International Journal of *Molecular Sciences*

MDPI

*Article*

# The phagocytosis of *Lacticaseibacillus casei* and its immunomodulatory properties on human monocyte-derived dendritic cells depend on the expression of Lc-p75, a bacterial peptidoglycan-hydrolase

**Márta Tóth[1,2,3], Szabolcs Muzsai[1,4], Krzysztof Regulski[5], Tímea Szendi-Szatmári[6], Zsolt Czimmerer[7], Éva Rajnavölgyi[1], Marie-Pierre Chapot-Chartier[5#] and Attila Bácsi[1,3*#]**

[1] Department of Immunology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, toth.marta@med.unideb.hu, muzsai.szabolcs@med.unideb.hu
[2] Doctoral School of Molecular Cell and Immune Biology, University of Debrecen, Debrecen, Hungary
[3] ELKH-DE Allergology Research Group, Debrecen, Hungary
[4] Gyula Petrányi Doctoral School of Clinical Immunology and Allergology, University of Debrecen, Debrecen, Hungary
[5] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France marie-pierre.chapot-chartier@inrae.fr
[6] Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, szatmari.timea@med.unideb.hu
[7] Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, czimmerer.zsolt@med.unideb.hu
* Correspondence: etele@med.unideb.hu
[#] Contributed equally to this work

**Citation:** Tóth, M.; Muzsai, Sz.; Regulski, K.; Szendi-Szatmári, T.; Czimmerer, Zs.; Rajnavölgyi, É.; Chapot-Chartier, M-P.; Bácsi, A. The phagocytosis of *Lacticaseibacillus casei* and its immunomodulatory properties on human monocyte-derived dendritic cells depend on the expression of Lc-p75, a bacterial peptidoglycan-hydrolase. *Int. J. Mol. Sci.* **2022**, *23*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date



**Abstract:** The human gut symbiont *Lacticaseibacillus (L.) casei* (previously *Lactobacillus casei*) is under intense research because of its wide range of immunomodulatory effects on the human host. Dendritic cells (DCs) are crucial players in the direct and indirect communication with lactobacilli in the gastrointestinal tract. Here, we demonstrate that human monocyte-derived DCs (moDCs) are able to engulf *L. casei* BL23, in which the intact bacterial cell wall and morphology have a key role. The absence of bacterial cell wall-degrading enzyme, Lc-p75, in *L. casei* cells causes remarkable morphological changes, which have important consequences in the phagocytosis of *L. casei* by moDCs. Our results show that the Lc-p75 mutation induced defective internalization and impaired pro-inflammatory and T cell polarizing cytokine secretion by bacteria-exposed moDCs. The T helper (Th) 1 and Th17 cell activating capacity of moDCs induced by the mutant *L. casei* was consequently reduced. Moreover, inhibition of the phagocytosis of wild-type bacteria showed similar results. Taken together, these data suggest that formation of short bacterial chains helps to exert the potent immunomodulatory properties of *L. casei* BL23.

**Keywords:** lactobacillus, dendritic cells, phagocytosis, immunomodulation

## 1. Introduction

Lactobacilli are Gram-positive lactic acid bacteria (LAB) that are natural inhabitants of the human gastrointestinal tract. They are also widely used in fermented food production, and certain strains are commercialized as probiotics with health-promoting properties. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[1]. A wide range of beneficial effects is attributed to probiotics, including maintaining the gut epithelial barrier integrity [2], preventing the adhesion of pathogenic bacteria [3,4], and secreting vitamins [5] and metabolites [6]. Furthermore, probiotics contribute to the development of the immune system and modulate immune responses [7–9].

Bacteria must directly or indirectly interact with both non-immune and immune cells of the host to exert their beneficial effects. Considering the unique and complex functional activities of dendritic cells (DCs) in modulating both innate and adaptive immunity, they became first-line candidates for characterizing the strain-specific actions of various probiotic bacteria, such as lactobacilli. DCs express unique sets of surface-bound and intracellular pattern recognition receptors (PRRs), which sense various microbe-associated molecular patterns (MAMPs) [10]. As professional phagocytes, DCs can internalize microbes upon recognition of MAMPs. This results in the activation of signaling cascades, after which DCs produce soluble factors that shape the innate and adaptive immune responses; further, as professional antigen-presenting cells, DCs present peptide antigens to naïve T cells mediating their activation and polarization [11].

Bacterial peptidoglycan (PG) molecules play an essential role in bacterial life and host-microbe interactions. The synthesis and degradation of PG are tightly regulated by bacterial enzymes, including peptidoglycan-hydrolases (PGHs), which can cleave the PG macromolecule at well-defined sites [12]. It has previously been reported that p40 and p75 enzymes belong to PGHs with pleiotropic effects on the bacterial cells. The two proteins are localized in the bacterial cell wall or released to the environment freely [13] or bound to extracellular vesicles [14]. It has been demonstrated that these enzymes play a role in *L. casei* growth and cell division [13,15]. Interestingly, *L. casei* p40 (Lc-p40) and p75 (Lc-p75) mutant strains remarkably differ in their morphology. Lacking Lc-p40 causes only minor changes; bacteria take a curved shape, while the absence of Lc-p75 leads to the formation of long-chain, auto-aggregation phenotype [13,15] . Moreover, Lc-p75 is endowed with γ-D-glutamyl-L-lysyl-endopeptidase activity and its inactivation causes altered composition of PG fragments [15] serving as MAMPs and modulating the host's immune responses. Bacterial PG, a major structural cell wall component, originating from pathogens [16] or probiotic [17] bacteria induce DC activation. However, the impacts of bacterial PG alterations on DC activation and functions are less studied.

In this study, we aimed to explore the DC-modulatory properties of *L. casei* BL23 and its ΔLc-p75 mutant derivative. With the help of the chain-forming ΔLc-p75 mutant, we showed that phagocytosis is a critical element in the modulation of DC functions by the probiotic bacteria. We observed that impaired phagocytosis of the mutant bacteria by human monocyte-derived DCs (moDCs) alters their T cell polarizing ability. These observations may contribute to a deeper understanding of the modulatory mechanisms exerted by probiotic bacteria. Furthermore, they might help generate probiotic bacterial strains, which can have remarkable therapeutic potential in different diseases via the modulation of immune responses.

## 2. Results

### *2.1. Lc-p75 deficiency does not influence the moDC-activating potential of* L. casei *but affects the inflammatory cytokine secretion by bacteria-exposed moDCs*

First, we investigated the effects of wild-type (WT) *L. casei* and its mutant counterpart, defective in Lc-p75 cell wall-degrading enzyme, on moDC activation. ΔLc-p75 mutant was obtained by deletion of the gene encoding the major PGH in *L. casei* BL23 [15]. As expected, the ΔLc-p75 mutant strain has modified cell wall structure and morphology compared to WT *L. casei* strain as showed by confocal microscopy (Figure 1A).

To assess the impact of Lc-p75 inactivation on the inflammatory-provoking capacity of moDCs, we studied the CD83 expression and inflammatory cytokine and chemokine secretion by moDCs treated with WT and ΔLc-p75 *L. casei* strains for 24 hours at a ratio of 1:4. Membrane-bound CD83 is strongly expressed by mature, gut-bacteria activated moDCs [18,19]. In our experimental system, *L. casei* strains also increased the expression level of CD83; however, no statistically significant difference was observed between WT and mutant strains with respect to the degree of CD83 elevation (Figure 1B). In contrast, WT bacteria induced a significantly higher amount of TNF-α, IL-1β, and IL-6 cytokines than their defective counterpart (Figure 1C). Interestingly, we did not detect any significant differences in IL-8 (Figure 1D) chemokine secretion by moDCs activated with the WT or PGH mutant bacteria. To exclude that the differences in moDC activating abilities of WT and ΔLc-p75 *L. casei* strains are the consequence of the different levels of treatment-induced cell death, we measured the frequency of dead cells in bacteria-exposed moDC cultures by flow cytometry. Remarkably, we could detect only an insignificant proportion of dead cells in moDC cultures incubated with either WT or mutant bacterial strains, similar to that observed in unstimulated moDC cultures (Figure S1).

These results collectively indicate that *L. casei* mutant with defective cell wall structure and altered morphology promotes an attenuated inflammatory response by moDCs. By contrast, the appearance of the activation marker CD83 on bacteria-exposed moDCs is not sensitive to the lack of bacterial Lc-p75 PGH.

