SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Phagocytic efficiency of human monocyte-derived dendritic cells is affected by the peptidoglycan modifications in *Lactobacillus casei* BL23 and tumor-derived soluble factors

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DEBRECEN, 2023

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10.00 a.m. on 16th of June 2017

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The PhD Defense takes place at Department of Internal Medicine, Building A, University of Debrecen, at 12.00 p.m. on 17th of April 2023.

1. INTRODUCTION

Loads of external and internal factors challenge daily the host throughout life. According to the nature of the exposing factors which can be harmless such as microbiota and harmful like pathogens and transformed self-cells, the immune system needs to react with a proper response against them.

Since dendritic cells (DCs) have a unique ability to migrate to the secondary lymphoid organs and activate T cells, they form a bridge between innate and adaptive immunity and can shape the developing T cell responses according to the nature of the antigens. Therefore, DCs in the periphery have to make direct contact with the antigens in which different endocytic processes play a central role.

Symbiotic bacteria such as Lactobacillus and their metabolites interact with the host cells in different ways. The direct interaction between the bacterium and the host cells often relies on the bacterial peptidoglycan (PG) layer. Alterations in the PG structure by enzymes such as acetyltransferases or peptidoglycan hydrolases (PGH) largely affect their modulatory potentials.

Similarly, transformed cancer cells manipulate the immune responses against themselves. Since DCs have a complex immunological activity, modifying their differentiation and functions by the cancer cells via secreted factors seems to be crucial for the evasion of DC-mediated immune responses.

Considering the complicated relationship between the microbiota or cancer cells and the DCs, the potential mechanisms are under intense research. However, the bacterial species-specific actions and the unique immunomodulatory properties of the different cancer cells present a great challenge in these studies.

1.1. General aspects of the microbiota

The emerging importance of microbiota can be well-characterized by the fact, that over the past decade $1.7 billion was spent on human microbiota research regarding its medical aspects. These microorganisms reside on the human skin and inside the body on the mucosal surfaces, including in the oral cavity, gastrointestinal (GI) tract, the airways, and reproductive tract. Among these tissues, the GI tract harbors the most abundant microbiota.

Microorganisms established different types of relationships with the host. Symbiotic species live in mutualism or commensalism with the host, whilst pathobionts are potentially pathogenic species and can cause diseases in immunocompromised people or in dysbiosis when healthy microbiota composition is disrupted, for example upon excessive antibiotic treatments.

It has long been demonstrated that symbiotic microorganisms exert many beneficial effects on the host including maintaining and reinforcing the intestinal epithelium integrity, restraining the adhesion of pathogens, and assisting in the digestion resulting in energy accumulation, vitamin and metabolite production. Additionally, they support immune system development and manipulate immune responses.

1.2. Microbiota contribution to the homeostasis

The host's encounter with the microbiota-derived metabolites begins already in the womb; maternal microbial factors shape the fetus' immune system and affect the development of potential pathological conditions later in life, such as asthma. The pioneer species first colonizing the newborn's skin and mucosal surfaces depend on the type of delivery. Adult microbiota composition is relatively stable as compared to children's microbiota. However, with changing the diet, increasing pollution and antibiotic use adult's microbiota can also be drastically altered.

Radical disruption of the diversity and composition of the individual microbiota i.e., dysbiosis can lead to serious consequences implying the importance of the symbiotic microbes in the host's life.

Microbiota-derived molecules serve as ligands for numerous pattern recognition receptors (PRRs) and locally act on intestinal epithelial cells (IECs) and immune cells or influence the functions of extraintestinal organs. It is well-known that muramyl-dipeptide (MDP) the smallest active fragment of the Gram+ bacterial PG is a ligand for the cytosolic NOD2 receptor highly expressed by Paneth cells and $CD11c^+$ myeloid cells controlling the microbiota composition and inflammatory cytokine secretion.

1.3. Antigen sampling by $CD11c^{high}$ intestinal DC subpopulations

Symbiotic bacteria have to make direct or indirect interactions with host cells to exert their modulatory effects.

Acquisition of the antigen by the DC subpopulations can take place by multiple mechanisms. CX_3CR1^+ DCs are able to extend their dendrites between the IECs and directly sample the luminal content of the gut. Similarly, it has been demonstrated that $CD103^+$ DCs translocate to the epithelium and sample antigen from the lumen via their dendrites in the ileum and activate T cells. $SIRP\alpha^-$ conventional DCs can cross-present viral antigens derived from apoptotic epithelial cells and migrate to the T-cell zone in the mesenteric lymph node (MLN). Additionally, indirect mechanisms assist in the antigen sampling. McDole et al. reported that

goblet cells deliver low molecular weight soluble antigens to the underlying $CD103^+$ DCs. Furthermore, IgG-coated antigens can also pass through the epithelial cells via the neonatal Fc receptor. Non-migratory CX_3CR1^+ macrophages (Mfs) can also hand over acquired antigen to $CD103^+$ DCs via gap junction transfer and induce oral tolerance.

1.4. Ingestion processes after antigen sampling by the DCs

Tissue-resident, immature DCs (IDCs) are equipped with the machinery required for all known endocytic processes: phagocytosis, macropinocytosis for the large particles and dynamin-dependent and independent endocytosis for small antigens. During the DC maturation, endocytic activity rapidly decreases, and the antigen processing and presentation are becoming more prominent.

Phagocytosis and macropinocytosis play a role in the engulfment of particles larger than 500 nm size such as bacteria and apoptotic cells. Moreover, both processes are actin-dependent, but their regulation and the involved receptors and molecules are greatly different. The phagocytic process is highly regulated and initiated by the recognition of the opsonized material via cell surface receptors, generally Fc, complement receptors and PRRs like Dectin-1, and proceeds with the engagement of other receptors inducing the membrane distortion and phagosome generation. Macropinocytosis or "cell drinking" is constitutively active in professional antigen presenting cells (APCs) thus, in the absence of threats, macropinocytosis assists in maintaining the DC-dictated peripheral tolerance via self-peptide presentation. It involves the engulfment of extracellular fluid together with particles like bacteria. Under inflammation or in the presence of infection, DCs mostly rely on macropinocytosis in the antigen uptake. Moreover, it has been reported that macropinocytosis is necessary for MHCII-mediated antigen presentation by the DCs.

Particles smaller than 500 nm like bacterial cell wall components can be engulfed by macro- or micropinocytosis and other endocytic processes, like clathrin- or caveolin-dependent endocytic processes which can be dynamin-dependent or independent. It has been reported that MDP entry in the Mfs uses a clathrin- and dynamin-dependent endocytic pathway, which is necessary for NOD2 signaling. In a study carried out this year, Popescu et al. have demonstrated that the internalization of the *Bacillus anthracis* PG polymer and larger fragments of various sizes takes place mostly via actin-dependent pathways. However, dynamin-dependent endocytosis also contributes to the engulfment by 20-30 %. In another investigation, it has been shown that epithelial cells are able to use two independent pathways for the uptake of cell wall

purified from *Streptococcus pneumoniae*. These studies demonstrate that the internalization pathways can vary between human cells.

1.5. Structure and composition of the PG

PG is the largest constituent of the Gram+ bacterial cell wall, consisting of long-glycan strands with repeating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) linked with β-1,4 bonds. The NAM and NAG residues can be O-acetylated or N-deacetylated. The long strands are cross-linked by short peptide chains. The most common peptide sequence is composed of L- and D-amino acids in the following order: L-alanine, D-glutamine, L-lysine, D-alanine, D-alanine. The third amino acid in the rod-shaped Gram+ and all Gram- bacteria can be alternatively meso-diaminopimelic acid or L-ornithine. The first L-alanine forms a lactyl bond with the carboxyl group of NAM. However, the structure and the composition of the mature PG are strikingly species-specific.

