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Immunomodulation of *Lactobacillus rhamnosus* GG (LGG)-derived soluble factors on antigen-presenting cells of healthy blood donors

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Lactobacillus rhamnosus GG (LGG) cells have been shown to promote type-1 immune responsiveness; however knowledge of immunomodulation of soluble factors secreted by LGG is limited. This is the first study to investigate whether LGG soluble factors promote a comparable immune responsiveness as the bacterial cells. Both treatments – LGG conditioned medium with (CM + LGG) or without (CM) LGG cells, in this study increased expression of several toll-like receptors (TLRs) in all studied cell types and antigen presentation-associated receptor HLA-DR in macrophages and “intermediate” monocytes; but decreased that of activation markers on monocytes and macrophages and production of IL-10, IL-12 and TNF α in macrophages. In co-culture with mononuclear cells, CM increased Th1-type cytokine profile but not as pronounced as CM + LGG. This study suggests that LGG soluble factors exert similar immunomodulatory effects as the intact cells, but cells may be required for optimal type-1 immune responsiveness polarizing capacity of this probiotic strain.

Conditioned medium of lactobacilli, which is cell-free supernatant containing soluble factors, has been shown to enhance lactobacilli but inhibit invasive *Escherichia coli*, adhere to intestinal epithelial cells, intestine mucus and gastric mucin^{1,2}; and elicit antibacterial activity against pathogens such as *Salmonella* species^{3–5}. Soluble factors refer to metabolites, proteins, DNA and cell-wall constituents^{2,6}.

Lactobacillus rhamnosus GG (LGG) (ATCC 53103) is perhaps the most researched lactobacilli in the world; but some of its soluble factors have only been recently identified, for example, p75 and p40 proteins⁷, porcine serpine protease inhibitor, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cell wall-associated hydrolase, transcriptional regulator and phosphoglycerate kinase⁸. These factors play an important role in the physiology of LGG^{8–12} and have been found beneficial to the gut, for example, diminishing apoptosis of intestinal epithelium^{7,13}, enhancing intestinal crypt survival¹³ and preserving cytoskeletal integrity of intestine¹⁴. However, knowledge of the immunomodulatory effects exerted by LGG soluble factors is lacking. Our previous study shows that LGG cells activate antigen-presenting cells (APCs) of healthy blood donors to promote type-1 immune responsiveness¹⁵. Herein, this study aimed to investigate whether LGG soluble factors promote the same immune responsiveness as the cells, and if LGG cells alter the immunomodulatory properties of the soluble factors. Elucidating the immunomodulatory effects of LGG soluble factors will help to draw evidence-based guidelines of this strain for the treatment development for various diseases such as drug synthesis and vaccine development.

Results

TLR mRNA expression in DCs, macrophages and monocytes. TLR mRNA levels of DCs, macrophages and monocytes are shown in Fig. 1. They were detected after 24-hour incubation with CM or CM + LGG. Both CM and CM + LGG increased TLRs 1, 4, 5, 6, 7, 8 and 9 mRNA levels of DCs and macrophages.