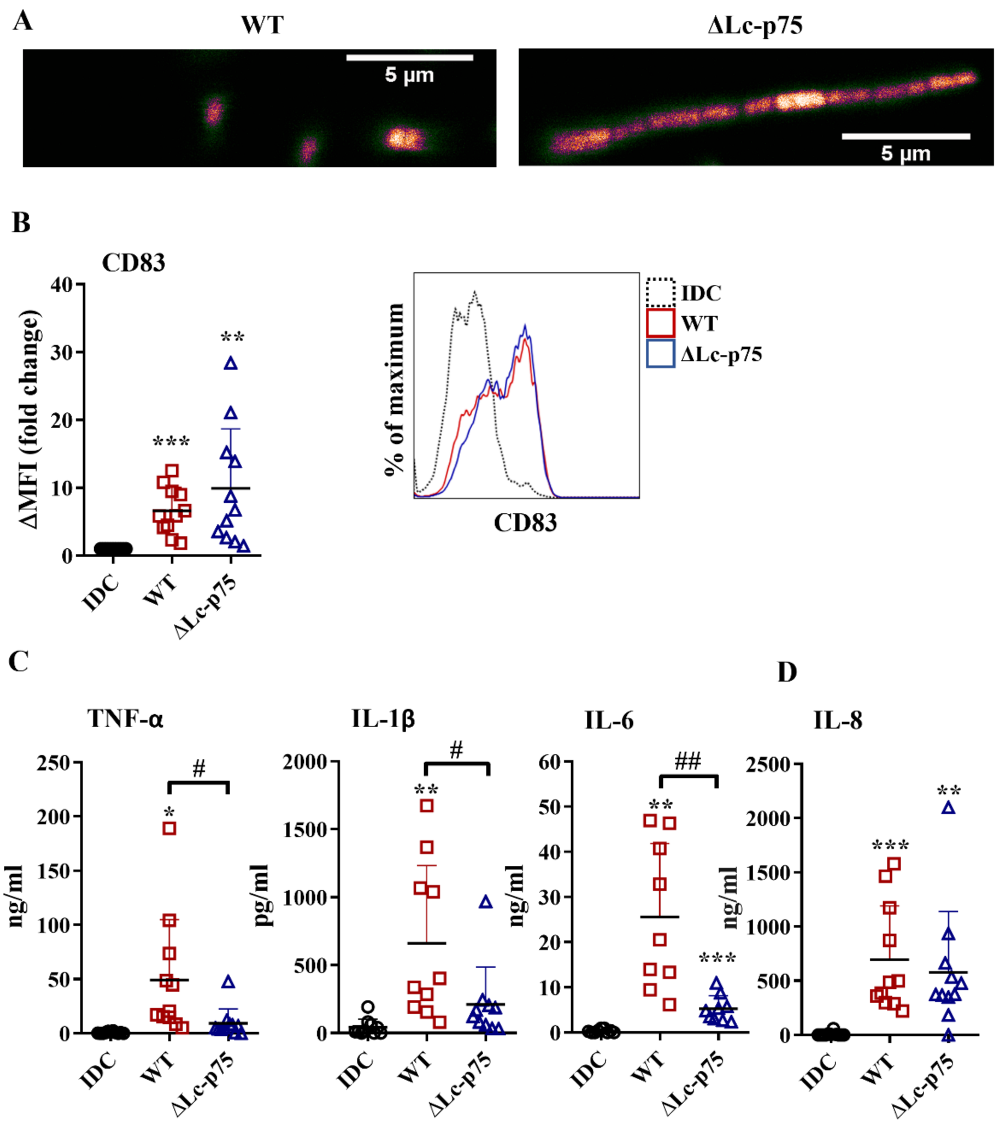

**Figure 1**. The absence of Lc-p75 PGH affects bacterial morphology, and the inflammatory mediators' production but not the CD83 expression by bacteria-activated moDCs. Figure A represents the confocal microscopic pictures of mCherry-expressing wild-type (WT) and ΔLc-p75 *L. casei* strains. Five-day moDCs were co-incubated with WT and PGH mutant bacteria at a ratio of 1:4 for 24 h. The expression of CD83 (B) was measured by flow cytometry. Fold change of median fluorescent intensities (ΔMFI) to the control, non-treated cells (immature DC /IDC/) was calculated from 6-9 independent experiments ±SD. Histogram overlays show one representative experiment. Each dot is representative of one donor. The concentrations of pro-inflammatory TNF-α, IL-1β, IL-6 cytokines (C), and IL-8 chemokine (D) were measured by ELISA from the supernatant of activated moDCs and control, non-activated cells (IDC). Figures show the mean of 9-11 independent experiments ±SD. Statistical analysis was performed by the Student's paired two-tailed t-test with significance defined as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared to IDC. The difference between WT and ΔLc-p75 was statistically significant defined as # $P < 0.05$ and ## $P < 0.01$.

### *2.2. Lc-p75-deficiency in L. casei does not influence the antigen presentation and co-stimulation but induces less T cell polarizing cytokine secretion by bacteria-exposed moDCs*

After recognizing pathogens, the highly phagocytic, immature DCs transform into a cell type that has the characteristics required for the activation and polarization of Th cells in different directions. One of the unique features of dendritic cells is the migration to the secondary lymphoid organs to activate naïve Th cells and provide co-stimulation simultaneously with the antigen presentation via MHCII molecules [20]. Therefore, the increased level of HLA-DQ and CD80 and CD86 co-stimulatory molecules is considered a good marker of stimulated, capable-of-T-cell-activation status of the DCs. After a 24-hour incubation the WT *L. casei* and ΔLc-p75 mutant derivative induced the up-regulation of HLA-DQ, CD80 and CD86 expression of moDCs, regardless of the mutation (Figure 2A).

Besides inflammatory cytokine and chemokine secretion, DCs also secrete soluble mediators to shape the differentiation of various T cell subsets. Therefore, we measured the T cell polarizing IL-10, IL-12, and IL-23 production by moDCs after a 24-hour activation with *L. casei* strains. Importantly, WT *L. casei* induced more robust IL-10, IL-12, and IL-23 secretion by moDCs than their PGH defective counterpart (Figure 2B).

These results indicate that the ΔLc-p75 mutant *L. casei* strain causes dampened T cell polarizing cytokine secretion by moDCs and consequently may influence their T cell activating capacity. In contrast, expression of antigen-presenting and co-stimulatory molecules on bacteria-exposed moDCs does not depend on the presence of bacterial Lc-p75 enzyme.

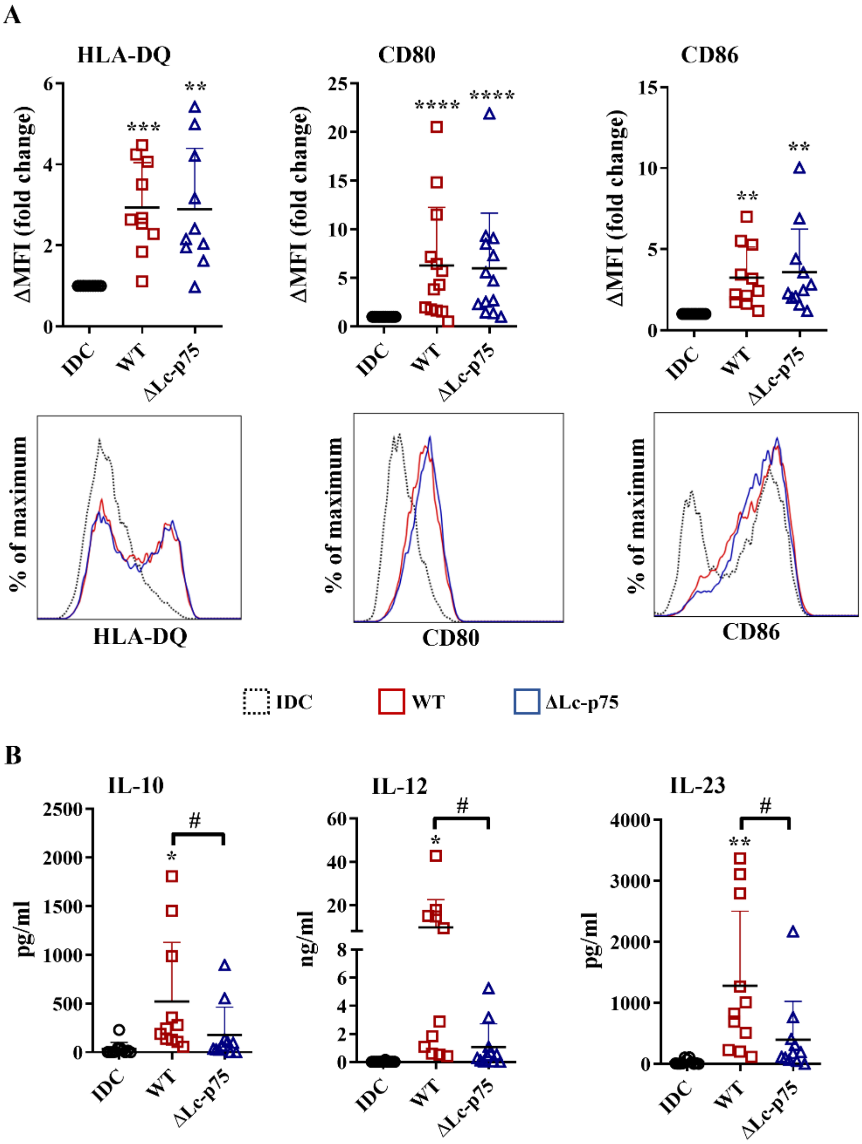


**Figure 2**. Deletion of Lc-p75 PGH in *L. casei* BL23 does not affect the antigen-presenting and co-stimulatory capacity of bacteria-exposed moDCs but causes decreased T cell polarizing cytokine secretion by these cells. Five-day moDCs were stimulated with live wild-type (WT) *L. casei* BL23 and ΔLc-p75 mutant strain at a ratio of 1:4 for 24 h. The expression of HLA-DQ as well as co-stimulatory CD80 and CD86 molecules (A) was measured by flow cytometry. The fold change of the median fluorescent intensities (ΔMFI) compared to the control cells (IDC) was calculated from 10-13 independent experiments ±SD. Histogram overlays show one representative experiment. The concentrations of T cell polarizing IL-10, IL-12, and IL-23 cytokines (B) were measured by ELISA from the supernatant of activated moDCs and control, non-treated cells (IDC). Figures show the mean of 10-11 independent experiments ±SD. Each dot represents one donor. Student's paired two-tailed t-test was used in the statistical analysis with significance defined as * P <0.05, ** P <0.01 and *** P <0.001 compared to IDC and # P <0.05 between WT and ΔLc-p75.

### *2.3. Activation and polarization of autologous T lymphocytes by bacteria-exposed moDCs depend on the presence of Lc-p75 in L. casei BL23*

To study whether the decreased T cell polarizing cytokine secretion could influence the outcome of T lymphocyte responses mediated by the moDCs, we co-cultured bacteria-activated moDCs with autologous peripheral blood lymphocytes (PBL) for 3 days. T cell responses were monitored using IFNγ-, IL-17- and IL-4-specific ELISPOT assays. Unstimulated moDCs and PBL were used as negative controls. Higher numbers and a larger area of the spots were observed in cases of IFN-γ- and IL-17-producing T cells when the moDCs were pre-stimulated with *L. casei* strains compared to unstimulated moDC or PBL controls. Pre-activation of moDCs with the *L. casei* ΔLc-p75 mutant resulted in significantly lower numbers and a smaller area of spots in case of IFN-γ-secreting T cells compared to pre-treatment with WT bacteria (Figure 3A). Similarly, significantly lower number of IL-17A secreting T cells and less intense IL-17A production was induced by moDCs pre-activated with ΔLc-p75 mutant bacteria compared to pre-treatment with WT bacteria (Figure 3B). In contrast, pre-stimulation of moDCs with *L. casei* strains resulted in a significant reduction in the spot numbers and the area coverage representing IL-4 production compared to control, untreated moDCs, independently of the PG mutation (Figure S2).

These results indicate that the Lc-p75 defective *L. casei* induces reduced Th1 and Th17 responses compared to WT *L. casei* BL23.