Microbiota-derived PG or PG muropeptides present various favorable effects on the host. Previously, it has been reported that microbiota PG fragments were released systemically from the gut lumen into the circulation in the absence of infection and were detected even in the bone marrow. The transferred PG components were bioactive because they could induce NOD1-dependent NF-κB activation in HEK293 cells.

Release of the PG fragments involves different mechanisms. Both pathogenic and symbiotic Gram+ bacteria constantly secrete membrane vesicles which contain PG components. Furthermore, in *Helicobacter pylori,* type IV secretion systems can transport radiolabeled PG from the bacterium to the cytoplasm of the host cells. In a study with *Lactobacillus (L.) acidophilus*, apart from NOD2, PG is recognized by many types of PRRs including NOD1, NOD-leucine rich repeat and pyrin domain-containing (NLRP) 3, NLRP1, and PG-binding proteins. These receptors sense PG fragments with various structures, sizes, and compositions, including disaccharide-di-, tri-, tetra- or pentapeptides i.e., muropeptides.

1.6. The role of PGHs in the bacterium's life

The precise cleavage of the muropeptides is dependent on bacterium species-specific enzymes so called PGHs which contribute to the PG synthesis, turnover, recycling, and degradation. Due to the action of the PGHs, muropeptides are generated which can be utilized in the PG turnover and recycling or released to the environment.

These enzymes perform a variety of functions: they control the separation of the daughter cells by cleaving the septum during proliferation. The absence of AmiA, AmiB, AmiC enzymes

causes filamentation of *Escherichia coli*. Furthermore, they determine the shape and size of the bacterium; during growth PGHs ensure that the PG polymer can stretch around the bacterium and provide space for the integration of new PG. In *Bacillus subtilis*, single inactivation of the cwlO or lytE endopeptidases causes shorter bacterial size, whereas the double mutant is non-viable. They also control the length of the glycan strands and the degree of the peptide cross-linking. These physical features influence the rigidity of the cell wall which can mean the survival of the bacterium in unfavorable conditions. In addition, it has been demonstrated that *Pseudomonas aeruginosa* can deliver Tse1 and Tse3 PGHs by type VI secretion system to the enemy cell competing for the niche.

Beside their role in the degradation of PG, PGHs can be released into the environment by symbiotic bacteria. It has previously been reported that Msp1 (p40) and Msp2 (p75) enzymes from *L. rhamnosus* (renamed to *Lacticaseibacillus rhamnosus*) can prevent cytokine-induced apoptosis through stimulation of Akt protein in mouse and human IECs. Moreover, it has been observed that p40 can limit epithelial cell injury and ameliorate inflammation in colitis via activation of epidermal growth factor (EGF) receptor. Besides, EGF receptor-dependent APRIL expression in IECs is also mediated by the *L. rhamnosus*-derived p40 and leads to IgA production. The homologues of these proteins were later found in other two bacteria, *L. casei* and *L. paracasei*.

The p75 enzyme of the *L. casei* BL23 (Lc-p75) has a remarkable effect on the PG structure and bacterial morphology. Lc-p75 is one of the main PGHs with γ-D-glutamyl-L-lysyl-endopeptidase activity. In the absence of Lc-p75, the proportion of disaccharide-dipeptide and acetylated disaccharide-dipeptide PG subunits are significantly reduced in Lc-p75 mutant *L. casei*. Additionally, bacteria form long-chains because the septum between the daughter cells is not cleaved.

1.7. The Lactobacillus casei group (LCG)

Lactobacilli are Gram+, rod-shaped, facultative anaerobic bacteria belonging to the lactic acid bacteria (LAB) and efficiently colonizing the vertebrate intestinal system. The members of the LCG are widely used in daily life in dairy products or in fermented food productions. Nevertheless, they are one of the most studied probiotic bacteria due to their health-related applications.

In the human small intestines, they contribute to the colonization resistance and make direct and indirect contacts with the host cells. These interactions result in benefits to the human host

including enhanced brain functions and reduced depressive behavior, stronger barrier integrity, anti-cancer activities and immunomodulation.

1. 8. Tumor-mediated actions affect the phagocytic efficiency and functions of myeloid cells

Myeloid-derived DCs and Mfs are professional phagocytic cells that are able to engulf tumor cells and present tumor-derived antigens. One of the possible mechanisms hijacking immune recognition by tumor cells is the inhibition of their uptake by phagocytic cells. Among many phagocytosis inhibitory proteins or "don't eat me" signals, including CD200, CD47 and the newly discovered CD24 are expressed by the tumor cells, whereas their interaction partners i.e., CD200R, SIRPα and Siglec10 are expressed by the phagocytic Mfs and DCs. The engagement can mediate the block of the inflammatory response and the uptake of the tumor cells. The pattern of the expressed don't eat me signals is probably tumor specific.

Another escape mechanism of the tumor cells is modulating the differentiation and functions of immune cells bearing suppressive features. To do so, monocytes are excellent candidates due to their extremely high plasticity. In the tumor microenvironment monocytes are differentiated into tumor-associated macrophages or DCs.

2. AIMS OF THE STUDY

2.1. Part I. Analyzing the effects of human symbiont L. casei BL23 and its PG modifications on human monocyte-derived (moDC)-mediated inflammatory and effector T cell responses

The first interface between the symbiotic bacterium and host cells is the bacterial PG. Therefore, alterations in the PG structure may induce modified responses by the host's immune cells. In our work, we aspired to explore the modulatory mechanisms of PG-manipulated *L. casei* BL23 on human moDCs. We aimed to investigate:

- the inflammation-inducing potential of wild-type and Lc-p75 mutant *L. casei* BL23,
- the effect of wild-type and Lc-p75 mutant *L. casei* BL23 on the moDC's Th cell-stimulatory capacity,
- the impact of the PG's structural differences on its moDC-activating capacity,
- the importance of the morphological appearance in the induction of the moDC activation and the role of phagocytosis in the *L. casei*'s immunomodulation.

2.2. Part II. Studying the effects of different tumor cell line-derived soluble factors on the differentiation program and phagocytic potential of moDCs

Tumor-derived soluble factors influence the differentiation and functions of the DCs. However, the *in vitro* experimental systems to investigate and compare their modulatory effects are still incomplete. Our goal was to compare the effects of different adenocarcinoma- and melanoma cell line-derived soluble factors on the differentiation and function of moDCs as the followings:

- characterizing the phenotypic appearance of different tumor-promoted moDCs,
- investigating the phagocytic potential of moDCs differentiated in the presence of adenocarcinoma- and melanoma-derived conditioned media,
- finding potential correlations between the expression of different DC markers and phagocytic properties of the moDC.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

L. casei BL23 and its ΔLc-p75 mutant derivative obtained by deletion of lcabl_02770 gene encoding the Lc-p75 PGH were used in this study provided by Dr. Marie-Pierre Chapot-Chartier. They were inoculated from frozen glycerol stocks on MRS agar plates and cultured 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 fluorescent strains were cultured with 5 μg/ml erythromycin (Merck KGaA, Darmstadt, Germany) added to 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 the dilution series of liquid bacterial culture and colony counting.