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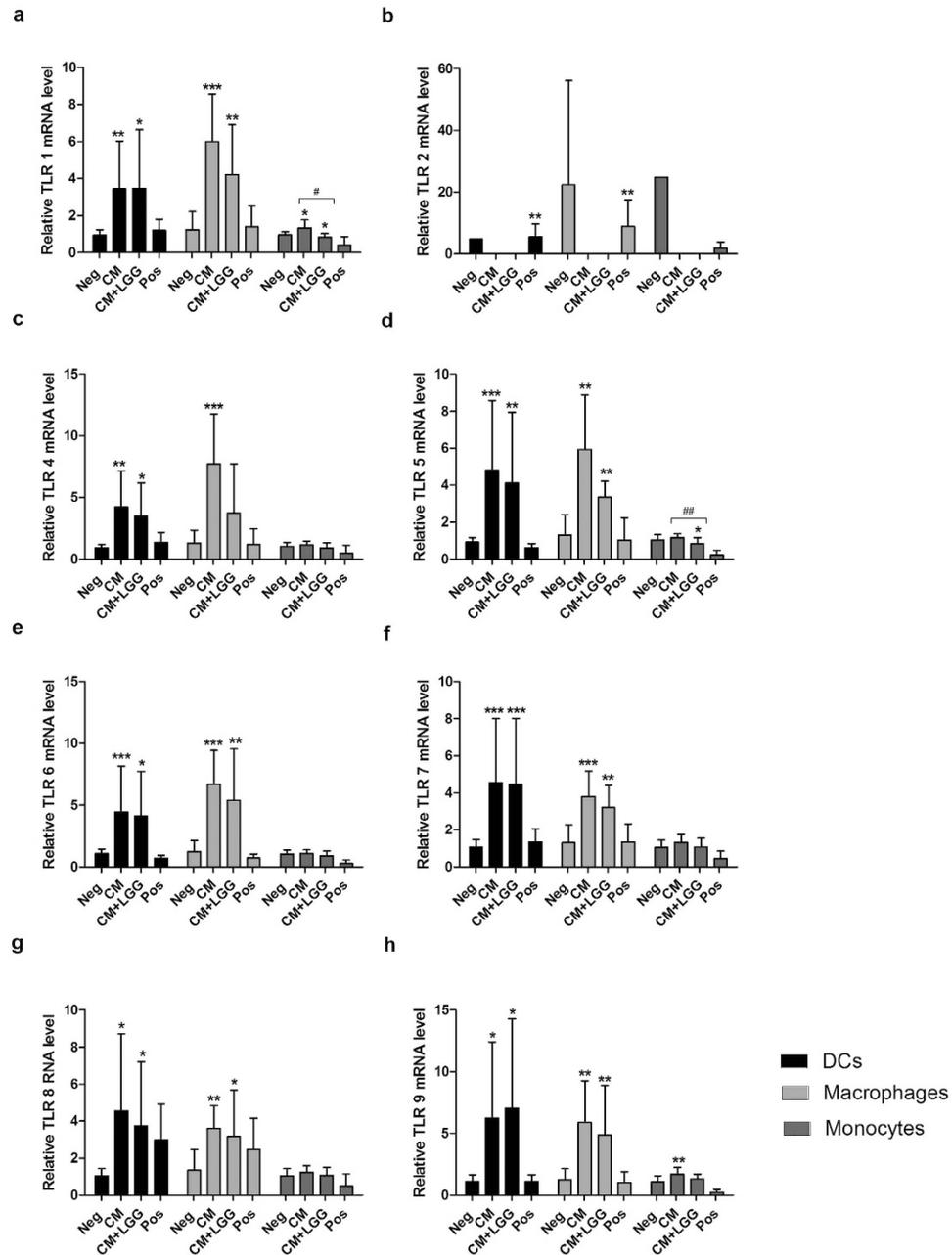


Figure 1. Toll-like Receptor (TLR) expression of dendritic cells (DCs), macrophages and monocytes. TLRs 1, 2, 4, 5, 6, 7, 8, and 9 expressions of monocytes, monocyte-derived DCs and macrophages of healthy blood donors ($n \geq 4$) treated with LGG conditioned medium (CM); or LGG conditioned medium with LGG cells (CM + LGG); or 1000 U/ml recombinant human (rh) IFN γ , 1000 ng/ml lipopolysaccharides (LPS) and 1 μ g/ml R848 (positive control; Pos); or alone (negative control; Neg) were analyzed by qPCR after 24-hour incubation. Results are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in comparisons between the treated cells and corresponding negative controls. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ in comparisons between CM-treated and CM + LGG-treated cells.

CM increased TLRs 1 and 9 mRNA levels of monocytes but CM + LGG decreased TLRs 1 and 5 mRNA levels of monocytes. Moreover, CM and CM + LGG decreased TLR 2 mRNA levels of DCs, macrophages and monocytes, which were below the detection limit. TLRs 1 and 5 mRNA levels of CM + LGG-treated monocytes were significantly lower than those of CM-treated monocytes.