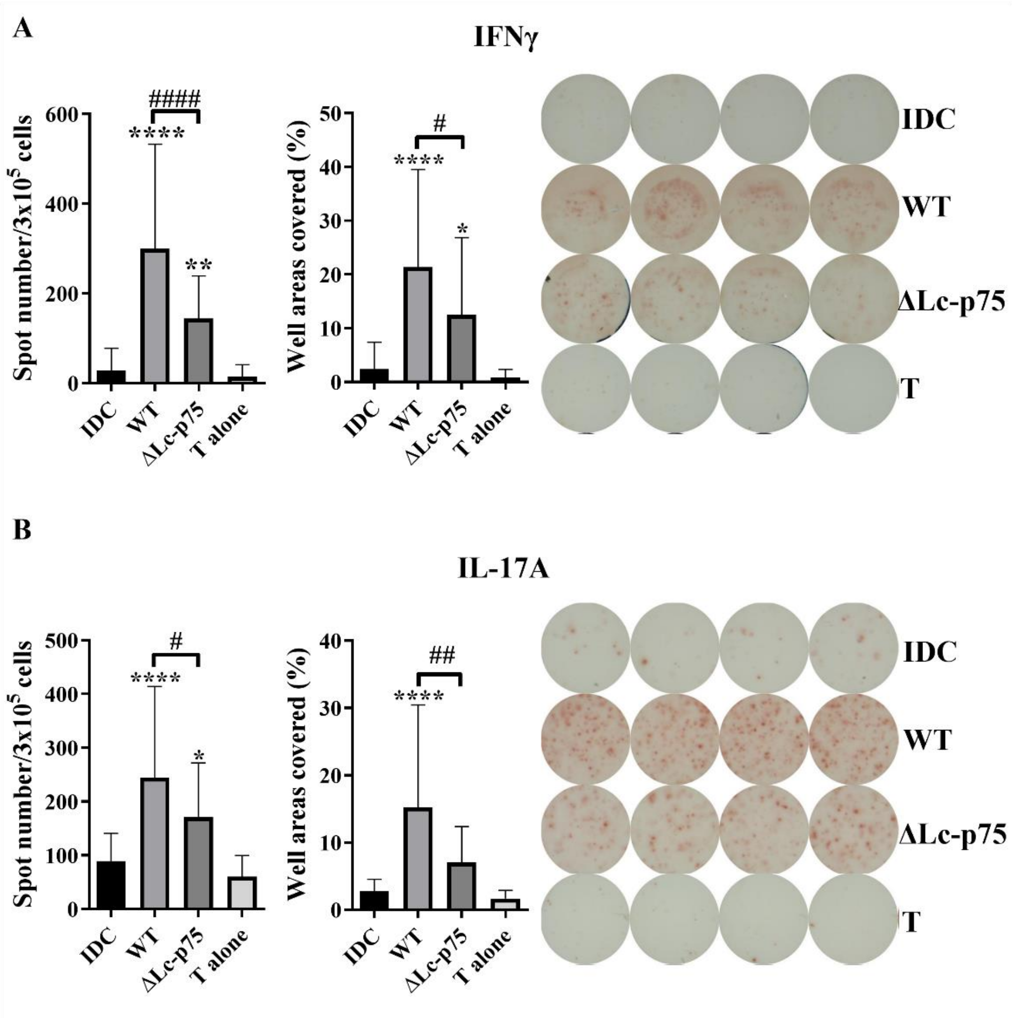


**Figure 3.** The T cell polarizing potential of moDCs is modulated by Lc-p75 inactivation in *L. casei* BL23. Five-day moDCs activated with wild-type (WT) and ΔLc-p75 mutant *L. casei* BL23 were washed and then co-cultured with autologous T cells from freshly isolated PBL at a ratio of 1:20 for 3 days. The frequency of cytokine-producing T lymphocytes was measured by IFN-γ (A) or IL-17A (B) ELISPOT assays. The spot number was counted, or the area covered by the spots was calculated by a computer-assisted ELISPOT image-analyzer. The mean value of spot numbers and well areas covered were calculated from 5 independent experiments with 4-6 parallel wells ±SD. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. Significance

defined as * P <0.05, ** P <0.01 and **** P <0.0001 compared to control, non-treated cells (IDC). The differences between WT and ΔLc-p75 were statistically significant defined as # P<0.05 and ## P <0.01 and #### P<0.0001.

### *2.4. Peptidoglycan derived from ΔLc-p75 mutant or WT L. casei BL23 exhibits similar moDC-activating potential*

Based on the results above, we raised the question whether the differences between WT and mutant *L. casei* strains in moDC activation are the direct consequences of the modified bacterial cell wall structure or linked indirectly to the altered bacterial morphology. Therefore, we purified PGs derived from the WT and ΔLc-p75 mutant strains and tested their moDC-stimulating activity. PG preparations induced elevated pro-inflammatory (Figure 4A) and T cell polarizing (Figure 4B) cytokine secretion by moDCs independently of the targeted PGH mutation. Furthermore, PGs from both *L. casei* strains caused significant elevation in CD83, HLA-DQ, CD80 and CD86 expression (Figure S3A) as well as in IL-8 production (Figure S3B). Importantly, these changes were not dependent on the targeted PGH mutation, similarly to the results obtained with the live bacteria

Taken together, these results demonstrate that the purified PGs from WT and mutant *L. casei* BL23 strains does not show any significant differences in their DC activating potentials.

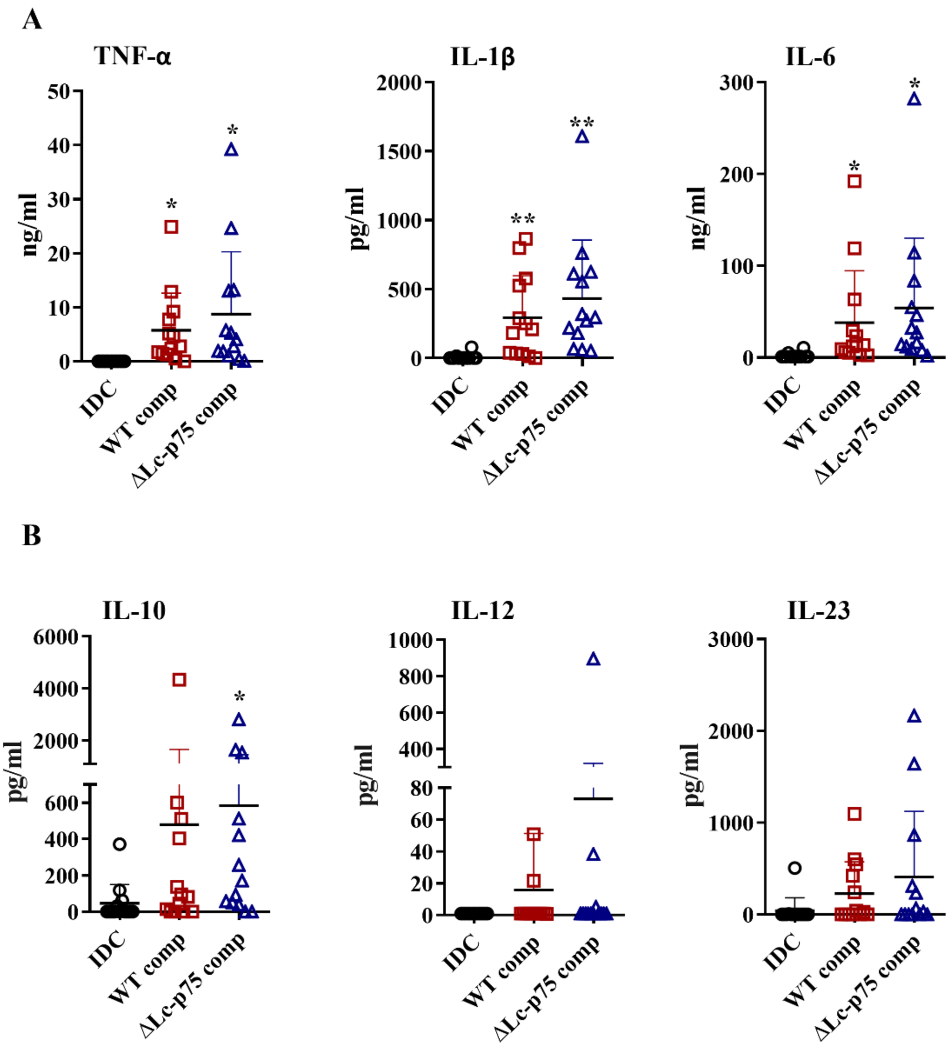

**Figure 4.** PG derived from wild-type (WT) *L. casei* BL23 and ΔLc-p75 mutant strains induces enhanced cytokine secretion by moDCs independently of the PGH mutation. Five-day moDCs were treated with 10 μg/ml PG components derived from WT and Lc-p75 mutant strains for 24 h. The concentrations of TNF-α, IL-1β, IL-6 pro-inflammatory cytokines (A) and of IL-10, IL-12 and IL-23 T cell polarizing cytokines (B) were determined by ELISA. The mean of the concentrations was calculated from 11-13 independent experiments. Each dot represents one donor. Student's paired two-tailed t-test was used in the statistical analysis with significance defined as * P <0.05 and ** P <0.01 compared to the control, non-activated cells (IDC).

### *2.5. Altered bacterial morphology leads to impaired phagocytosis by moDCs*

Since we could not detect any differences in moDC activation between WT and ΔLc-p75 mutant *L. casei*-derived purified PGs, we hypothesized that the attenuated DC stimulation induced by the ΔLc-p75 mutant *L. casei* BL23 is the consequence of their impaired phagocytic uptake by moDCs.

For the phagocytosis assay, *in-vitro* differentiated 5-day moDCs were co-incubated with bacteria of each strain expressing mCherry fluorescent protein, for 3 or 24 h. Within both incubation periods at 37°C, moDCs were shown to internalize fluorescent bacteria, consequently becoming mCherry positive. The percentage of these mCherry positive phagocytic cells could be quantified by flow cytometry and was compared to control samples incubated on ice or in the absence of bacteria.

We found that WT bacteria were internalized with higher frequency by moDCs than mutant bacteria after 3 (Figure 5A, 5B) and 24 (Figure 5C, 5D) hours. The control samples incubated on ice exhibited a low percentage of mCherry positive cells. The significantly higher frequency of mCherry positive moDCs co-incubated with WT *L. casei* BL23 at 37°C indicates active, temperature-dependent internalization of bacteria within a 3-hour incubation period (Figure 5A, 5B) and after 24 hours of coincubation (Figure 5C, 5D). We confirmed the moDC-mediated uptake of WT and ΔLc-p75 mutant (Figure 5E) bacteria by confocal microscopy displayed with Z-stack image's orthogonal views.

It has previously been reported that the $CD1a^+$ and $CD1a^-$ subsets of *in vitro* generated moDCs differ in their *Escherichia coli*-internalizing capacity [21]. However, in another study, Seshadri et al. demonstrated that the absence of CD1a protein on the surface of moDCs does not cause any defect in the engulfment of *Escherichia coli (E. coli)* cells [22]. Similarly, we did not observe any significant differences between the internalizing capacity of $CD1a^-$ and $CD1a^+$ moDCs activated with WT or ΔLc-p75 mutant *L. casei* BL23 (Figure 6A, 6B).

These observations indicate that the *L. casei* PGH's targeted mutation modifies the fate of bacteria through the inhibition of their efficient uptake by phagocytic moDCs. This phenomenon may be the consequence of the chain-forming property of the mutant bacterial strain. The absence or presence of the cell surface CD1a molecule does not influence the phagocytosis of *L. casei* strains by moDCs.