3.2. Extraction of peptidoglycan

PG was extracted from *L. casei* strains as previously described with some modifications. Cells from a 500 ml exponentially growing culture (OD_{600}, 0.3) were chilled on ice and collected 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 treated with Pronase (2 mg/ml) for 90 min at 60°C, by α-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% hydrofluoric acid overnight at 4°C to remove wall polysaccharides. After centrifugation, the pellet (containing PG) was washed several times with 0.25 M Tris-HCl (pH 7.0) and deionized H_2O. The final pellet was lyophilized and stored at -20°C.

3.3. Human monocyte separation and differentiation to dendritic cells

Leukocyte-enriched buffy coats were obtained from healthy blood donors and drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen,

Hungary) in accordance with the written endorsement 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). Written informed consent was collected from the blood donors prior to blood donation; their data were processed and stored in accordance with the directives of the European Union.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using a Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Monocytes were purified from PBMCs by magnetic cell separation by applying CD14-specific antibody-coated microbeads and a VarioMACS magnet following the manufacturer's instruction (MiltenyiBiotec, Bergisch Gladbach, Germany). The autologous monocyte depleted peripheral blood lymphocytes (PBL) fraction was used as a T cell source in the ELISpot assays.

For the experiments with wild-type (WT) and Lc-p75 mutant *L. casei*, isolated monocytes were seeded at 1×10^6/ml concentration in serum-free AIM-V medium (Thermo Fisher Scientific, Waltham, MA, USA) complemented 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. Half of the medium was changed on day 2, supplemented with 100 ng/ml IL-4 and 80 ng/ml GM-CSF. When indicated, moDCs were pretreated with 15 μM Cytochalasin D (CyD, Merck KGaA) dissolved in dimethyl-sulfoxide (DMSO, Serva Electrophoresis GMBH, Germany) for 30 min to block the phagocytosis. DMSO was used as vehicle control.

Purified monocytes were seeded for four days at a density of 1.5×10^6/ml concentration in RPMI1640 medium (Merck KGaA) supplemented with 10% fetal calf serum (FCS, Thermo Fisher Scientific) and 1% antibiotic/antimycotic solution (Hyclone, Shrewsbury, MA, USA) or in the presence of tumor cell line-derived conditioned media (TU-CM) at a ratio of 1:1. The medium also contained 100 ng/ml IL-4 (PeproTech) and 80 ng/ml GM-CSF (Gentaur Molecular Products). When indicated, 0.25 μM dexamethasone (Merck KGaA) was added to the monocyte cultures generating monocyte-derived dexamethasone DCs (dexDCs) which are considered to be regulatory DCs.

3.4. Maintenance of the tumor cell lines and generation of tumor cell line-derived conditioned media

MDA-MB231 (human breast adenocarcinoma), HeLa (human cervical adenocarcinoma), HT29 (human colorectal adenocarcinoma), WM278 (primary melanoma), WM1617 (metastatic counterpart of WM278 melanoma), WM983A (primary melanoma) and WM983B (metastatic

counterpart of WM983A melanoma) were maintained in RPMI 1640 medium complemented with 10% FCS and 1% antibiotic/antimycotic solution.

Supernatants were discarded from the cell lines; cells were washed, and media were replaced by fresh ones. Cultures were rested for 48 h. TU-CM were harvested and spun at 3000 rpm for 5 min. The collected TU-CM was used in the differentiation process of monocytes to dendritic cells.

3.5. Activation of moDCs with bacteria or their derived PG fragments

On day 5 of *in vitro* moDC differentiation, bacteria were washed twice with cold PBS and were added to the moDCs at a ratio of 1 (moDC): 4 (bacteria) for the indicated incubation periods. Untreated moDCs (IDC-immature DC) were used as a control.

Purified bacterial PG samples were resuspended in deionized water and sonicated with Branson Sonifier 450 until they became clear. 10 μg/ml PG fragments were used to activate the moDCs for 24 h. Each experiment was repeated with at least three independent donors.

3.6. Analysis of the cell surface marker expression and viability of dendritic cells by flow cytometry

After a 24-h stimulation of moDCs by bacteria, cells were stained with fluorescence-conjugated monoclonal antibodies: CD83-fluorescein isothiocyanate (FITC), CD80-FITC, CD86-phycoerythrin (PE) and human leukocyte antigen- (HLA) DQ-FITC (BioLegend, San Diego, CA, US) or left unlabeled as a control.

For the TU-CM experiments moDCs were stained with anti-human CD14-FITC, CD209/DC-SIGN-PE, CD1a-FITC, CD1d-Peridinin-Chlorophyill-Protein (PerCP), CD86-PE, PD-L1-PE, HLA-ABC-FITC, and HLA-DR-FITC.

Non-specific antibody binding was blocked using heat-inactivated mouse serum. The moDC population was gated according to the forward/side scatter parameters. Fluorescence intensities were measured by an ACEA NovoCyte 2000R cytometer (Agilent, Santa Clara, CA, US); data analyzes were performed using the FlowJo vX.0.7 software (Tree Star Inc., Ashland, OR, US). Viable and nonviable cells were dissected by flow cytometry after labeling the freshly collected, non-fixed cells with 0.5 μg/ml 7-Amino-actinomycin D (7-AAD) (Merck KGaA).

3.7. Cytokine and chemokine measurements by enzyme-linked immunosorbent assays (ELISA)

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

3.8. Enzyme-linked immunospot (ELISpot) assays

Bacteria-stimulated moDCs were counted, washed and co-cultured with autologous PBL at a ratio of 1:20 for 3 days at 37°C in a 5% CO_2 atmosphere in AIM-V medium. On day 4, cells were harvested, counted, and subjected to IFNγ, IL-17A and IL-4 Ready Set Go ELISpot assays according to the instructions of the manufacturer (eBioscience, San Diego, CA, USA). Unstimulated moDCs and PBL cultures alone served as negative controls.

Identification of the cytokine release was performed by biotinylated IFNγ, IL-4, or IL-17A-specific antibodies in the presence of horseradish peroxidase enzyme (HRP) conjugated to streptavidin. Then the colorigenic substrate, 3-amino-9-ethylcarbazole (AEC Substrate Set, BD Biosciences) was added to the plates. Air-dried plates were evaluated by a computer-assisted ELISpot image analyzer (Series 1 ImmunoSpot Analyzer, ImmunoSpot Version 4.0 Software Academic, Cellular Technology Limited, Shaker Heights, OH, USA).

3.9. Phagocytosis assays by flow cytometry

To assess the differences between the uptake of WT and Lc-p75 mutant *L. casei* by moDCs, the cells and mCherry-expressing bacteria were co-incubated at a ratio of 1:4 for 3 or 24 h at 37°C in a 5% CO_2 atmosphere or on ice as a control. After the incubation periods, moDCs were labeled with allophycocyanin (APC)-conjugated anti-CD1a monoclonal antibody (BioLegend, San Diego, CA, US).

To test the phagocytic capacity of the different TU-CM-educated moDCs, the cells and mCherry-expressing WT *L. casei* were co-incubated at a ratio of 1:4 for 4 h at 37°C and 4°C. Unstimulated moDCs and dexDCs were used as controls.

Fluorescent intensities were measured using ACEA NovoCyte 3000 RYB flow cytometer (Agilent). Data were analyzed by the FlowJo vX.0.7 software. The moDC population was gated according to the forward/side scatter parameters by excluding the non-internalized bacteria. The frequency of phagocytic cells was determined as a percentage of mCherry-positive cells or according to the median values of mCherry fluorescence intensity (MFI). Similarly, the ratio of

the bacteria internalization was determined in the $CD1a^+$ and the $CD1a^-$ moDC subpopulations as a percentage of mCherry positive cells.