Expression of activation markers and intracellular cytokines among monocytic subsets.

Mean Fluorescence Intensities (MFIs) of activation markers and intracellular cytokines of CM-treated or CM + LGG-treated “classical” CD14hiCD16-, “intermediate” CD14hiCD16lo and “non-classical” CD14loCD16lo monocytic subsets are shown in Fig. 2. Within “classical” CD14hiCD16- monocytic subset, both CM and CM + LGG decreased CD11b MFI but CM + LGG increased TNF α MFI. IL-12 MFI was significantly higher in

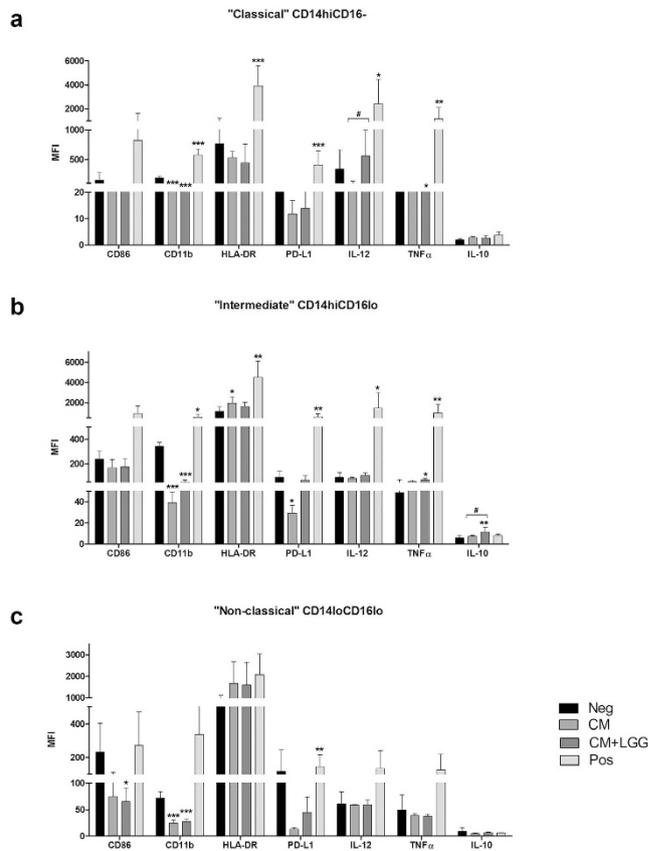


Figure 2. Expression of activation markers and intracellular cytokines among monocytic subsets. Mean fluorescence intensities (MFIs) of activation markers and intracellular cytokines among monocytic subsets of healthy blood donors ($n \geq 4$) treated with LGG conditioned medium (CM); or LGG conditioned medium with LGG cells (CM + LGG); or 1000 U/ml recombinant human (rh) IFN γ , 1000 ng/ml lipopolysaccharides (LPS) and 1 μ g/ml R848 (positive control; Pos); or alone (negative control; Neg) were determined by flow cytometer after 24-hour incubation. (a) “classical” CD14^{hi}CD16⁻, (b) “intermediate” CD14^{hi}CD16^{lo}, and (c) “non-classical” CD14^{lo}CD16^{lo} monocytic subsets were studied. Results are presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in comparisons between treated monocytes and negative control. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ in comparisons between CM-treated and CM + LGG-treated monocytes.

CM + LGG-treated than in CM-treated monocytes. Within “intermediate” CD14^{hi}CD16^{lo} monocytic subset, CM decreased CD11b and PD-L1 MFIs but increased HLA-DR MFI. CM + LGG decreased CD11b MFI but increased TNF α and IL-10 MFIs. IL-10 MFI of CM + LGG-treated monocytes was significantly higher than that of CM-treated monocytes. Within “non-classical” CD14^{lo}CD16^{lo} monocytic subset, CM + LGG significantly decreased CD86 MFI while both treatments reduced CD11b MFI.