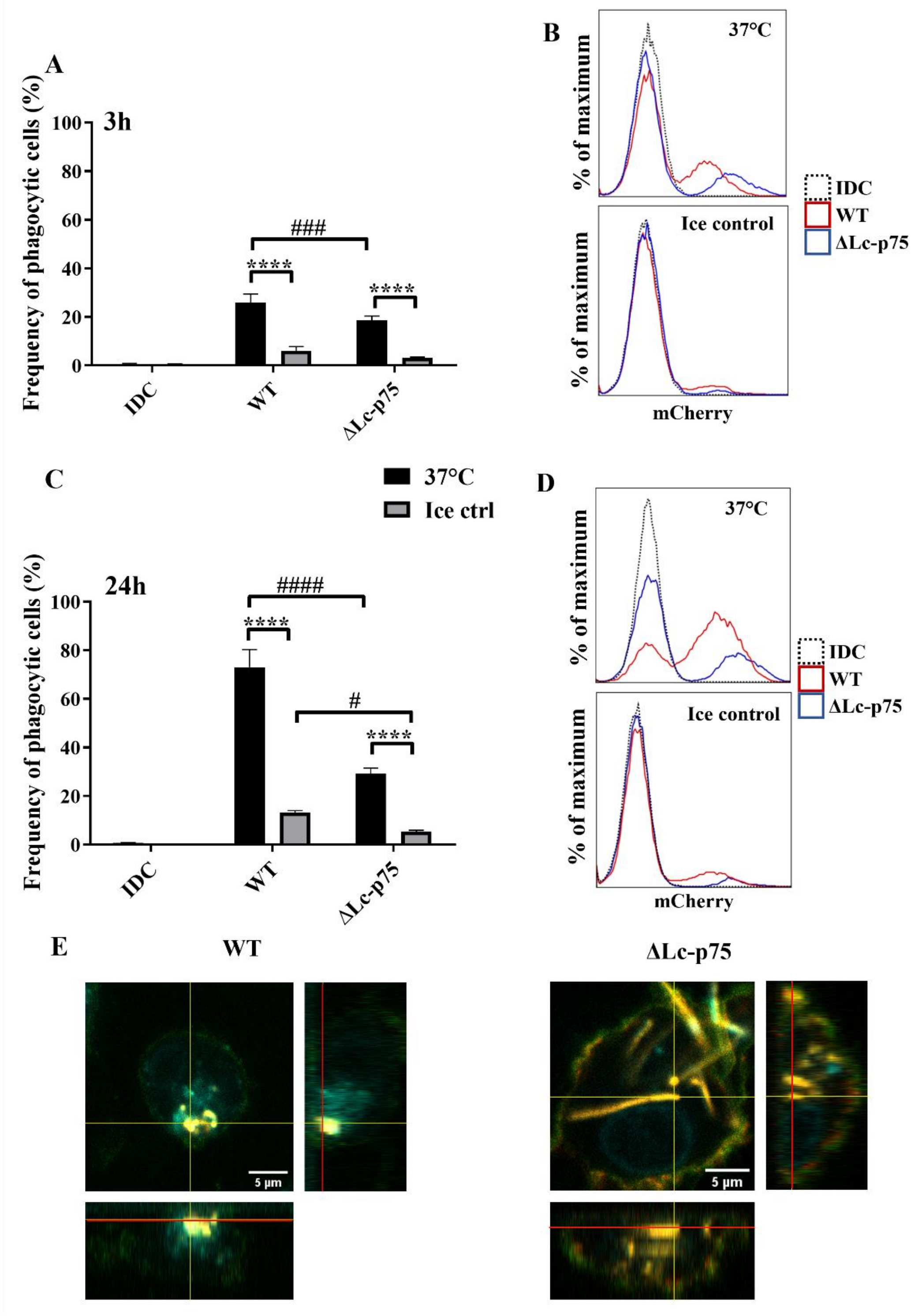


**Figure 5.** Inactivation of Lc-p75 in *L. casei* BL23 alters the phagocytic efficiency of moDCs. *In vitro* differentiated five-day moDCs were co-incubated with mCherry-labeled WT *L. casei* BL23 and ΔLc-p75 mutant for 3 and 24 h at a ratio of 1:4 at 37°C and on ice as a control. Phagocytic activity of moDCs was measured by detecting mCherry fluorescent protein-positive-moDCs with flow cytometry after 3 hours (A, B) and after 24 hours (C, D). IDC means non-activated moDCs. Histogram overlays show the results of one representative experiment. On the flow cytometric figures, the mean values of 3 independent experiments are presented ±SD. For confocal microscopic pictures (E) moDCs were labeled with HLA-DQ (green), CD1a (red) and DAPI (cyan); mCherry was indicated

with yellow. Orthogonal views are demonstrated of one Z-stack image. On the bottom is Z-projection in the X-Z direction, on the right is the Z-projection in the Y-Z direction. The red lines indicate the Z-depth of the optical slice and the orthogonal planes of the X-Z and Y-Z projections, respectively. Two-way ANOVA followed by Tukey's multiple comparison tests was used in the statistical analysis. Significance defined as **** P <0.0001 showing the differences between the samples incubated at 37ºC and on ice. The difference between WT and ΔLc-p75 was statistically significant defined as # P <0.05, ### P <0.001 and #### P <0.0001.

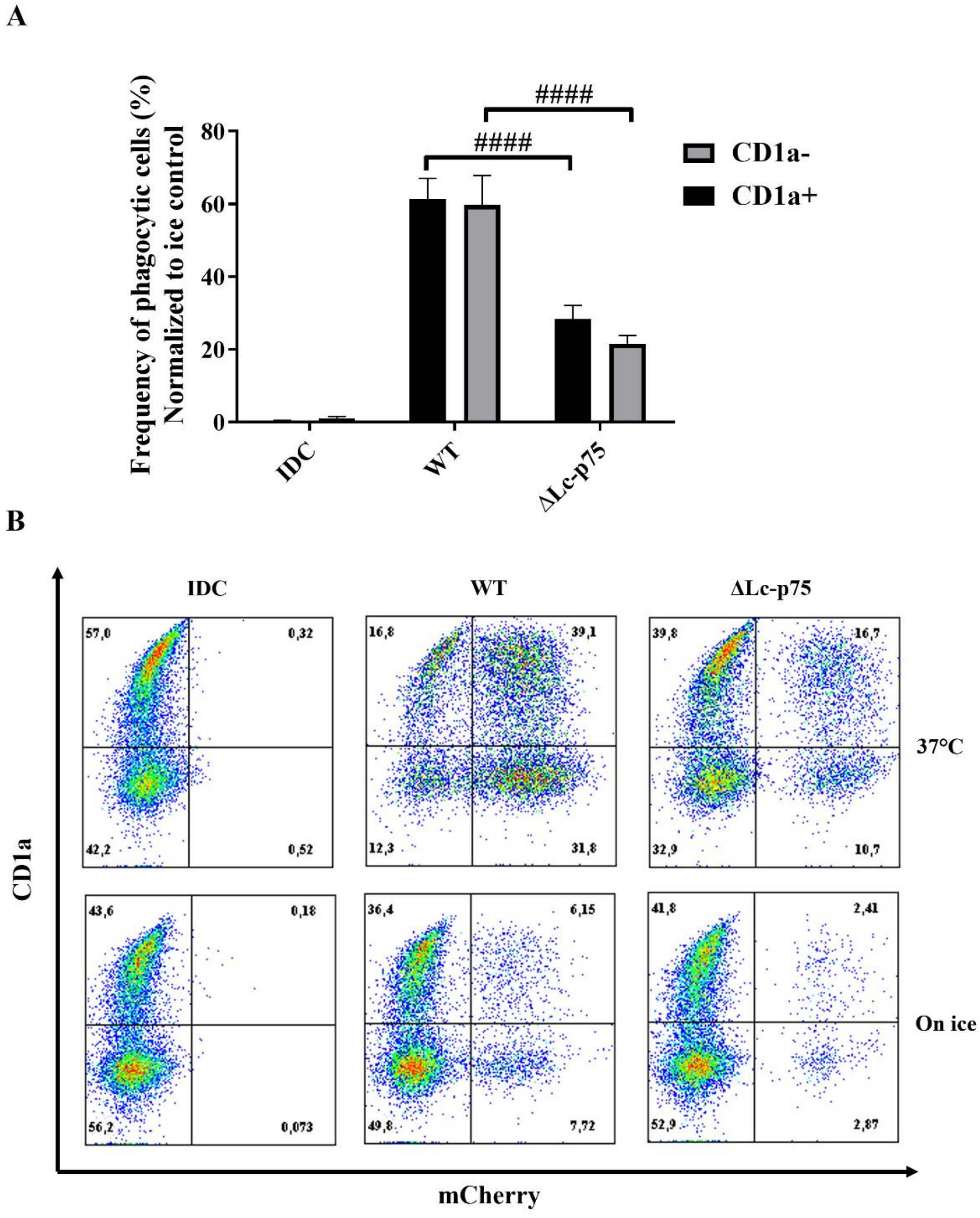


**Figure 6.** Phagocytic efficiency of moDCs activated with *L. casei* bacteria is independent of the CD1a expression. Five-day moDCs were stimulated with mCherry-labeled WT *L. casei* BL23 and ΔLc-p75 mutant bacteria for 24 h at 37ºC and on ice. The phagocytic capacity of $CD1a^+$ and $CD1a^-$ subsets was measured by detecting mCherry-positive moDCs with flow cytometry (A). IDC represents the sample without any bacterial treatment. The frequency of phagocytic cells is normalized to ice control and the mean values of 3 independent experiments are presented ±SD. Figure B demonstrates

dot plots from one experiment showing the CD1a and mCherry expression of moDCs. Two-way ANOVA followed by Tukey's multiple comparison tests was used in the statistical analysis. Differences between WT and ΔLc-p75 were statistically significant, defined as #### P <0.0001.

### *2.6. Inhibition of phagocytosis diminishes the effects triggered by WT L. casei BL23 on moDC functions*

To further investigate whether phagocytosis is the essential step in activating moDCs by WT *L. casei* BL23, we inhibited actin polymerization, the early steps of phagocytosis by cytochalasin D (CyD). Firstly, we determined the phagocytic activity of moDCs by flow cytometry and confocal microscopy. The CyD treatment induced a significant decrease in the WT bacteria's engulfment by moDCs compared to the non-treated moDCs (Figure 7A, 7B). To check that impairment in the phagocytosis of WT bacteria is not the consequence of increased cell death caused by CyD, we measured the frequency of the dead DCs by 7AAD staining. Importantly, we could not detect any significant cell death in the CyD-treated and non-treated samples after co-incubation with WT *L. casei* BL23 (Figure S4A). Confocal microscopic montage pictures and the orthogonal views revealed that moDCs phagocytosed the WT bacteria after 24 h (Figure 7C). However, after the CyD pre-incubation, mCherry-positive bacteria were attached only to the cell surface of moDCs (Figure 7D).

Next, we tested whether the inhibition of the phagocytosis has a blocking effect on cytokine secretion by human moDCs. We found that impairment in the bacterial engulfment caused a highly significant decrease in pro-inflammatory TNF-$\alpha$, IL-1$\beta$, IL-6 cytokine secretion (Figure 8A). Furthermore, CyD treatment attenuated the IL-10, IL-12, and IL-23 T cell polarizing cytokine release by *L. casei*-activated moDCs (Figure 8B) and consequently reduced the number of IFN$\gamma$- and IL-17A-producing T cells in DC-T cell cocultures (Figure S4B). It should be noted that CyD treatment induced significantly increased IL-8 chemokine production by moDCs even in the absence of bacteria (Figure S4C), which is in good agreement with previous results obtained with human retinal pigment epithelial cells [23]. Importantly, CyD pre-treatment did not influence the cell surface expression of CD83, HLA-DQ (Figure 9A, 9B) and the co-stimulatory CD80 and CD86 molecules (Figure 9C).

Overall, these results demonstrate that bacteria internalization is an essential step in *L. casei* BL23-induced moDC activation, indicating that the reduced phagocytic uptake of ΔLc-p75 mutant *L. casei* BL23 may result in its decreased moDC-activating capacity.