3.10. Confocal microscopy

To identify the localization of the internalized bacteria inside the moDCs, confocal microscopic analysis was performed. Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with 40x water immersion objective (NA 1.2) was applied to illustrate the uptake of WT *L. casei* by TU-CM-conditioned moDCs after 4 h and the phagocytosis of WT and Lc-p75 mutant *L. casei* cells by moDCs after 24 h. Z-stack images were collected at 1 μm intervals from the bottom to the top of the cells. Images, montage, and orthogonal views were visualized using ImageJ 1.53c (Wayne Rasband, National Institute of Health, USA) based on Java 1.8.1_172.

3.11. Bioinformatical analyses for generation of heatmaps and correlograms

Bioinformatical analyses were performed using R (version 4.1.3) and RStudio (version 1.4.1717). The expression data of cell surface markers and phagocytosis data of the TU-CM experiments were included in the dataset. Heatmaps were visualized with *pheatmap* function of *pheatmap* package (version 1.0.12). Values were normalized using z-score and scaled by the columns. Correlation matrices were calculated using *cor* function of the *base* RStudio package (version 4.1.3). Correlograms from correlation matrices were plotted by *ggcorrplot* library (version 0.1.3) with *ggcorrplot* function.

3.12. Statistical analyses

Plotting the data and statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software Inc., San Diego, CA, USA). Two groups were compared with paired, two-tailed Student's t test. Comparison of more than two groups was performed by One-way ANOVA followed by Tukey's post hoc test. Comparison of two independent variables was executed by Two-way ANOVA followed by Tukey's post hoc test. Results are displayed as mean ± standard deviation (SD). Differences were statistically significant at $p < 0.05$. Significance was determined as * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$ or as # $p < 0.05$; ## $p < 0.01$, ### $p < 0.001$; #### $p < 0.0001$.

4. RESULTS

4.1. Part I. Analyzing the effects of human symbiont L. casei BL23 and its PG modifications on human moDC-mediated inflammatory and effector T cell responses

4.1.1. L. casei ΔLc-p75 mutant bacteria can not alter the moDC activation program but influence the inflammatory cytokine production by moDCs

Bacterial PG, a major structural cell wall component originating from pathogens or probiotic bacteria triggers DC activation. Therefore, we investigated firstly the moDC-activating capacity of WT and ΔLc-p75 mutant *L. casei*. CD83 is a membrane-bound molecule whose expression is strongly correlated to the maturation status of moDCs. In our system, both *L. casei* strains induced the appearance of CD83 molecule on the surface of the moDCs regardless of the bacterial PGH mutation.

Next, we tested the pro-inflammatory cytokine and chemokine secretion by bacteria-exposed moDCs. In contrast to the CD83 expression, we found significant differences between the WT and mutant *L. casei* strains in the induction of TNF-α, IL-1β and IL-6 secretion by moDCs. Co-incubation with WT bacteria induced significantly higher inflammatory cytokine release from moDCs. Interestingly, the production of the inflammatory chemokine, IL-8 did not show the same pattern as the cytokines; IL-8 secretion was triggered by both *L. casei* strains at the same efficiency.

These results suggest, that Lc-p75 deficient *L. casei* with abnormal cell wall structure and morphology induces a diminished inflammatory response by moDCs.

4.1.2. Absence of Lc-p75 in L. casei does not influence the antigen presentation and co-stimulation but promotes an attenuated T-cell response by bacteria-exposed moDCs

Immature, tissue-resident dendritic cells after pathogen recognition transform into powerful antigen-presenting, mature DCs which have the characteristics essential for the activation and polarization of Th cells in distinct directions. During the migration to the secondary lymphoid organs, DCs process the pathogens and increase the expression of MHCII and co-stimulatory molecules to activate naïve Th cells. Hence, the elevated level of HLA-DQ and CD80 and CD86 co-stimulatory molecules is considered a valuable marker of the stimulated, capable-of-T-cell-activation status of the DCs. In our experimental system, both *L. casei* strains induced increased expression of HLA-DQ, CD80, and CD86 molecules on the moDC surface independently of the bacterial PGH mutation.

Besides the antigen presentation and co-stimulation, moDCs have to secrete soluble mediators to determine the direction of the naïve T-cell polarization. Similar to the inflammatory cytokines, WT bacteria caused elevated IL-10, IL-12 and IL-23 T-cell polarizing cytokine secretion by moDCs as compared to their mutant counterpart.

To get insight into how the discrepancy between the cell surface molecule expression and T-cell polarizing cytokine secretion is translated to the Th-cell responses, we cocultured bacteria-exposed moDCs with autologous PBLs. After 3 days T cells were subjected to IFNγ-, IL-17A- and IL-4 specific ELISpot assays. We found that moDCs exposed to both *L. casei* strains increased the IFNγ- and IL-17A-producing T-cell numbers and the percentage of spot-covered well areas in comparison with unstimulated moDCs. Prestimulation of moDCs with Lc-p75 mutant *L. casei* led to the reduction of IFNγ-secreting T-cell number and smaller area of spots compared to preactivation with WT *L. casei*. Similarly, a significantly lower IL-17A-secreting T-cell number and less extensive spot-covered well area were promoted by moDCs pretreated with Lc-p75-defective bacteria in comparison with WT bacteria-exposed moDCs. In contrast, a significant reduction in the IL-4-producing T-cell number and area coverage were induced by moDCs activated with *L. casei* strains compared to unstimulated moDCs regardless of the PGH mutation. These results indicate that the Lc-p75 defective *L. casei* causes dampened Th1 and Th17 responses via the attenuated T-cell-polarizing cytokine secretion by the moDCs.

4.1.3. Purified peptidoglycan extracted from WT or Lc-p75 mutant L. casei demonstrates similar moDC-activating potential

Lc-p75 deficiency causes modified composition and structure of the peptidoglycan, and aberrant bacterial morphology. Based on the above-detailed results, we raised the question whether the different moDC-activating potential of WT and Lc-p75 mutant strains is the straightforward consequence of the altered cell wall structure or indirectly connected to the transformed, long-chain forming morphology. Hence, we performed peptidoglycan purification derived from WT and Lc-p75 mutant *L. casei* strains and examined their moDC-activating capacity. Elevated pro-inflammatory TNF-α, IL-1β and IL-6 production was observed by moDCs activated with PG preparations from both *L. casei* strains. Similarly, PG from *L. casei* strains induced increased T-cell polarizing IL-10, IL-12, and IL-23 secretion by moDCs regardless of the PGH mutation.

Moreover, moDCs activated with PG derived from WT and Lc-p75 mutant bacteria expressed a higher level of CD83, HLA-DQ, CD80 and CD86 and produced more IL-8 than the unstimulated moDCs independently of the targeted Lc-p75 mutation, similarly to the results

obtained with live bacteria. Taken together, these results demonstrate that the alteration in PG structure due to the Lc-p75 mutation does not cause any differences in the purified PGs' moDC-activating capacity.