Expression of activation markers and intracellular cytokines in macrophages. MFIs of activation markers and intracellular cytokines of CM-treated or CM + LGG-treated macrophages are shown in Fig. 3. Both treatments increased HLA-DR MFI but decreased MFIs of activation markers CD80, CD86, CD163, CD206, CD64 and CD209. They also reduced MFIs of M1-type cytokines IL-12, TNF α , Th1 polarization-associated transcription factor T-bet; and M2-type cytokine IL-10. CD80 MFI of CM + LGG-treated macrophages was significantly higher than that of CM-treated macrophages.

Cytokine secretion profiles of DC-PBMC co-cultures. Cytokine secretion profiles of co-cultures of treated DCs and mononuclear cells are shown in Fig. 4. Both treatments increased type-1 immune response-associated cytokines (IL-1 α , IFN γ and TNF α) and Th17-associated cytokines (GM-CSF, IL-17F and IL-6). They decreased Th2 signature cytokine (IL-4) but increased IL-25. A pronounced increase was seen in the production of regulatory cytokine IL-10 with CM + LGG treated DCs but not with CM-treated DCs. Levels of IL-1 α , IFN γ , TNF α , IL-12(p70), IL-23, GM-CSF, IL-17F and IL-10 were significantly higher in cultures with CM + LGG-treated DCs than in CM -treated DCs.

Discussion

To the best of our knowledge, this is the first study to demonstrate the immunomodulatory effects of LGG soluble factors on APCs of human blood donors. The results indicate that the soluble factors released during LGG growth

