	Lpc-37	*L. casei*	Contig	3.08	46.3	Low
L. casei UW4	UW4	*L. casei*	Contig	2.63	46.4	Low
L. casei 12A	12A	*L. casei*	Contig	2.93	46.3	Low
L. casei 5b	5b	*L. casei*	Contig	3.02	46.3	Low
L. casei	N87	*L. casei*	Contig	3.00	47.9	High
L. casei	867_LCAS	*L. casei*	Contig	3.09	47.9	High
L. casei	DPC6800	*L. casei*	Contig	3.05	46.4	Low
L. casei	Lc1542	*L. casei*	Contig	2.92	46.5	Low
L. casei	1316.rep1_LPAR	*L. casei*	Scaffold	2.86	46.5	Low
L. casei	1316.rep2_LPAR	*L. casei*	Scaffold	2.79	46.4	Low
L. casei	844_LCAS	*L. casei*	Scaffold	2.79	46.4	Low
L. casei	BM-LC14617	*L. casei*	Scaffold	3.04	46.3	Low
L. casei	Lbs2	*L. casei*	Scaffold	3.27	47.9	High
L. casei DSM 20011 = JCM 1134	JCM 1134	*L. casei*	Contig	2.78	47.7	High
Lactobacillus paracasei ATCC 334	ATCC 334	*L. paracasei*	Complete genome	2.92	46.6	Low
L. paracasei subsp. *paracasei* 8700:2	8700:2	*L. paracasei*	Complete genome	3.03	46.3	Low
L. paracasei N1115	N1115	*L. paracasei*	Complete genome	3.06	46.5	Low
L. paracasei subsp. paracasei JCM 8130	JCM 8130	*L. paracasei*	Complete genome	3.02	46.6	Low
L. paracasei	CAUH35	*L. paracasei*	Complete genome	2.97	46.3	Low
L. paracasei	L9	*L. paracasei*	Complete genome	3.08	46.3	Low
L. paracasei	KL1	*L. paracasei*	Complete genome	2.92	46.6	Low
Lactobacillus zeae DSM 20178 = KCTC 3804	DSM 20178	*L. zeae*	Scaffold	3.12	47.7	High
Lactobacillus rhamnosus GG	GG (ATCC 53103)	*L. rhamnosus*	Complete genome	3.01	46.7	Low

of microorganisms ameliorating symptoms of immune diseases and help developing functional probiotics for the treatment of immune disorders.

DATA ACCESS

The *L. casei* LC5 genome sequencing project has been deposited at GenBank under the accession number CP017065. The BioProject and BioSample designation for this project is PRJNA340077 and SAMN05631198, respectively. This strain has been deposited in the Korean Collection for Type Cultures (deposit ID: KCTC 12398BP).

AUTHOR CONTRIBUTIONS

Y-DN and SL designed and coordinated all the experiments. T-JL and JK performed cultivation and DNA preparation. JK and W-HC performed genome assembly, gene prediction, gene annotation, and comparative genomic analysis. Y-DN, W-HC, TW, and JK wrote the manuscript. All authors have read the manuscript and approved.

FUNDING

This work was supported by a grant from Korea Food Research Institute (project no. E0170602-01).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.