4.1.4. Modified bacterial morphology leads to defective phagocytosis by moDCs

Since the PGs purified from the WT and Lc-p75 mutant *L. casei* showed similar moDC-activating capacity, we hypothesized that the diminished moDC activation triggered by the live Lc-p75 mutant bacteria is the aftermath of their impaired engulfment by moDCs. It has previously been shown that moDCs are able to internalize different Lactobacillus strains. In line with this, moDCs were able to phagocytose fluorescent mCherry-expressing WT and Lc-p75 mutant bacteria as shown by one Z-stack image of confocal microscopic picture.

The fluorescent *L. casei* strains were used for the 3-h and 24-h phagocytosis assays, during which moDCs were co-incubated with WT and Lc-p75 mutant bacteria. After the incubation periods moDCs internalized both bacteria at 37°C, consequently becoming mCherry-positive; the frequency could be quantified by flow cytometry. We found remarkable differences in the moDCs' phagocytic capacity at 37°C and on ice, which represents active, temperature-dependent uptake of fluorescent bacteria after 3 and 24 h. Importantly, a significant deviation was observed between WT and Lc-p75 mutant *L. casei*; WT bacteria were phagocytosed with higher intensity by moDCs than Lc-p75 defective bacteria after 3 h or 24 h.

In 2007, Gogolak et al reported, that the $CD1a^+$ and $CD1a^-$ subpopulations of *in vitro* generated moDCs differ in their *E. coli*-internalizing ability. However, in our experimental system, lacking CD1a molecule on the surface of moDCs did not cause insufficient uptake of *L. casei* bacteria; $CD1a^+$ and $CD1a^-$ cells internalized WT and Lc-p75 mutant *L. casei* bacteria to a similar extent.

These results suggest that the targeted mutation of *L. casei* Lc-p75 alters the mutant bacteria's fate due to their blocked phagocytosis by moDCs regardless of the cell surface expression of the CD1a molecule. This observation can be linked to the long-chain morphology of the mutant *L. casei* strain.

4.1.5. Blocked uptake of WT L. casei induces diminished inflammatory and T-cell polarizing moDC responses

To further examine that the internalization of WT bacteria is an essential element in moDC activation, actin polymerization, which is the initial step of phagocytosis was inhibited by cytochalasin D (CyD). Firstly, we detected the engulfment of mCherry-expressing WT *L. casei*

by CyD-treated and non-treated moDCs using flow cytometry and confocal microscopy. Our results showed an extreme drop in the frequency of mCherry-positive, CyD-treated moDCs. The montage and the orthogonal views of one Z-stack image of the confocal microscopic pictures revealed that moDCs without any CyD treatment could uptake WT bacteria. In contrast, CyD-pretreated moDCs could not engulf WT bacteria. Moreover, CyD treatment caused remarkable morphological changes in moDCs, in agreement with a previous study, in which CyD treatment generated tubular protrusions and invaginations in neutrophil granulocytes.

Next, we analyzed whether the inhibition of the WT bacteria uptake had a blocking effect on moDCs' functional responses. Firstly, we tested the pro-inflammatory TNF-α, IL-1β and IL-6 secretion by moDCs. We found that CyD-pretreatment caused a highly significant decline in the pro-inflammatory cytokine secretion by moDCs. Similarly, CyD-treated moDCs produced significantly less T-cell-polarizing IL-10, IL-12, and IL-23 cytokines by moDCs, and consequently diminished IFNγ- and IL-17A-producing T-cell numbers in the moDC-T-cell cocultures.

In addition to this, we tested the effect of CyD pretreatment on cell surface marker expression and IL-8 chemokine secretion by moDCs. Previously we could not find differences in these functions between WT and Lc-p75 mutant bacteria-activated moDCs. Similarly, CyD-treated and WT bacteria-stimulated moDCs expressed CD83, HLA-DQ and co-stimulatory CD80 and CD86 molecules at a similar level as DMSO- and WT bacteria-treated moDCs. The same was true for the IL-8 production by CyD-treated and non-treated, WT *L. casei*-exposed moDCs. However, it is worth noting that CyD treatment alone also triggered significantly elevated IL-8 secretion, related to previous results obtained with human retinal pigment epithelial cells.

Overall, these results indicate that bacteria engulfment is a crucial step in WT *L. casei*-triggered moDC activation, representing that the attenuated phagocytic uptake of Lc-p75 mutant *L. casei* may lead to diminished moDC-activating capacity and consequently reduced Th1 and Th17 cell responses.

4.2. Part II. Studying the effects of different tumor cell line-derived soluble factors on the differentiation program and phagocytic potential of moDCs

4.2.1. TU-CMs alter the phenotypic properties of moDCs

It is well known that different tumor types can alter the differentiation program and T-cell-polarizing activities of myeloid cells such as DCs. Therefore, we analyzed the typical differentiation markers including CD14, CD209, CD1a, and CD1d, as well as the molecules that participated in T-cell activation (HLA-ABC, HLA-DR, CD86 and PD-L1) of TU-CM-educated moDCs.

CD14 expression is continuously decreasing during the moDC differentiation process, while CD209/DC-SIGN is increasing. We found that control moDCs showed reduced CD14 and elevated CD209 expression. However, dexDCs maintained the CD14 expression on their surface, while expressed CD209. TU-CM tendentiously upregulated the expression of CD14; significant differences were observed between HT29-CM- and WM278-CM-conditioned moDCs and the control moDCs. Interestingly, WM983A primary melanoma cell line supernatant significantly decreased the CD14 expression by moDCs as compared to dexDCs and the other primary melanoma cell line, WM278-conditioned moDCs. Besides, CD209 expression was generally reduced by TU-CM on the moDC surface in a non-significant manner. Two exceptions were found: WM983A-CM and its metastatic counterpart, WM983B-CM significantly down-regulated the expression of CD209 by moDCs.

Next, we aimed to test how the TU-CM influence the CD1a and CD1d expression of moDCs. Members of the CD1 family are readily expressed by the moDCs. In our system, TU-CM along with the dexamethasone significantly decreased the CD1a expression by moDCs; however, MDA-CM could only non-significantly attenuate the moDCs' CD1a expression. In contrast, CD1d expression was not altered by any TU-CM-exposed moDCs as compared to RPMI-moDCs. We found only one significant difference between dexDC and WM278-CM-exposed moDCs in the CD1d expression.

For presenting the antigens to the T cells, moDCs increase the expression of MHCI and MHCII molecules. Therefore, in the next step we analyzed the expression of HLA-ABC and HLA-DR molecules by TU-CM-educated moDCs. We found, that neither HLA-ABC, nor HLA-DR expression was changed to any TU-CM exposure on the surface of moDCs as compared to RPMI-moDCs. In contrast, significant differences were observed between dexDCs and WM983A-CM and its metastatic counterpart WM983B-CM-conditioned moDCs in HLA-ABC expression. In addition, dexamethasone treatment induced significantly increased HLA-

DR expression by moDCs compared to RPMI-moDCs and all the tumor cell line-educated moDCs except HT29.

Besides the antigen-presenting molecules, DCs increase the expression of co-stimulatory and co-inhibitory molecules upon T-cell activation. Hence, in the next step we studied the expression of co-stimulatory CD86 and co-inhibitory PD-L1 expression by TU-CM-exposed moDCs. CD86 expression was decreased due to dexamethasone treatment of moDCs. In HT29 and HeLa adenocarcinoma cell lines and WM278 and WM983A primary melanoma cell CM-treated moDCs, we observed a significantly higher CD86 expression than in dexDCs. PD-L1 expression was completely diminished by dexamethasone-exposed moDCs. Adenocarcinoma cell line-CM caused significantly elevated PD-L1 expression by moDCs as compared to dexDCs. Interestingly, the two primary melanoma cell lines behaved differently; WM278 induced significantly increased PD-L1 expression as compared to WM983A-derived CM by moDCs. Similarly, their metastatic counterparts showed similar effects on the PD-L1 expression to the primary melanomas.