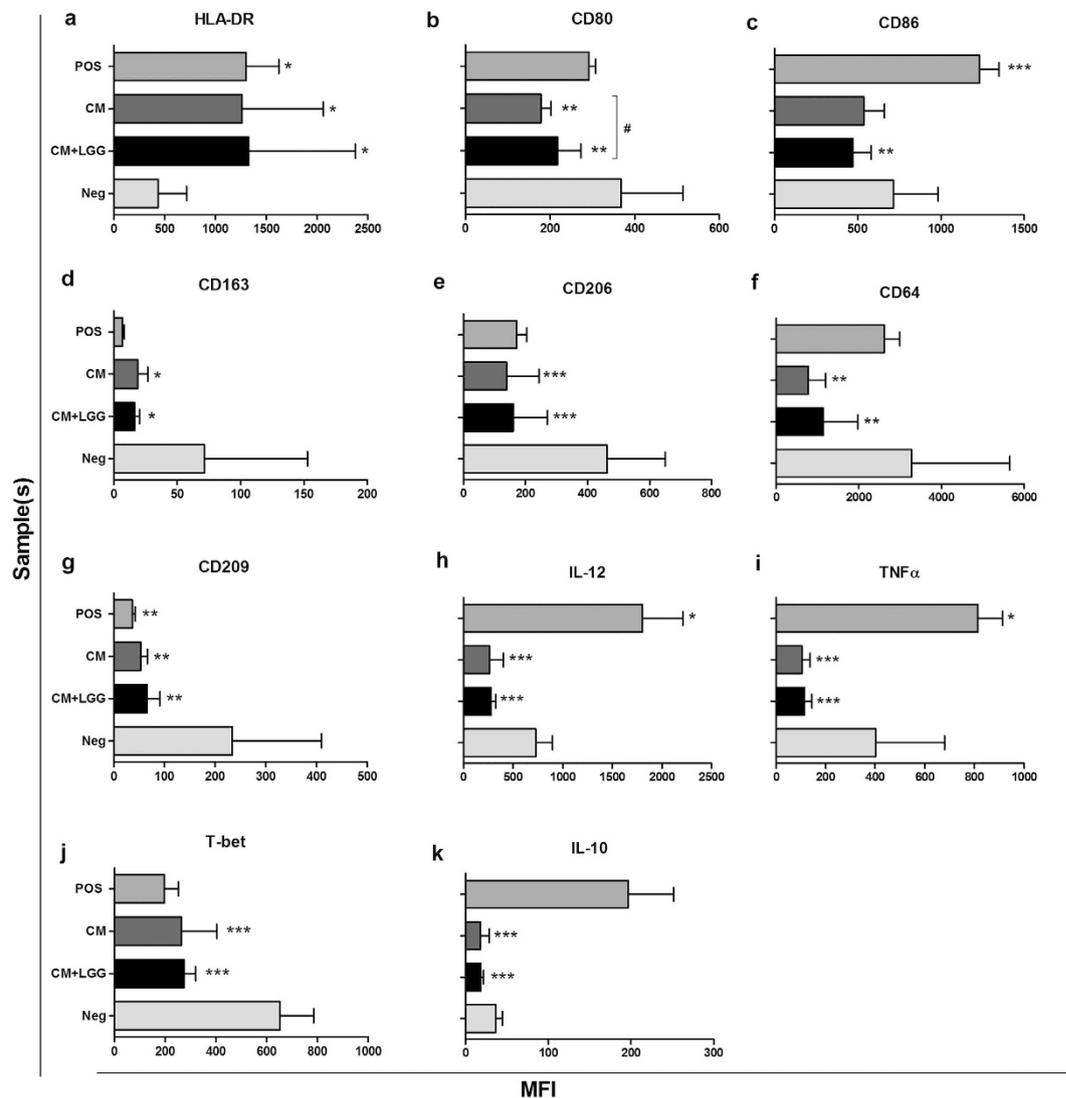


Figure 3. Expression of activation markers and intracellular cytokines in macrophages. MFIs of activation markers and intracellular cytokines in monocyte-derived macrophages ($n \geq 4$) stimulated with LGG conditioned medium (CM); or LGG conditioned medium with LGG cells (CM + LGG); or 1000 U/ml recombinant human (rh) IFN γ , 1000 ng/ml lipopolysaccharides (LPS) and 1 μ g/ml R848 (positive control; Pos); or alone (negative control; Neg) were analyzed by flow cytometer after 24-hour incubation. Results were presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in comparisons between treated macrophages and negative control. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ in comparisons between CM-treated and CM + LGG-treated macrophages.

have a similar pattern but a relatively modest influence on APC activation and immunoresponse polarization as the viable bacterial cells per se.

In our previous study, we showed that LGG cells reduced TLR 2 expression of DCs, macrophages and monocytes and TLR 8 expression of macrophages of healthy blood donors¹⁵. In the present study, LGG soluble factors seemed to exert an opposite effect that they significantly increased TLRs 1, 4, 5, 6, 7, 8 and 9 expressions of DCs and macrophages, and TLRs 1 and 9 expressions of monocytes. Down-regulation of TLR mRNA levels has been indicated to be related to the ligation of those particular receptors¹⁹. In this case, the up-regulation of certain TLR expressions could be a general response due to failure of ligation of several, but not all, of the corresponding TLRs on the cell surface. The alteration of TLR expressions on the key APCs could influence the subsequent innate responsiveness to pathogen-associated molecular patterns (PAMPs)^{20,21}. Intracellular antiviral pathways and production of antiviral effectors such as interferons (IFNs) and pro-inflammatory cytokines may be affected. Further investigations are, however, required.

One specifically interesting finding in this study is the significant reduction in the expression of several activation and inflammation markers on monocytes and macrophages, particularly the significant decrease in the expression of the adhesion molecule CD11b on all studied monocytic subsets. Increase in CD11b expression on circulating monocytes has been previously demonstrated to be associated with atherogenic inflammation and