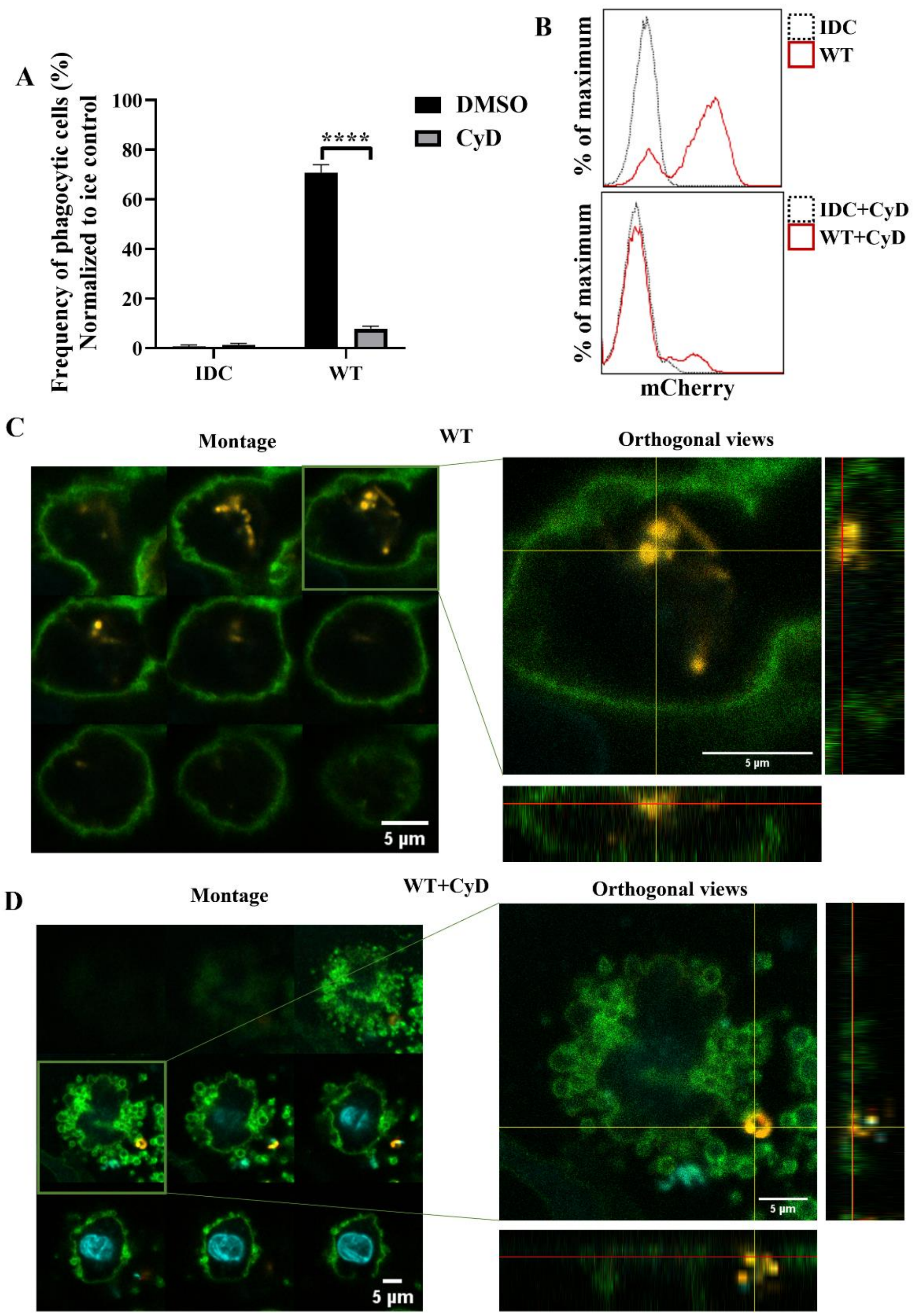


**Figure 7.** Inhibition of the cytoskeleton rearrangement causes impaired phagocytosis of wild-type *L. casei* BL23 by moDCs. Five-day moDCs were preincubated with 15 µM Cytochalasin D (CyD) or the vehicle control DMSO for 30 min. Then, moDCs were activated with four-times more mCherry-expressing wild-type bacteria for 24 h. Phagocytosis of the WT bacteria by moDCs was detected by flow cytometry and confocal microscopy. The frequency of the phagocytic moDCs at 37°C was normalized to ice control and was calculated from 3 independent experiments ±SD (A). IDC means the non-activated moDC. Histogram overlays show one representative experiment (B). For the confocal microscopic pictures (C, D) the moDCs were labeled with HLA-DQ (green) and DAPI (cyan); mCherry was indicated with yellow. On figure left, montage pictures show the Z-stack images per 1 µm from the bottom to the top of the cell. On figure right the orthogonal views are demonstrated

of one Z-stack image. On the bottom is Z-projection in the X-Z direction, on the right is the Z-projection in the Y-Z direction. The red lines indicate the Z-depth of the optical slice and the orthogonal planes of the X-Z and Y-Z projections, respectively. Two-way ANOVA followed by Tukey's multiple comparison tests was used in the statistical analysis. The difference between the non-treated and CyD-treated moDCs was statistically significant, defined as **** P <0.0001.

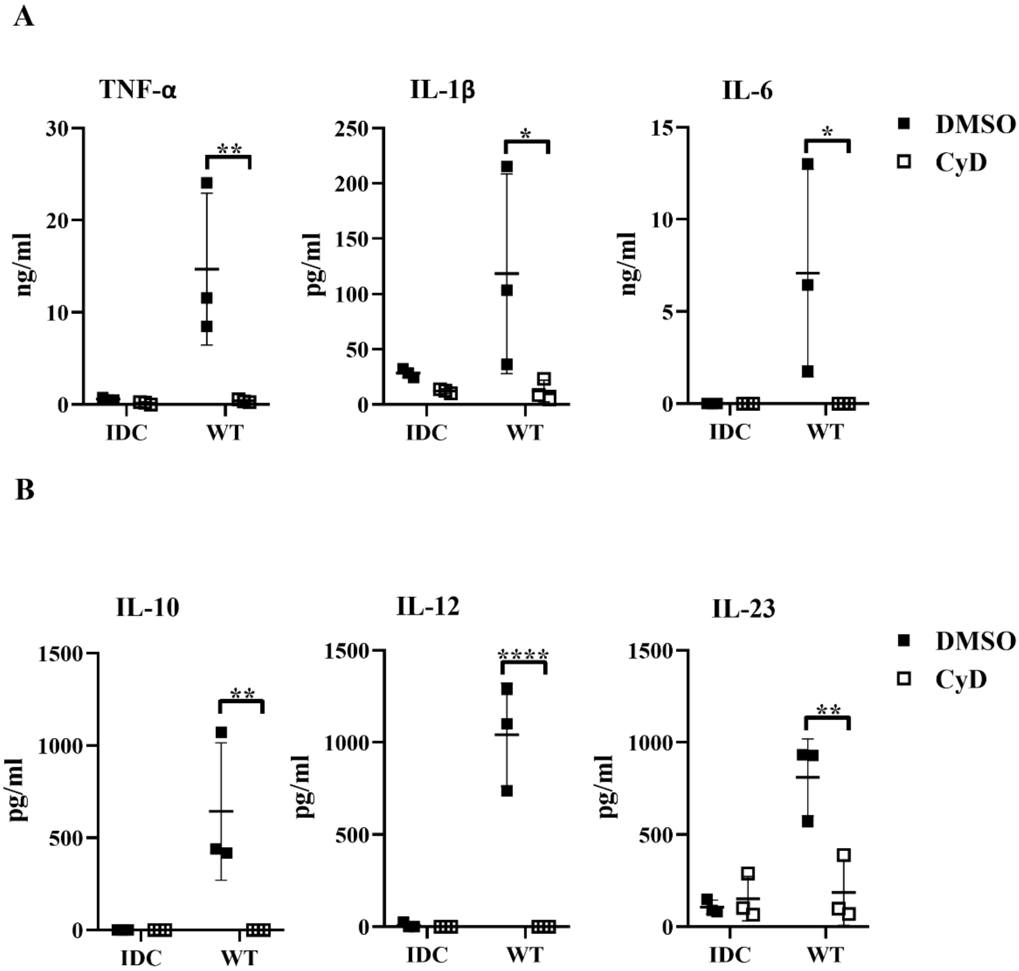


**Figure 8.** Blocked phagocytosis leads to reduced cytokine secretion by bacteria-exposed moDCs. Five-day moDCs were pre-incubated with 15 μM CyD or the vehicle control DMSO for 30 min. Then, moDCs were stimulated with WT *L. casei* at a ratio of 1:4 for 24 h. IDC means cells without bacterial treatment. The concentrations of pro-inflammatory TNF-$\alpha$, IL-1$\beta$, IL-6 cytokines (A) and the T cell polarizing IL-10, IL-12 and IL-23 (B) were determined from the supernatants of the WT bacteria activated moDCs and control IDCs. Figures represent the mean of 3 independent donors ±SD. Each dot represents one donor. Student's paired two-tailed t-test was used in the statistical analysis with significance defined as * P <0.05, ** P <0.01 and **** P <0.0001 between the CyD- and DMSO-treated moDCs.