4.2.2. Mediators derived from different tumor cell lines modify the phagocytic capacity of moDCs

Several studies demonstrate that probiotic bacteria, including different Lactobacillus species can have beneficial effects on different tumors such as human papillomavirus-induced cervical cancer, melanoma, or gastrointestinal cancers. However, little is known about how the different tumor-derived soluble mediators can influence the phagocytic activity of professional phagocytes like DCs. It was revealed in the flow cytometric measurements that moDCs internalized wild-type *L. casei* at 37°C but the phagocytosis was inhibited at 4°C. *L. casei* uptake with the highest frequency was observed by dexDCs; further significant differences were detected between the control moDCs and two melanoma cell lines, WM278- and its metastatic counterpart WM1617-educated moDCs. In contrast, any adenocarcinoma cell line did not change the *L. casei* uptake by moDCs. These results show that certain melanoma cell lines indirectly modify the *L. casei*-internalizing capacity of moDCs.

With the application of heatmaps we could make more specific statements regarding the relationships between markers/phagocytosis and the different TU-CM-educated moDCs dividing into two groups: adenocarcinoma and melanoma.

In the adenocarcinoma group, the relationship between the markers/phagocytosis and the different cell line-educated moDCs showed a diverse pattern, each cell line had its unique profile. MDA-CM moDCs had a positive link with CD1a expression and phagocytosis, while

had a strong negative relationship with CD14, HLA-DR and CD86 expression. HT29-CM-conditioned moDCs displayed high HLA-ABC, CD14 and CD209 expression but were strongly negatively connected to PD-L1 and phagocytosis. HeLa-CM-educated moDCs had an intense positive relationship with HLA-DR expression and a strongly negative one with CD1d expression.

Among melanoma cell lines, WM278-CM-moDCs had a positive relationship with every marker and the phagocytosis at various extents, while the other primary melanoma cell line, the WM983A-CM-educated moDCs had a negative link to all markers and phagocytosis, except a slight positive relation to CD86. Interestingly, WM278-CM- and its metastatic counterpart WM1617-CM-educated moDCs behaved completely differently regarding almost every marker and phagocytosis with the most prominent difference in the CD1a and CD86 expression. In contrast, the other melanoma pair, WM983A- and WM983B-CM acted similarly on the marker expression and phagocytosis by moDCs.

4.2.3. Correlation analyses reveal associations between the expression of different cell surface markers and phagocytosis by adenocarcinoma- or melanoma-derived CM-educated moDCs

To analyze the degree of the correlations between the markers and phagocytic activity, we performed correlation analyses of the two groups in R Studio.

In the group of adenocarcinoma-CM-conditioned moDCs strong positive correlations were found between the phagocytic activity and CD1a and PD-L1 expression. Interestingly, phagocytosis was negatively correlated with CD14, CD209, HLA-ABC and CD86. In addition, we found strong positive correlations between CD86 and CD14, CD86 and HLA-DR, HLA-ABC and CD14, HLA-ABC and CD209 and finally, CD14 and CD209. On the contrary, PD-L1 negatively correlated with CD1a, CD209 and HLA-ABC; CD86 and HLA-DR had a strong negative correlation with CD1a and CD1d. Moreover, HLA-ABC is anticorrelated with CD1a and CD1a with CD14.

Surprisingly, in the group of melanoma-CM-educated moDCs only positive correlations were found between the phagocytosis and cell surface marker expressions at various degrees; between CD86 and CD1a no correlation was observed.

Taken together, our results indicate that diverse tumor types are able to indirectly manipulate the phenotype and phagocytic activity of moDCs in different ways. Our results also emphasize that primary and metastatic melanoma cells act differently on the differentiation, phenotype, and phagocytic activity of moDCs.

5. DISCUSSION

The human body is continuously exposed to various external and internal stimuli, which can threaten the integrity of homeostasis such as microbiota members and transformed self-cells. Important task of the immune cells is the proper reaction to these various stimuli. Dendritic cells organize immune responses by bridging innate and adaptive immunity. DCs continuously sample the microenvironment by micropinocytosis, receptor-mediated endocytosis and phagocytosis and are able to mediate inflammatory or tolerogenic responses to different microbiota- or transformed cell-derived factors.

PG as an essential component of the bacterium plays a remarkable role in host-microbe interactions. Host cells can interact with PG or its fragments in different ways and respond to them. PG fragments can be generated by the actions of PGHs which cleave the PG macromolecule at explicit sites. Alterations in PG structure due to the absence of different PGHs can influence the host's response to the PG. In our work, we managed to generate an *L. casei* BL23 strain deficient in Lc-p75 PGH and study its moDC- and consequently the T-cell-activating potential.

Besides their prominent probiotic effects, LAB strains such as lactobacilli and their metabolites/components are able to activate dendritic cells, however, the outcomes are varied between studies. A study from Foligne et al demonstrated that *L. salivarius*, *L. rhamnosus* and *L. acidophilus* differentially activated bone marrow derived DCs. In another study, the effects of VSL#3 a probiotic cocktail containing *Bifidobacterium (B.) longum*, *B. infantis*, *B. breve*, *L. acidophilus*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum* and *Streptococcus salivarius* subsp. *thermophilus* on BMDCs were investigated at different bacterial cell numbers. It was revealed, that increasing VSL#3 bacterial load induced more mature BMDC indicated by elevated MHCII and co-stimulatory CD80, CD86, and CD40 expression. Christensen et al also demonstrated that *L. casei* subsp *alactus*-activated BMDCs did not produce IL-10, but secreted IL-12, IL-6, and TNF-α and expressed MHCII and CD86. In line with these data, our results showed that wild-type *L. casei* BL23 induced the maturation of moDCs indicated by the elevated expression of CD83, MHCII, and co-stimulatory molecules. Additionally, WT *L. casei*-activated moDCs secreted pro-inflammatory cytokines and chemokines, and T-cell polarizing cytokines as well. Moreover, WT *L. casei*-exposed moDCs induced the activation of IFNγ and IL-17A-producing T cells. These observations emphasize the strain- and condition-dependent immunomodulatory actions of different LAB strains and prove the difficulties of microbiota research.