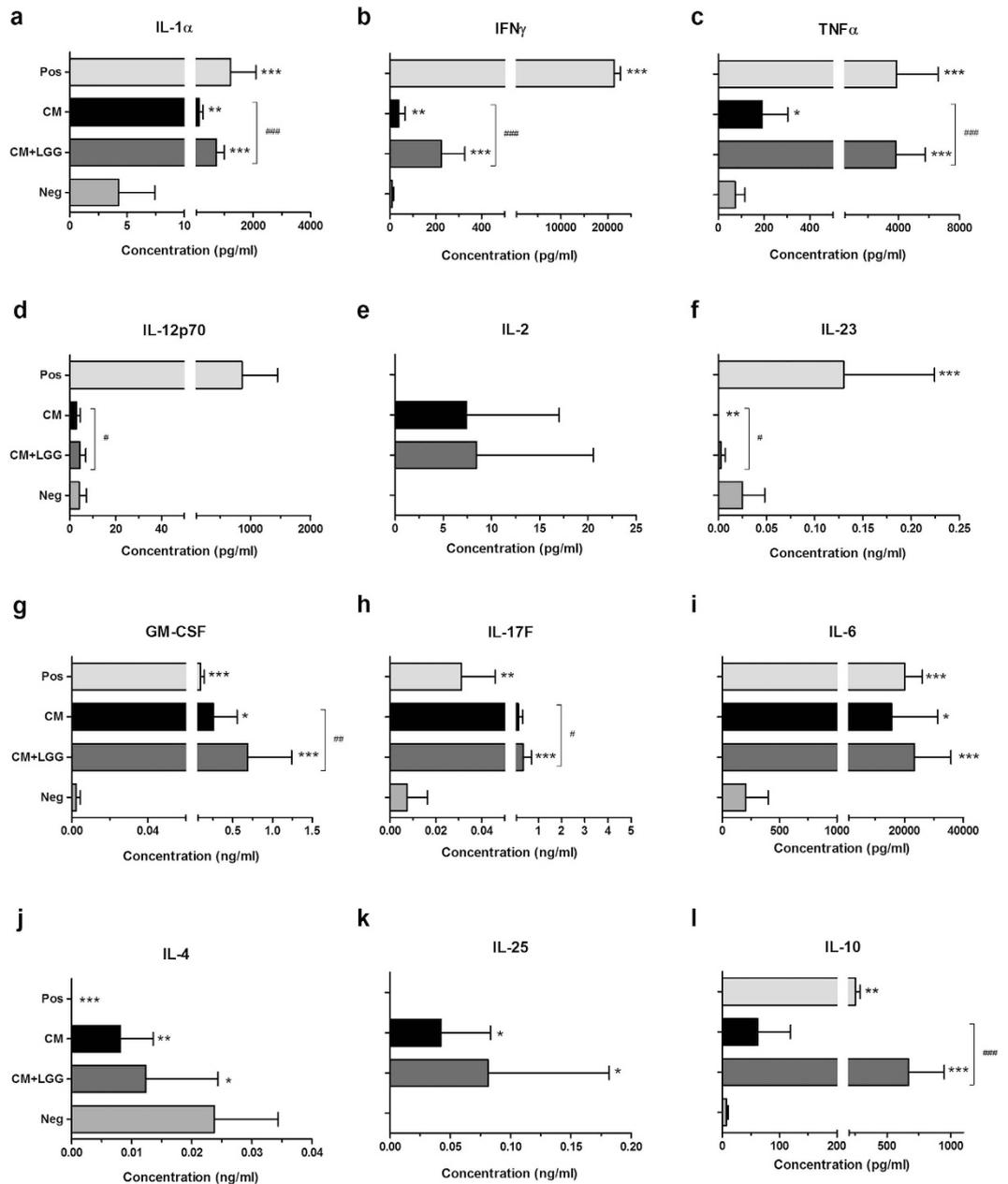


Figure 4. Cytokine secretion profiles of DC-peripheral blood mononuclear cell (PBMC) co-cultures. Cytokine production levels of co-cultures of autologous PBMCs and monocyte-derived DCs ($n \geq 4$) pre-stimulated 24 hours with LGG conditioned medium (CM); or LGG conditioned medium with LGG cells (CM + LGG); or 1000 U/ml recombinant human (rh) IFN γ , 1000 ng/ml lipopolysaccharides (LPS) and 1 μ g/ml R848 (positive control; Pos); or alone (negative control; Neg) were analyzed by multiplex assay. Results are shown as mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in comparisons between treated cells and negative control. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ in comparisons between CM-treated and CM + LGG-treated cells.