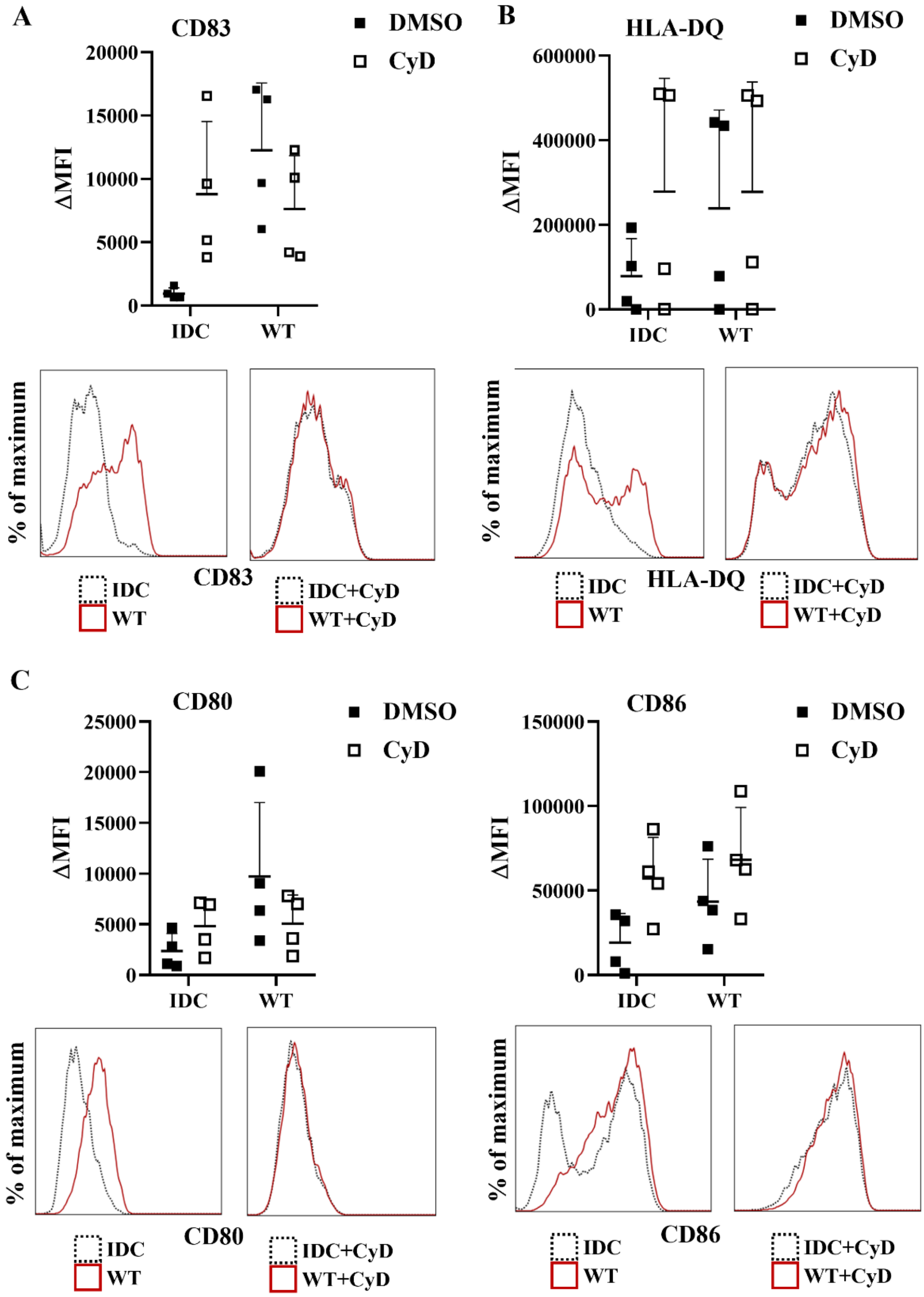


**Figure 9**. Cytochalasin D pre-treatment does not cause alterations in the expression of activation and co-stimulatory markers of moDCs. Five-day moDCs were preincubated with 15 µM CyD or its vehicle control DMSO for 30 min. Then, moDCs were stimulated with WT *L. casei* BL23 at a ratio of 1:4 for 24 h. Expression of CD83 (A), HLA-DQ (B) and co-stimulatory CD80 and CD86 (C) molecules was measured by flow cytometry. IDC represents cells without any bacterial treatment. Median fluorescent intensities (ΔMFI) were calculated from 4 independent experiments ±SD. Histogram overlays show one representative experiment. Each dot is representative of one donor. Two-way ANOVA followed by Tukey's multiple comparison tests was used in the statistical analysis.

## 3. Discussion

As a mucosal surface, the gastrointestinal tract is a typical entry of pathogens. However, on the other hand, the gut harbors a large number of commensal microorganisms, such as LAB. LAB exerts various beneficial effects on the human host; hence, an essential role of the gut immune system is to distinguish the pathogen-derived antigens from those derived from the microbiota. Due to their ability to precisely sense the local microenvironment, DCs can control both the immunogenic and tolerogenic responses according to the features of the engulfed antigens [24]. DCs can directly [25] or indirectly [26] sample the luminal content of the gut, such as bacteria or their derived components and metabolites. Furthermore, the unique, naïve T cell activating capacity of DCs is essential in inducing tolerance or adaptive immune responses against the microbiota members. Therefore, the interaction between LAB members such as lactobacilli and DCs is under extensive investigation. In this study, we investigated the effects of PG alterations in the cell wall of *L. casei* BL23 on moDCs activation and their T cell polarizing ability.

Our findings demonstrate that ΔLc-p75 mutant *L. casei* strain induces efficient maturation of moDCs, while it causes dampened cytokine secretion by these cells. This result is in line with previous observation showing that potentially probiotic bacteria induce expression of moDC maturation and activation markers as efficiently as pathogenic bacteria, but their ability to trigger cytokine production varies significantly from one bacterial strain to another [27]. Among the investigated pro-inflammatory soluble mediators, IL-8 was an exception, as we could not detect any significant differences in its secretion by moDCs activated with the WT or PGH mutant *L. casei* strains. This observation suggests that the gene expression of pro-inflammatory cytokines and IL-8 is differentially regulated in myeloid cells. Indeed, analysis of recruitment of p65 NF-kB to the corresponding promoters in U937 macrophages revealed that although the p65 recruitment to TNF-$\alpha$, IL-1$\beta$, and IL-6 promoters is inhibited by the nuclear IkB$\alpha$, recruitment to IL-8 promoter is not repressed. Furthermore, the IL-8 promoter is specifically associated with the S536-phosphorylated form of p65 NF-kB, whereas only the unphosphorylated p65 is recruited to promoters of TNF-$\alpha$, IL-1$\beta$, and IL-6 genes [28]. Another study reported that knockdown of regulator of G-protein signaling 16 (RGS16) by RNAi in human promonocytic cell line THP-1 upregulates TNF-$\alpha$, IL-1$\beta$, and IL-6 but not IL-8 [29]. Our finding that the Lc-p75 defective *L. casei* induces reduced Th1 and Th17 responses compared to WT bacteria was not surprising after our observation that mutant bacteria trigger lower production of IL-12 and IL-23, major cytokines required for polarization and maintaining the Th1 and Th17 subsets, respectively [30], by moDCs compared to their WT counterpart.

To answer the question whether the observed differences between WT and mutant *L. casei* strains in DC activation are the direct consequences of the modified bacterial cell wall constituents, we tested the moDC stimulating ability of purified PGs from the WT and ΔLc-p75 mutant strains. We found that the purified PGs from WT and mutant *L. casei* BL23 strains have a similar immunomodulatory effect on moDCs. It has long been known that PGs can be recognized by PRRs in the host cells and mediates their activation. In 1999, it was demonstrated that their recognition is TLR2-mediated [31]. However, soon after, Travassos et al. found that the intracellular Nod proteins are more potent candidates in the sensing of Gram+ bacterial PGs [32]. Therefore, PGs or their derived fragments have to enter the intracellular space of the host cell. Various mechanisms help the uptake of PGs such as phagocytosis, transporter-mediated translocation and micropinocytosis-like mechanisms [33] or the action of bacterial PGHs, which can generate PG fragments [34]. Based on our results, no matter how they enter the moDCs, purified PG components from ΔLc-p75 mutant or WT bacteria activate their receptors with the same efficiency.

Several studies demonstrated that DCs could phagocytose different *Lactobacillus* strains, which induce distinct DC activation patterns. A recent study showed that human moDCs were able to uptake *L. rhamnosus* JB-1 bacteria after 48 hours that caused poor co-stimulatory molecule expression and weak cytokine production [35]. In another publication, mucus adhesin-expressing *L. reuteri* strains were efficiently phagocytosed by moDCs [19]. In agreement with these data, our phagocytosis results revealed that human moDCs were able to uptake *L. casei* BL23. Moreover, our results showed that the long-chain-forming mutant bacteria were weakly internalized by moDCs.

The regulation of the phagocytic process is multifaceted, and is determined by the involved receptors, duration of the binding, and the physical characteristics, such as the size of the engulfed material [36]. It was previously shown that bacterial aggregates were weakly phagocytosed by polymorphonuclear leukocytes (PMN), influencing the killing ability of PMNs [37]. Thus, the properties of phagocytosis may consequently determine the nature of developing responses. Indeed, it has been illustrated that in addition to the signaling initiated by TLR2 receptor, bacterial phagocytosis and Nod proteins are also important for macrophage responsiveness to Gram-positive bacteria [38]. Our results showed that the long-chain-forming mutant of *L. casei* BL23 could induce only a weak pro-inflammatory and T cell polarizing cytokine secretion by human moDCs as compared to WT bacteria. The importance of phagocytosis in cytokine production was further confirmed by its inhibition with cytochalasin D, which strongly reduced the concentration of secreted pro-inflammatory and T cell polarizing cytokines. The observed phenomenon was similarly to that case when moDCs were stimulated with the long-chain-forming ΔLc-p75 mutant bacteria.

It has been demonstrated that there is a Nod2-mediated enhancement of TLR-induced TNF-$\alpha$, IL-12 and IL-10 production in DCs [39]. Nod2fs mutation, detected in patients suffering from Crohn's disease, results in a loss-of-function phenotype in human myeloid DCs. Dendritic cells derived from Crohn's disease patients homozygous for this mutation respond normally to purified TLR ligands but fail to up-regulate cytokine production in response to the treatment with a Nod2 ligand [39]. We assume that a similar phenomenon is taking place in moDCs exposed to the long-chain-forming ΔLc-p75 mutant bacteria. Signals triggered by TLR2 on the cell surface induce the production of pro-inflammatory and T cell polarizing cytokines, but this remains at a relative low level because of the lack of Nod2-mediated enhancement due to the less efficient uptake of mutant bacteria. However, this hypothesis needs further investigation.

Taken together, our results indicate that the immunomodulatory properties of *L. casei* BL23 bacteria are highly dependent on their phagocytosis by human moDCs. Internalization determines the cytokine secretion of moDCs, which overall can be translated to inflammation and also to Th1/Th17-mediated adaptive immune responses. The long-chain-forming ΔLc-p75 mutant *L. casei*, which are less likely to be phagocytized, induces only moderate DC and T cell responses. Our findings may be useful in the generation of new probiotic agents.

## 4. Materials and Methods

### *4.1. Bacterial strains and growth conditions*

*L. casei* BL23 [40] and its ΔLc-p75 mutant derivative obtained by deletion of gene *lcabl_02770* encoding the Lc-p75 PGH [15] were used in this study. They were inoculated from frozen glycerol stocks on MRS agar plates and cultivated at 37°C for 48 h to obtain isolated colonies. Single colonies were diluted in MRS broth (BD Difco, Fisher Scientific, Co.L.L.C, PA, US) and propagated for 16 h at 37°C until the beginning of the stationary phase. Fluorescent mCherry strains were obtained by transformation with a plasmid encoding red fluorescent mCherry protein (pTS-mCherry; kind gift of Jerry M. Wells, Wageningen University, the Netherlands). The red fluorescent strains were cultured with 5 μg/ml erythromycin (Merck KGaA, Darmstadt, Germany) included in MRS medium to select the plasmid. Optical density (OD) of 1 at 600 nm corresponds to 2.5 x $10^8$ cells per ml for the wild type *L. casei* BL23 strain, as calculated from dilution series of liquid bacterial culture and colony counting.

To verify that the same OD values for cell suspensions of the long-chain forming mutant or wild type strain correspond to similar numbers of bacterial cells, bacteria were cultivated for 16 h, then they were stained in suspension at $OD_{600nm}$ with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent DNA dye, and the bacteria-associated fluorescence was measured. We observed that for both strains under study, the $OD_{600nm}$ value was perfectly correlated with the fluorescence intensity level and thus with the cell number (Figure S5).

## *4.2. Extraction of peptidoglycan*

PG was extracted from *L. casei* strains as described previously [15] with some modifications. Cells from a 500 ml exponentially growing culture ($OD_{600}$, 0.3) were chilled on ice and harvested by centrifugation. Cells were suspended in deionized $H_2O$ and boiled for 10 min. They were then resuspended in 5% (w/v) SDS in 50 mM Tris-HCl pH 7.0 and boiled for 25 min. The pellet obtained by centrifugation at 20,000 g for 10 min, was resuspended in 4% (w/v) SDS in Tris-HCl buffer and boiled again for 15 min. Cell walls were recovered by centrifugation at 20,000 g for 10 min and washed six times with deionized $H_2O$ to remove SDS. The cell wall pellet was then successively treated with Pronase (2 mg/ml) for 90 min at 60°C, by $\alpha$-amylase (50 μg/ml) for 2 h at 37°C, by DNase (50 μg/ml) and RNase (50 μg/ml) for 4 h at 37°C, and lipase (50 μg/ml) and finally by trypsin (200 μg/ml) for 16 h at 37°C. The final pellet was then treated with 2% SDS in Tris-HCl (50 mM pH 7.0.) and washed with deionized $H_2O$. The insoluble pellet was treated with 48 % fluorhydric acid overnight at 4°C to remove wall polysaccharides (WPS) [41]. After centrifugation, the pellet (containing PG) was washed several times with 0.25 M Tris-HCl, pH 7.0 and deionized H2O. The final pellet was lyophilized and stored at -20°C.