One of many beneficial effects attributed to LAB is inhibiting the adherence, colonization, and biofilm formation of pathogens and pathobionts. The colonization resistance may be executed in many ways, including competition for nutrients and energy sources, by soluble factors like bile acids or antimicrobial peptides and proteins. In addition to this, it has previously been explored that some Lactobacillus strains have high adhesion and aggregation potential which may be efficient against pathogenic biofilm. Inactivation of Lc-p75 in *L. casei* BL23 led to the formation of long-chain, aggregated phenotype which induced significantly less inflammatory and T-cell-polarizing cytokine secretion by moDCs as compared to wild-type bacteria in our experimental system. Moreover, Lc-p75 defective *L. casei* caused reduced Th1 and Th17 responses via the attenuated T-cell-polarizing cytokine secretion by moDCs. Intriguingly, the expression of CD83, HLA-DQ, CD80, and CD86 molecules on the moDCs was not affected by the Lc-p75 mutation in *L. casei* BL23. It has been published that DCs from Crohn's disease patients bearing a Nod2fs mutation had a normal maturation indicated by the elevated level of CD80 and CD86 molecules after TLR stimulation. Besides, IL-12p70, TNF-α, and IL-10 production remained also unaffected by the DCs derived from CD patients upon TLR activation. However, NOD2 ligand MDP-exposed DCs patients failed to induce the expression of co-stimulatory molecules and the cytokine secretion was completely abrogated by the DCs. In contrast to this, in a paper in which the moDC-modulatory capacity of *L. reuteri* ATCC PTA 6475 and its CmbA, a mucus-binding protein inactivated counterpart was studied, it was found that wild-type and CmbA-mutant *L. reuteri* ATCC PTA 6475 induced the expression of CD83, CD80, CD86, and HLA-DR at a similar level, while WT *L. reuteri* promoted more TNF-α, IL-12 and IL-1β secretion by moDCs than its mutant counterpart. Similar to the cell surface markers, Lc-p75 mutation in *L. casei* BL23 did not influence the IL-8 production by moDCs in our experiments. In good agreement with this, in a study executed with human macrophages it was found that nuclear IκBα inhibited the release of TNFα, IL-1β, and IL-6 upon LPS stimulation, however, IL-8 production remained unaffected. Another study demonstrated that the inactivation of Regulator of G-protein Signaling 16 (RGS16) in THP-1 cell line induced elevated TNFα, IL-1β and IL-6 production but not IL-8. These results suggest that the regulation of the IL-8 gene expression differs from the inflammatory cytokines. Indeed, it has recently been published that ERK3 controls the IL-8 production by gut epithelial cells, but there is no available information about its contribution to the regulation of cytokine production.

To further analyze, that the observed discrepancy between live WT and ΔLc-p75 mutant *L. casei* in inflammatory-inducing capacity by moDCs is the direct outcome of the altered

bacterial PG components, we investigated the moDC-activating ability of purified PG from the WT and Lc-p75 mutant strains. Based on our results, we concluded that the modified PG in ΔLc-p75 mutant could activate its receptor on moDCs with similar efficiency as the fragments derived from WT bacteria. Indeed, analyses of PG components of pathogenic bacteria and *L. acidophilus* uncovered that disaccharide units regardless of the number of peptide residues can trigger innate immune responses.

Besides the altered composition of PG, the Lc-p75 defective *L. casei* BL23 strain has significant morphological alterations consisting of the formation of long cell chains, we concluded that its low/moderate DC-activating capacity may be the consequence of its impaired phagocytic uptake.

Many studies have shown that DCs can engulf various Lactobacillus strains. In a study with porcine APCs, *L. jensenii* TL2937 was efficiently phagocytosed and increased the production of IL-1β, IL-12p70, and IL-10 by moDCs. In another study, *L. rhamnosus* JB-1 bacteria were actively internalized by human moDCs causing weak co-stimulatory molecule expression and cytokine secretion. In accordance with these data, moDCs were able to efficiently phagocytose *L. casei* BL23; however, Lc-p75 mutant strain with long-chain forming morphology was weakly engulfed by moDCs. Similarly, it has been shown that wild-type *L. reuteri* ATCC PTA 6475 and ATCC 53608 strains were actively phagocytosed, but their CmbA and mucus-binding proteins deficient counterparts were less engulfed by moDCs.

The phagocytic process depends on various factors, including the participating receptors, duration and affinity of the binding and the physical features of the target like the size and shape. Hence, the properties of the internalization process may guide the nature of the emerging immune responses. In our experimental system, the long-chain-forming mutant counterpart of *L. casei* BL23 induced only a limited inflammatory and T-cell-polarizing cytokine production by moDCs as compared to WT *L. casei*. The relevance of phagocytosis was also corroborated by its blockade with cytochalasin D, which caused a robust reduction in the pro-inflammatory and T-cell-polarizing cytokine secretion by moDCs, similarly to the Lc-p75 mutant bacteria-activated moDCs. This observation is in good agreement with a study obtained with human monocytes activated by *Candida albicans* and *Saccharomyces cerevisiae*. Cytochalasin D strongly decreased the pro-inflammatory and IL-10 secretion by monocytes.

Based on our findings and the literature, we can interpret our results relevant to *in vivo* situations in the following ways: 1) Direct interaction of the moDCs with *L. casei* BL23 induces an inflammatory response, which is sufficient to promote adaptive, memory Th1 and Th17 responses and at the same time drives IL-10 secretion which is crucial for the maintenance of

tolerance against mutualistic microbes in the gut. 2) Aggregated, filamentous *L. casei* BL23 due to the successful evasion of its phagocytosis by moDCs induces reduced inflammation and adaptive immune responses to avoid excessive activation of the immune cells and thus harmful reactions to the healthy tissues.

Overall, these results demonstrated that the immunomodulatory mechanisms exerted by *L. casei* BL23 are strikingly dependent on their internalization by moDCs. The uptake defines the cytokine production by moDCs, which can be translated to the inflammatory response and to stimulatory Th1/Th17-driven adaptive immune responses. The long-chain-forming Lc-p75 mutant *L. casei* bacteria, which are less efficiently phagocytosed, cause moderate moDC and T cell responses, similar to the moDCs unable to phagocytose wild-type *L. casei* due to the action of cytochalasin D.

Beside their multifaceted role in the microbiota – host interaction, DC subsets form an important part of the tumor microenvironment due to their action as APCs and contribute significantly to the antitumor or tumor-promoting responses via orchestrating the T-cell activities. In our work, monocytes were differentiated in the presence of different tumor cell line-conditioned media, and we compared their effects on the cell surface marker expression and phagocytic efficiency of moDCs.

During the differentiation from $CD14^+$ monocytes, DCs begin to express CD209/DC-SIGN and parallelly they lose the CD14 from their surface via epigenetic changes. However, $CD14^+$ DCs can be found normally in the dermis, either induced by stromal cell-derived factors or at increased frequency in many diseases including rheumatoid and psoriatic arthritis and in various types of cancers. In line with these data, melanoma, and adenocarcinoma-derived soluble factors could induce the expression of CD14 to varying degrees, while CD209 expression remained unaffected except WM983A, primary melanoma cells and their metastatic counterpart WM983B cells.

Besides CD209, CD1a is increasingly expressed on the surface of moDCs upon differentiation. Since CD1a presents lipid or glycolipid antigens to T cells, tumor cells readily reduce its expression by the DCs thereby evading the tumor antigen presentation. All the examined tumor CM in our experiments downregulated the expression of CD1a by the DCs.

Furthermore, it has been demonstrated that tumors and tumor-derived molecules decrease the capacity of peptide antigen presentation and co-stimulation by the DCs. In our experiments neither HLA-ABC, HLA-DR nor CD86 expression was altered significantly by the tumor-derived factors in comparison with non-treated control cells indicating that these moDCs retained their ability to activate T cells.

Antigen processing by APCs requires the uptake of the antigens by any endocytic pathway. Owing to the largely expressed “don’t eat me” signals by the tumor cells, they successfully evade the phagocytosis and the subsequent tumor antigen presentation by the APCs. In a clinical approach these phagocytosis inhibitory signals such as CD47-SIRPα and PD-1-PD-L1 serve as phagocytosis checkpoint therapies against different tumor types including solid tumors and lymphomas as well. In our experimental setting, phagocytosis of the *L. casei* BL23 was not blocked by the TU-CM, and parallel they tended to elevate the frequency of PD-L1^{high} moDCs except WM983A. Despite PD-L1 negatively affects phagocytosis we found a positive correlation between phagocytosis and PD-L1 expression by both adenocarcinoma- and melanoma-CM-exposed moDCs.