reflect the activity level of the disease^{22,23}. Our unpublished data show that viable LGG cells (1×10^8 CFU/day) significantly suppressed the atherosclerosis in ApoE $^{-/-}$ mice with Western-diet induced atherosclerosis (Chan *et al.*, unpublished). The treatments also consistently reduced other maturation and activation markers on the APCs but increased the expression of HLA-DR. Increase in HLA-DR expression indicates an increase in antigen presentation capacity. Antigen presentation by immature APCs theoretically promotes the formation of tolerogenic responses although no evidence has been found in the co-culture experiments^{24–26} with autologous mononuclear cells. Moreover, both treatments decreased the expression of Th1 polarization-associated transcription factor T-bet in macrophages, implying a decrease in the expression of another signature M1-type cytokine IFN γ ²⁷, which was not measured in the present study.

Instead, DCs pretreated with CM alone or CM with LGG cells significantly increased the secretions of pro-inflammatory type-1 cytokines (IL-1 α , IFN γ and TNF α) in the PBMC co-cultures. While the effect was

evident with both pretreatments it was significantly greater with DCs pretreated with the cocktail of CM and viable LGG cells. This implies the importance of the cellular structures of LGG over its secreted metabolic products for pro-inflammatory effects, which is supported by previous studies^{28,29}. These studies have suggested the importance of, for example, fimbriae or pilus adhesin on LGG for adhesion, close interaction between LGG and host cells, and modulation of inflammatory responsiveness and innate immune gene expression; lipoteichoic acid (LTA) for interaction with various pattern recognition receptors (PRRs); and exopolysaccharides (EPS) for modulation of adaptation of the bacterial cells.

In summary, the present study demonstrates in an *in vitro* model of APCs that the immunomodulation of LGG conditioned growth media shows a similar pattern as that of the viable probiotic strain per se. Both treatments decreased the constitutive expressions of several TLRs in all studied APCs, and activation markers and cytokine expressions of monocytes and macrophages. Based on the cytokine secretion profile of autologous co-cultures, the treated DCs appeared to promote type-1 pro-inflammatory responsiveness; and the influence was significantly higher with than without the viable cells. All these findings are of significant relevance to *in vivo* physiological responses, for example, manipulating intestinal microbial communities; suppressing adherence of pathogens to intestines; activating anti-apoptotic genes in intestinal epithelial cells; fortifying intestinal barrier; regulating intestinal homeostasis; and modulating systemic immunity^{30–33}.

Conclusion

In conclusion, the soluble factors released by LGG during its growth have similar immunomodulatory effects as the intact cells, but the latter seem to be required for the optimal type-1 immune responsiveness polarizing capacity of this probiotic strain. These results can provide an evidence-based insight for further clinical development of this strain, such as drug synthesis and vaccine development, for various diseases.

Methods

Derivation of dendritic cells and macrophages from monocytes. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors ($n \geq 4$) (Hong Kong Red Cross Blood Transfusion Service, Hong Kong, China) by density gradient centrifugation over Ficoll-Plaque™ Plus (GE Healthcare Life Sciences, Piscataway, NJ, USA) and seeded in complete RPMI-1640 medium (LONZA, Basel, Switzerland) for 2 hours at 37 °C to isolate monocytes. Extra PBMCs were frozen for later experiments. Isolated monocytes were derived to immature DCs in the presence of 40 ng/ml recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) and 40 ng/ml rh interleukin (IL)-4 (both from PeproTech EC, Ltd, London, UK); or to immature macrophages in the presence of 40 ng/ml rh GM-CSF. Cells were incubated for 7 days with fresh supplemented medium added every 2 days. Protocol was modified from the studies of Lacey (2012) and Mohamadzadeh (2005)^{16,17}, and approved by the University of Hong Kong. All methods were carried out in accordance with the approved guidelines.