## *4.3. Human monocyte separation and differentiation to dendritic cells*

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized leukocyte-enriched buffy coats applying a Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Monocytes were separated from PBMCs by magnetic cell separation using CD14-specific antibody-coated microbeads, according to the manufacturer's instruction (MiltenyiBiotec, Bergisch Gladbach, Germany). After separation on a VarioMACS magnet, the homogeneity of the isolated $CD14^+$ monocyte fraction was greater in all experiments than 90-95% checked by flow cytometry. The autologous monocyte-depleted PBL fraction was used in the ELISPOT assays as a T cell source.

Isolated monocytes were seeded at 1x$10^6$/ml concentration in serum-free AIM-V medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 ng/ml IL-4 (PeproTech EC, London, UK) and 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium) and were differentiated to moDCs for 5 days. On day 2, half of the medium was changed complemented with 100 ng/ml IL-4 and 80 ng/ml GM-CSF. When indicated moDCs were preincubated with 15 μM Cytochalasin D (CyD, Merck KGaA) dissolved in dimethyl-sulfoxide (DMSO, Serva Electrophoresis GMBH, Germany) for 30 min to inhibit the phagocytosis. DMSO was used as a vehicle control.

## *4.4. Stimulation of moDCs with bacteria or their PG fragments*

Bacteria were washed twice with cold PBS and were added to the moDCs on day 5 of *in vitro* differentiation at a ratio of 1 (moDC): 4 (bacteria) for 3 or 24 h. Control cells were left untreated (IDC).

Purified bacterial PG samples were resuspended in deionized water and sonicated with Branson Sonifier 450 until they became clear. MoDCs were treated with 10 μg/ml PG fragments for 24 h.

Each experiment was repeated with at least three independent donors.

### *4.5. Analysis of the expression of cell surface markers and cell viability of dendritic cells by flow cytometry*

WT and Lc-p75 mutant Lactobacilli were washed with PBS and added to the five-day moDCs for 24 h at a ratio of 1:4. In parallel, purified lactobacterial PG fragments were resuspended in deionized water and sonicated. MoDCs were treated with 10 μg/ml PG fragments for 24 h. On day 6 cells were stained with fluorescence-conjugated monoclonal antibodies: CD83-fluorescein isothiocyanate (FITC), CD80-FITC, CD86-phycoerythrin (PE) and HLA-DQ-FITC (BioLegend, San Diego, CA, US) or left unlabeled as a control. Non-specific antibody binding was inhibited using heat-inactivated mouse serum. The moDC population was gated according to the forward/side scatter parameters. Fluorescence intensities were measured by ACEA NovoCyte 2000R cytometer (Agilent, Santa Clara, CA, US); data analyzes were performed by the FlowJo vX.0.7 software. Viable and nonviable cells were dissected by flow cytometry after staining the freshly collected, non-fixed cells with 0.5 μg/ml 7-Amino-actinomycin D (7-AAD) (Merck KGaA).

### *4.6. Cytokine measurements by enzyme-linked immunosorbent assays (ELISA)*

Supernatants of stimulated moDCs were collected after 24 h and the concentration of IL-1β, IL-6, TNF-α, IL-12p70, IL-10, IL-23 and IL-8 was measured by using BD OptEIA ELISA kits according to the manufacturer's instructions (Becton Dickinson, BD Biosciences, US). OD was detected at 450 nm using Synergy™ HT Multi-Detection Microplate reader (Bio-Tek Instruments, VT, USA) and KC4 software v3.4.

### *4.7. ELISPOT assays*

Bacteria-stimulated moDCs were counted, washed with PBS and co-cultured with autologous PBL at a ratio of 1:20 in serum-free AIM-V medium for 3 days at 37°C in a 5% CO2 atmosphere. On day 4, the cells were collected, counted, and subjected to IFNγ, IL-17A and IL-4 Ready Set Go ELISPOT assays according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Briefly, 300 000 cells/well were incubated in CTL-Test medium (Cellular Technology Limited, Cleveland, OH, USA) for 40-48 h at 37°C in MultiScreen-HTS PVDF plates (Millipore S.A., Molsheim, France) pre-coated with capture antibodies specific for IFNγ, IL-4, or IL-17A. Together with the IL-4- and IL-17A-specific capture antibody, 0.5 μg/ml purified anti-human CD3 antibody (BD Biosciences) was added to the coating buffer for the mitogenic stimulation of $CD3^+$ T cells. The detection of the cytokine release was performed by biotinylated IFNγ, IL-4, or IL-17A-specific antibodies in the presence of HRP conjugated to avidin. Soon after the colorigenic substrate, 3-amino-9-ethylcarbazole (AEC Substrate Set, BD Biosciences) was added. The color development was stopped by tap water, and air-dried plates were analyzed by a computer-assisted ELISPOT image analyzer (Series 1 ImmunoSpot Analyzer, ImmunoSpot Version 4.0 Software Academic, Cellular Technology).

### *4.8. Phagocytosis assay by flow cytometry*

The moDCs and mCherry-expressing bacteria were co-incubated at a ratio of 1:4 for 3 or 24 h at 37°C in a 5% CO2 atmosphere or on ice as a control. After the incubation period, moDCs were stained with allophycocyanin (APC)-labeled anti-CD1a mAb (BioLegend, San Diego, CA, US). Cell analysis was performed with ACEA NovoCyte 3000 RYB flow cytometer (Agilent). Data analyzes were performed by the FlowJo vX.0.7 software (Tree Star Inc., Ashland, OR, US). The moDC population was gated according to the forward/side scatter parameters by excluding the non-internalized, free bacteria. The phagocytosis ratio was determined as a percentage of mCherry positive cells or according to the median values of mCherry fluorescence intensity (MFI). Similarly, the frequency of phagocytic cells was determined in the CD1a positive and the CD1a negative moDC subpopulations as a percentage of mCherry positive cells.

### *4.9. Confocal microscopy*

Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with 40x water immersion objective (NA 1.2) was used to image the phagocytosis of *L. casei* cells by moDCs after 24 h. For the excitation of DAPI a 405 nm diode laser; for FITC the 488 nm line of an Argon ion laser; for mCherry a 543 nm He-Ne laser and for APC a 633 nm He-Ne laser was applied. Fluorescence emissions of DAPI and FITC were detected in the wavelength range of 410-485 and 490-610 nm, respectively, while the detection of mCherry was performed with 575-695 nm band-pass filter. For the detection of APC, 640-745 nm band-pass filter was used. Z-stack images were collected at 1 μm intervals from the bottom to the top of the cells.

### *4.10. Statistical analysis*

Analyses were performed by Microsoft Excel and GraphPad Prism v8.0 (GraphPad Software Inc., San Diego, CA, US). Comparison between two groups was performed by paired, two-tailed Student's t-test. One-way ANOVA followed by Tukey's post-hoc test was used for the comparisons of more than 2 groups. Two-way ANOVA followed by Tukey's post-hoc test was applied for the comparisons of two independent variables. The results were shown as mean ± standard deviation (SD). Differences were considered to be statistically significant at $P < 0.05$. Significance was indicated as * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; **** $P < 0.0001$ or as # $P < 0.05$; ## $P < 0.01$, ### $P < 0.001$; #### $P < 0.0001$.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, **Figure S1**: Activation with *L. casei* strains or PGs derived from them does not alter the viability of the moDCs. **Figure S2**: Exposure to *L. casei* BL23 reduces the number of IL-4-producing T cells primed by moDCs. **Figure S3**: PGs derived from *L. casei* BL23 WT and mutant bacteria induce the expression of CD83, HLA-DQ, CD80, and CD86 and IL-8 secretion by moDCs at similar levels. **Figure S4:** CyD treatment does not increase the death of moDCs but decreases the number of IFN-γ and IL-17A producing T cells after *L. casei* activation and elevates the IL-8 production by moDCs regardless of bacterial presence. **Figure S5:** Correspondence between $OD_{600nm}$ values of bacteria suspension and number of bacteria as measured by DAPI staining for *L. casei* BL23 and its derivative ΔLc-p75 mutant.

**Author Contributions:** Conceptualization, M.T., É.R., M-P.C-C. and A.B.; methodology, M.T., Sz.M., K.R., Sz-Sz.T.; software, M.T.; validation, É.R., M-P.C-C. and A.B.; formal analysis, M.T.; investigation, M.T. and Sz.M.; resources, É.R, M-P.C-C. and A.B.; data curation, M.T.; writing—original draft preparation, M.T.; writing—review and editing, Zs. Cz., É.R., M-P.C-C. and A.B.; visualization, M.T.; supervision, É.R., M-P.C-C. and A.B.; project administration, M.T., É.R., M-P.C-C. and A.B.; funding acquisition, É.R., M-P.C-C. and A.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** K.R. was the recipient of a doctoral fellowship from INRAE (MICA Department) and from a FEMS short-term research fellowship. This research was funded by TÁMOP-4.2.2.A-11/1/KONV-2012 0023 project implemented through the New Hungary Development Plan, and the National Research, Development and Innovation Office (NK 101538 and NN 114423 to É.R. and K 125337 to A.B.). The work was also supported by GINOP-2.3.2-15-2016-00050 project (A.B.). The project is co-financed by the European Union and the European Regional Development Fund.

**Institutional Review Board Statement:** 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 (OVSzK 3572-2/2015/5200) and the Regional and Institutional Research Ethical Committee of the University of Debrecen, Faculty of Medicine (Debrecen, Hungary).