It has been illustrated by multiple studies that certain bacterial strains from the microbiota including *B. longum*, *Enterococcus faecium*, Faecalibacterium species and *Akkermansia muciniphila* were linked to the increased efficiency of anti-PD-1-PD-L1 therapy in melanoma patients and in mouse experiments. Moreover, it has recently been reported that four Bifidobacterium species, *B. bifidum*, *B. longum*, *B. lactis* and *B. breve* could be detected in the colonic tumor tissues and enhanced the efficacy of the anti-CD47 immunotherapy. In both cases, DCs were the targeted cells by the microbiota members, however the precise interactions between the bacteria, tumor cells and DCs remained unclear.

Taken together, we provided a comprehensive analysis of the differentiation and functions of several tumor-derived soluble factor-exposed moDCs facilitating the *in vitro* differentiation of tumor-conditioned moDCs. Correlation analyses showed tumor type-specific actions on the differentiation and phagocytic potential of moDCs. The exerted immunomodulatory actions did not depend on the tissue origin or the stage of tumor growth.

6. SUMMARY OF THE NEW FINDINGS

"All disease begins in the gut" said Hippocrates more than 2000 years ago and it is now becoming increasingly clear that he was right, owing to the numerous studies attempted to investigate the relationship between microbiota and the human host. Many beneficial and modulatory actions are attributed to the mutualistic bacteria in our gastrointestinal tract. Disruption of their delicate balance can lead to serious diseases, including inflammatory disorders, autoimmunity and even cancer.

Considering the complex organizing functions of the different DC subsets, it is evident that these cell types are excellent targets for studying the symbiotic bacteria-exerted immunomodulatory effects, which are commonly context- and strain-dependent. In our work, we aimed to set up an *in vitro* experimental system in which the moDC-modulatory properties of *L. casei* and its altered PG can be analyzed. We found that the presence of WT *L. casei* induced more prominent inflammatory and Th1 and Th17 responses by moDCs than the mutant *L. casei*-induced ones. The morphological differences between the wild-type and mutant bacterial strains were behind these observations. Phagocytic efficiency of moDCs was impaired in the presence of the long-chain forming mutant bacteria like in the actin-polymerization-inhibited WT *L. casei*-exposed moDCs.

Phagocytosis is an essential element in the interaction between transformed cells and immune cells. Cancer cells efficiently evade the recognition and uptake by the immune cells due to their strong direct and indirect immunomodulatory actions, in which DCs are also excellent targets. Cancer cells are able to manipulate the differentiation and functions of all DC subtypes favoring the generation and maintenance of a suppressive microenvironment. However, the exerted immunomodulatory activities by the cancers are context and tumor-type specific. In our work, we attempted to compare the regulatory actions of different adenocarcinoma- and melanoma cell line derived soluble factors on the differentiation and functions of moDCs. Our results stress the differences in the impacts of distinct tumor cell-lines in the regulation of moDC differentiation and phagocytic activity regardless of origin and primary or metastatic activity.

Collectively, by targeting the moDCs by external factors such as altered microbiota members or different cancer cell-derived factors, the moDC-mediated immune responses can be fundamentally changed. These collected observations may help in the deeper understanding of the complex interactions between the innocent microbiota members and DCs, as well as the dangerous cancer cells and the DCs.

7. PUBLICATIONS

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Registry number: DEENK/2/2023.PL
Subject: PhD Publication List

Candidate: Márta Tóth
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
MTMT ID: 10059109

List of publications related to the dissertation

1. Burai, S., Kovács, R., Molnár, T., **Tóth, M.**, Szendi-Szatmári, T., Jenei, V., Bíró-Debreceni, Z., Brisco, S., Balázs, M., Bácsi, A., Koncz, G., Türk-Mázló, A.: Comprehensive analysis of different tumor cell-line produced soluble mediators on the differentiation and functional properties of monocyte-derived dendritic cells.
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Total IF of journals (all publications): 80,08
Total IF of journals (publications related to the dissertation): 9,96

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

09 January, 2023

DEBRECENI EGYETEM
Egyetemi és Nemzeti Könyvtár

8. KEYWORDS

Microbiota, Lactobacillus, peptidoglycan, peptidoglycan-hydrolase, monocyte-derived dendritic cell, inflammation, phagocytosis, T cell, adenocarcinoma, melanoma, tumor cell line-derived soluble factors, differentiation

9. ACKNOWLEGDEMENTS

One page is not enough to express my deep thanks to those, who helped me during this period of my life.

Firstly, I would like to thank my supervisor, *Prof. Dr. Attila Bácsi* for his continuous and essential support finalizing my PhD years. He always helped me with his outstanding professional knowledge and brilliant insights through the emerging troubles. His door was always open for the scientific and personal problems even the smallest ones.

I am also very grateful to my former supervisor, *Prof. Dr. Éva Rajnavölgyi*, who allowed my MSc studies and fostered my PhD and pre-doctoral studies. She introduced me to the scientific world and taught how to present our results in a precise way.

I also say thanks to the previous Head of the Department, *Prof. Dr. Tamás Bíró* for the encouragement going to the US for a short-study program.

I am very thankful to *Dr. Donna L. Farber* for allowing me to work in her laboratory in the Columbia University and *Dr. Pranay Dogra* for teaching me that the errors in the RStudio do not necessarily mean bad.

I would like to express my special thanks to my present (and forever) roommate and friend *Dr. Anett Türk-Mázló* that she was attaching me like her MSCl cells attach to the flask in every Microsoft error and making the workdays feel like home. I also would like to thank *Dr. Krisztián Bene*, my friend and former roommate for the laughing, and experiences we shared together with Anett.

I am also very grateful to the past member of the Bácsi's lab, *Dr. Brahma V. Kumar*, my friend and "American connection". Additionally, I am also thankful for the present members of the lab *Dr. Aliz Varga*, *Dr. György Hajas* and *Szabolcs Muzsai* and the honorary member of the lab, *Dr. Adrienn Gyöngyösi* for the talks about scientific and real-life problems.

I would like to thank all my colleagues, my students, and collaborators especially *Dr. Marie-Pierre Chapot-Chartier* and *Dr. Krzysztof Regulski* for fixing every problem I ever had, the support and advice. I would like to say special thanks to *Katalin Orosz-Tóth* for teaching me the precise lab work, her continuous support and encouragement starting the PhD. I am also very grateful to *Erzsike Nagyné Kovács*, *Zsuzsanna Bíró-Debreceni* and *Andrea Justyákné Tóth* for their excellent assistance and advice in the lab work.

The acknowledgement would not be complete without expressing my deep thanks to my partner, *Dr. Zsolt Czimmerer*, who taught me that everything starts with the genes. He inspires me every day and with his completely different scientific vision, he always encourages me to improve myself.

Finally, I would like to deeply thank to my “non-work” friends, my supporting family, my mother *Márta*, my father *István*, my brother *István Jr.*, my aunt *Kati* for their patience and love and our dog, *Netti,* who helped me reduce (and sometimes intensify) the stress.

The work was supported by TAMOP-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 (NKFIH PD132570, NK 101538, NN 114423, K 125337, K 125224 and K 142930). The work was also supported by GINOP-2.3.2-15-2016-00050 project. The project is co-financed by the European Union and the European Regional Development Fund.