Bacterial strain and stimulation of antigen-presenting cells (APCs). LGG (ATCC 53103) was cultivated in De Man, Rogosa, Sharpe (MRS) broth (LAB M Limited, Lancashire, UK) anaerobically at 37 °C to logarithm-phase. Cell-free conditioned medium (CM) was prepared by centrifugation at 3000 rpm for 15 minutes at 4 °C and dual filtration through 0.2 µm Acrodisc Syringe Filters (Life Sciences, MI, USA). Immature DCs, macrophages and monocytes were stimulated with CM or CM in the presence of LGG (CM + LGG) for 24 hours.

Detection of toll-like receptor (TLR) expression by quantitative real-time PCR (qPCR). TLRs 1, 2, 4, 5, 6, 7, 8 and 9 mRNA levels of DCs, macrophages and monocytes were determined by qPCR with modified protocol and sequences of primers and probes from the study of Flacher *et al.*¹⁸. Data were acquired on ABI StepOne Plus Real-Time PCR System (Life Technologies, NY, USA).

Detection of expressions of activation markers and intracellular cytokines of monocytes and macrophages by flow cytometry. Brefeldin A solution (eBioscience, CA, USA) was added to monocytes and macrophages cultures (10 µg/ml) 15 hours before the cells were harvested. Cells were stained with appropriate anti-human monoclonal antibodies such as CD14-FITC (eBioscience), CD16-BD Horizon PE-CF594 (BD Biosciences, New Jersey, USA), CD86-PE (eBioscience), CD11b-PE/Cy7 (eBioscience), HLA-DR-APC/Cy7 (BioLegend, CA, USA), PD-L1-APC (eBioscience), CD80-PerCP3Flour[®]710 (eBioscience), CD163-APC (eBioscience), CD206-APC/Cy7 (Biolegend), CD64-PE (Biolegend), CD209-PE/Cy7 (eBioscience), T-bet-PerCP/Cy5.5 (eBioscience), IL-12(p40)-PE (eBioscience), IL-10-Alexa Fluor 647 (eBioscience) and TNFα-PerCP-Cy5.5 (BD Pharmingen™) following manufacturer's protocol. Data were acquired on FACSAria III flow cytometer (BD Biosciences) and analyzed with FlowJo Version 7.6 (Ashland, OR, USA) based on 20,000 cells.

Detection of cytokine levels by multiplex assay. Treated DCs were washed with PBS and incubated with autologous PBMCs in a 1:20 ratio for 3 days. Supernatant was collected and stored at –20 °C for cytokine detection. Levels of IL-1α, IFNγ, TNFα, IL-12(p70), IL-2, IL-23, GM-CSF, IL-17F, IL-6, IL-4, IL-25 and IL-10 were determined by Luminex[®] 200™ with xPONENT 3.1 software (Luminex Corporation, Texas, USA) following manufacturer's protocol and analyzed by MILLIPLIX Analyst Version 3.5.5.0 (Vigene Tech Inc., Carlisle, MA, USA).

Statistical Analysis. Results were shown as mean ± standard deviation (SD) and analyzed with Kruskal-Wallis test, Mann-Whitney test, analysis of variance and two-tailed Student's t test. CM-treated and CM + LGG-treated cells were compared with the negative control and with each other. P-values below 0.05 were considered as significant. Statistical calculations were performed using GraphPad Software Prism Version 6.04 (San Diego, CA, USA) and SPSS Version 19 for Windows (Chicago, IL, USA).

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Author Contributions

Contributions of F.L.Y.F. were on experimental design, performing experiment, data analysis and writing manuscript. Contributions of P.K. were mainly on commenting and proof-reading the manuscript. H.E. was the corresponding author of this manuscript. Contributions of H.E. were mainly on providing chemicals and reagents and commenting the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

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