**Informed Consent Statement:** 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.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We thank Dr. Attila Kiss for his technical help and Erzsébet Nagyné Kovács for her excellent assistance. We greatly appreciate Dr. Krisztián Bene's Dr. Anett Türk-Mázló's and Dr. Aliz Varga's kind help in reviewing the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

## References

1. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514, doi:10.1038/nrgastro.2014.66.
2. Ren, C.; Zhang, Q.; De Haan, B.J.; Faas, M.M.; Zhang, H.; De Vos, P. Protective effects of lactic acid bacteria on gut epithelial barrier dysfunction are Toll like receptor 2 and protein kinase C dependent. *Food Funct.* **2020**, *11*, 1230–1234, doi:10.1039/c9fo02933h.
3. Baümler, A.J.; Sperandio, V. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* **2016**, *535*, 85–93, doi:10.1038/nature18849.
4. Campana, R.; Van Hemert, S.; Baffone, W. Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. *Gut Pathog.* **2017**, *9*, 1–12, doi:10.1186/s13099-017-0162-4.
5. LeBlanc, J.G.; Levit, R.; Savoy de Giori, G.; de Moreno de LeBlanc, A. Application of vitamin-producing lactic acid bacteria to treat intestinal inflammatory diseases. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 3331–3337, doi:10.1007/s00253-020-10487-1.
6. Resta, S.C. Effects of probiotics and commensals on intestinal epithelial physiology: Implications for nutrient handling. *J. Physiol.* **2009**, *587*, 4169–4174, doi:10.1113/jphysiol.2009.176370.
7. Yan, F.; Polk, D.B. Probiotics and immune health. *Curr. Opin. Gastroenterol.* **2011**, *27*, 496–501, doi:10.1097/MOG.0b013e32834baa4d.
8. Vieira, A.T.; Teixeira, M.M.; Martins, F.S. The role of probiotics and prebiotics in inducing gut immunity. *Front. Immunol.* **2013**, *4*, 1–12, doi:10.3389/fimmu.2013.00445.
9. Rochat, T.; Bermúdez-Humarán, L.; Gratadoux, J.J.; Fourage, C.; Hoebler, C.; Corthier, G.; Langella, P. Anti-inflammatory effects of Lactobacillus casei BL23 producing or not a manganese-dependant catalase on DSS-induced colitis in mice. *Microb. Cell Fact.* **2007**, *6*, 1–10, doi:10.1186/1475-2859-6-22.
10. Niedergang, F.; Didierlaurent, A.; Kraehenbuhl, J.P.; Sirard, J.C. Dendritic cells: The host Achille's heel for mucosal pathogens? *Trends Microbiol.* **2004**, *12*, 79–88, doi:10.1016/j.tim.2003.12.011.
11. Moretti, J.; Blander, J.M. Insights into phagocytosis-coupled activation of pattern recognition receptors and inflammasomes. *Curr. Opin. Immunol.* **2014**, *26*, 100–110, doi:10.1016/j.coi.2013.11.003.
12. Vermassen, A.; Leroy, S.; Talon, R.; Provot, C.; Popowska, M.; Desvaux, M. Cell wall hydrolases in bacteria: Insight on the diversity of cell wall amidases, glycosidases and peptidases toward peptidoglycan. *Front. Microbiol.* **2019**, *10*, doi:10.3389/fmicb.2019.00331.
13. Bäuerl, C.; Pérez-Martínez, G.; Yan, F.; Polk, D.B.; Monedero, V. Functional analysis of the p40 and p75 proteins from lactobacillus casei BL23. *J. Mol. Microbiol. Biotechnol.* **2011**, *19*, 231–241, doi:10.1159/000322233.
14. Bäuerl, C.; Coll-Marqués, J.M.; Tarazona-González, C.; Pérez-Martínez, G. Lactobacillus casei extracellular vesicles stimulate EGFR pathway likely due to the presence of proteins P40 and P75 bound to their surface. *Sci. Rep.* **2020**, *10*, 1–12, doi:10.1038/s41598-020-75930-9.
15. Regulski, K.; Courtin, P.; Meyrand, M.; Claes, I.J.J.; Lebeer, S.; Vanderleyden, J.; Hols, P.; Guillot, A.; Chapot-Chartier, M.P.

Analysis of the peptidoglycan hydrolase complement of lactobacillus casei and characterization of the major $\gamma$-D-Glutamyl-L-Lysyl-endopeptidase. *PLoS One* **2012**, *7*, doi:10.1371/journal.pone.0032301.

16. Wolf, A.J.; Underhill, D.M. Peptidoglycan recognition by the innate immune system. *Nat. Rev. Immunol.* **2018**, *18*, 243–254, doi:10.1038/nri.2017.136.
17. Drakes, M.; Blanchard, T.; Czinn, S. Bacterial probiotic modulation of dendritic cells. *Infect. Immun.* **2004**, *72*, 3299–3309, doi:10.1128/IAI.72.6.3299.
18. Bene, K.; Varga, Z.; Petrov, V.O.; Boyko, N.; Rajnavolgyi, E. Gut microbiota species can provoke both inflammatory and tolerogenic immune responses in human dendritic cells mediated by retinoic acid receptor alpha ligation. *Front. Immunol.* **2017**, *8*, doi:10.3389/fimmu.2017.00427.
19. Bene, K.P.; Kavanaugh, D.W.; Leclaire, C.; Gunning, A.P.; MacKenzie, D.A.; Wittmann, A.; Young, I.D.; Kawasaki, N.; Rajnavolgyi, E.; Juge, N. Lactobacillus reuteri surface mucus adhesins upregulate inflammatory responses through interactions with innate C-Type lectin receptors. *Front. Microbiol.* **2017**, *8*, 1–16, doi:10.3389/fmicb.2017.00321.
20. Lim, T.S.; Goh, J.K.H.; Mortellaro, A.; Lim, C.T.; Hämmerling, G.J.; Ricciardi-Castagnoli, P. CD80 and CD86 Differentially Regulate Mechanical Interactions of T-Cells with Antigen-Presenting Dendritic Cells and B-Cells. *PLoS One* **2012**, *7*, 1–8, doi:10.1371/journal.pone.0045185.
21. Gogolak, P.; Rethi, B.; Szatmari, I.; Lanyi, A.; Dezso, B.; Nagy, L.; Rajnavolgyi, E. Differentiation of CD1a- and CD1a+ monocyte-derived dendritic cells is biased by lipid environment and PPAR$\gamma$. *Blood* **2007**, *109*, 643–652, doi:10.1182/blood-2006-04-016840.
22. Seshadri, C.; Shenoy, M.; Wells, R.D.; Hensley-McBain, T.; Andersen-Nissen, E.; McElrath, M.J.; Cheng, T.-Y.; Moody, D.B.; Hawn, T.R. Human CD1a Deficiency Is Common and Genetically Regulated. *J. Immunol.* **2013**, *191*, 1586–1593, doi:10.4049/jimmunol.1300575.
23. Gao, M.; Wu, S.; Ji, J.; Zhang, J.; Liu, Q.; Yue, Y.; Liu, L.; Liu, X.; Liu, W. The influence of actin depolymerization induced by Cytochalasin D and mechanical stretch on interleukin-8 expression and JNK phosphorylation levels in human retinal pigment epithelial cells. *BMC Ophthalmol.* **2017**, *17*, 4–9, doi:10.1186/s12886-017-0437-z.
24. Coombes, J.L.; Powrie, F. Dendritic cells in intestinal immune regulation. *Nat. Rev. Immunol.* **2008**, *8*, 435–446, doi:10.1038/nri2335.
25. Rescigno, M.; Urbano, M.; Valzasina, B.; Francolini, M.; Rotta, G.; Bonasio, R.; Granucci, F.; Kraehenbuhl, J.P.; Ricciardi-Castagnoli, P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2001**, *2*, 361–367, doi:10.1038/86373.
26. Niess, J.H.; Reinecker, H.C. Dendritic cells in the recognition of intestinal microbiota. *Cell. Microbiol.* **2006**, *8*, 558–564, doi:10.1111/j.1462-5822.2006.00694.x.
27. Latvala, S.; Pietilä, T.E.; Veckman, V.; Kekkonen, R.A.; Tynkkynen, S.; Korpela, R.; Julkunen, I. Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World J. Gastroenterol.* **2008**, *14*, 5570–5583, doi:10.3748/wjg.14.5570.
28. Ghosh, C.C.; Ramaswami, S.; Juvekar, A.; Vu, H.-Y.; Galdieri, L.; Davidson, D.; Vancurova, I. Gene-Specific Repression of Proinflammatory Cytokines in Stimulated Human Macrophages by Nuclear I$\kappa$B$\alpha$. *J. Immunol.* **2010**, *185*, 3685–3693, doi:10.4049/jimmunol.0902230.
29. Suurväli, J.; Pahtma, M.; Saar, R.; Paalme, V.; Nutt, A.; Tiivel, T.; Saaremäe, M.; Fitting, C.; Cavaillon, J.M.; Rüütel Boudinot, S. RGS16 restricts the pro-inflammatory response of monocytes. *Scand. J. Immunol.* **2015**, *81*, 23–30, doi:10.1111/sji.12250.
30. Dupage, M.; Bluestone, J.A. Harnessing the plasticity of CD4+ T cells to treat immune-mediated disease. *Nat. Rev. Immunol.* **2016**, *16*, 149–163, doi:10.1038/nri.2015.18.
31. Schwandner, R.; Dziarski, R.; Wesche, H.; Rothe, M.; Kirschning, C.J. Peptidoglycan- and lipoteichoic acid-induced cell

activation is mediated by Toll-like receptor 2. *J. Biol. Chem.* **1999**, *274*, 17406–17409, doi:10.1074/jbc.274.25.17406.

32. Travassos, L.H.; Girardin, S.E.; Philpott, D.J.; Blanot, D.; Nahori, M.A.; Werts, C.; Boneca, I.G. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep.* **2004**, *5*, 1000–1006, doi:10.1038/sj.embor.7400248.
33. Bastos, P.A.D.; Wheeler, R.; Boneca, I.G. Uptake, recognition and responses to peptidoglycan in the mammalian host. *FEMS Microbiol. Rev.* **2021**, *45*, 1–25, doi:10.1093/femsre/fuaa044.
34. Lebeer, S.; Vanderleyden, J.; De Keersmaecker, S.C.J. Host interactions of probiotic bacterial surface molecules: Comparison with commensals and pathogens. *Nat. Rev. Microbiol.* **2010**, *8*, 171–184, doi:10.1038/nrmicro2297.
35. Konieczna, P.; Schiavi, E.; Ziegler, M.; Groeger, D.; Healy, S.; Grant, R.; O'Mahony, L. Human dendritic cell DC-SIGN and TLR-2 mediate complementary immune regulatory activities in response to Lactobacillus rhamnosus JB-1. *PLoS One* **2015**, *10*, 1–14, doi:10.1371/journal.pone.0120261.
36. Goodridge DM, U.H. Information processing during phagocytosis. *Nat. Rev Immunol* **2017**, *176*, 139–148, doi:10.1038/nri3244.Information.
37. Alhede, M.; Lorenz, M.; Fritz, B.G.; Jensen, P.Ø.; Ring, H.C.; Bay, L.; Bjarnsholt, T. Bacterial aggregate size determines phagocytosis efficiency of polymorphonuclear leukocytes. *Med. Microbiol. Immunol.* **2020**, *209*, 669–680, doi:10.1007/s00430-020-00691-1.
38. Kapetanovic, R.; Nahori, M.A.; Balloy, V.; Fitting, C.; Philpott, D.J.; Cavaillon, J.M.; Adib-Conquy, M. Contribution of phagocytosis and intracellular sensing for cytokine production by Staphylococcus aureus-activated macrophages. *Infect. Immun.* **2007**, *75*, 830–837, doi:10.1128/IAI.01199-06.
39. Kramer, M. Impaired dendritic cell function in Crohn's disease patients with NOD2 3020insC mutation. *J. Leukoc. Biol.* **2006**, *79*, 860–866, doi:10.1189/jlb.0805484.
40. Acedo-Félix, E.; Pérez-Martínez, G. Significant differences between Lactobacillus casei subsp. casei ATCC 393 T and a commonly used plasmid-cured derivative revealed by a polyphasic study. *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 67–75, doi:10.1099/ijs.0.02325-0.
41. Vinogradov, E.; Sadovskaya, I.; Grard, T.; Chapot-Chartier, M.P. Structural studies of the rhamnose-rich cell wall polysaccharide of Lactobacillus casei BL23. *Carbohydr. Res.* **2016**, *435*, 156–161, doi:10.1016/j.carres.2016.10.002.