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

Characteristics of dendritic cell-mediated immunity modulated by the unique features of normal gut microbiota members and the retinoid milieu

by Krisztián Pál Bene

Supervisor: Prof. Dr. Éva Rajnavölgyi

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

DEBRECEN, 2017

Characteristics of dendritic cell-mediated immunity modulated by the unique features of normal gut microbiota members and the retinoid milieu

by Krisztián Pál Bene, MSc

Supervisor: Prof. Dr. Éva Rajnavölgyi, PhD, DSc

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the **Examination Committee**:	Prof. Dr. Sándor Sipka, MD, PhD, DSc
Members of the Examination Committee:	Prof. Dr. János Matkó, PhD, DSc
	Dr. Judit Szabó, MD, PhD

The examination took place at the Department of Immunology, Faculty of Medicine, University of Debrecen, at 11:00 a.m. on 04th of May, 2015.

Head of the **Defense Committee**:	Prof. Dr. László Fésüs, MD, PhD, DSc, MHAS
Reviewers:	Dr. Andrea Szegedi, MD, PhD, DSc
	Dr. Zoltán Pós, MD, PhD
Members of the Defense Committee:	Prof. Dr. János Matkó, PhD, DSc
	Dr. István Szatmári, PhD

The PhD defense takes place at the Lecture Hall of Department of Anatomy, Histology and Embryology, Faculty of Medicine, University of Debrecen, at 2.00 p.m. on 16th of June, 2017.

1. INTRODUCTION

The characteristics of an ongoing antigen specific immune response are determined by specificity, diversity and memory generated by the innate and the adaptive immunity. Dendritic cells (DCs) represent a relatively rare population of innate immune cells found in peripheral tissues however, they play a fundamental role in induction and polarization of specific immune responses against microbial, tumor and other non-self antigens.

After birth, the human body, preferentially large mucosal surfaces and the skin continuously encounter microbes. Development of pathogen-induced diseases can be prevented by the immune system and also by mutualistic effects mediated by some beneficial microbes such as *Lactobacillus reuteri* which compete with pathogens to adhere to the mucus layer of the mucosa by expressing mucus-binding proteins (MucBPs). To maintain a stable *L. reuteri* population in the gut, the contribution of the immune system involves DCs being crucial for inducing inflammation, immunological memory and tolerance against beneficial bacteria however, the details of these mechanisms are not completely understood.

Diet and the gut microbiota also impacts the local and the systemic immune systems at both health and disease as the gut milieu supplies a rich source of ligands such as retinoids for nuclear hormone receptors upon food supplementation and due to the presence of the gut microbiota. DCs act as key actors in the maintenance of the balanced regulation between the microbiota and the host however, our knowledge about the interplay of molecular interactions during diet involving vitamin (Vit)-A supplementation, the presence of gut microbiota species in the course of an ongoing human immune system is still limited in both health and diseases.

To solve a part of these uncovered mechanisms, we dedicated this work to discover the importance of MucBPs in shaping the immune response to *L. reuteri*. We also analyzed how the Vit-A derivate all-trans retinoic-acid (ATRA) enriched milieu may affect the differentiation, inflammatory properties and immunogenicity of human primary DCs in the presence of normal gut microbiota members.

1.1 Different dendritic cell subtypes exhibit unique effector and/or regulatory functional activities

The term of ‘dendritic cell’ describes a heterogeneous population of cells involving conventional (cDC), plasmacytoid (pDC) as well as monocyte-derived (moDC) DCs. All these DCs derive from a $CD34^+$ hematopoietic stem cell (HSC) progenitor in the bone marrow (BM) and can differentiate to common myeloid progenitor (CMP), which has the potential to differentiate to $CD14^+$ monocytes.

To analyze the functional properties of human DCs in depth, several *in vitro* protocols have been designed and applied for the characterization of various DC types. Inflammatory and tolerogenic DCs can be generated in vitro from monocytes, BM-derived cells and embryonic stem cells in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-4, tumor necrosis factor (TNF)-α and IL-10, transforming growth factor-beta (TGF)-ß or Vit-D. MoDCs can act as potential regulators of both systemic and mucosal immune responses and also drive a wide array of cell communications at gene, protein expression and cytokine levels.

1.2 Master transcription factors mediate DC differentiation and dictate the outcome of the provoked immune response

During the differentiation of DCs, the correct regulation of genes is essential to support the function of master transcription factors such as the nuclear hormone receptor PPARγ, retinoic-acid receptors (RARs), IRF4, IRF8 and Runx3, which also may contribute to the characterization of various DC subtypes. Nuclear hormone receptors bind to endogenous or exogenous lipophilic ligands and upon microbial stimuli allow DCs to shape immune responses. These receptors can be divided into four groups: homodimer steroid hormone receptors, retinoid X receptor (RXR) heterodimers (PPARs, RXRs, RARs, Liver X Receptors/LXRs, Vit-D Receptor/VDR), dimeric and monomeric orphan receptors. At steady state, nuclear receptors are present typically in the cytosol in an inactive form bound to heat-shock proteins or in the nucleus repressed by co-repressor complexes with histon-deacetylase activity. Upon ligand binding, the cytosolic and DNA-bound receptors release the heat-shock protein and the repressor complex, respectively.

The primary source of RXR and RAR nuclear receptor ligands is the intestine and the blood, but it is also produced endogenously. The natural ligand of RARs is ATRA, while RXRs are activated by 9-*cis* RA. RARs play a pivotal role in embryogenesis, wound healing, keratinization and the development of myeloid cells, the skeletal and the nervous systems. RARα isoform regulates the gene expression of GM-CSF, GM-CSFR and CD1 proteins moreover, RARα also up regulates PPARγ through CEBPε.

PPARγ is up regulated in the presence of IL-4 and GM-CSF also confirmed by *in vivo* studies and collaborates with retinoid receptors acting as a master transcriptional regulator upon human moDC differentiation and function. In the presence of its ligands, PPARγ becomes activated in moDCs and drives the expression of genes related to endogenous RA synthesis leading to the activation of RARα by endogenous ATRA. In particular, a set of genes involved in metabolism, lipid antigen processing and presentation, invariant natural killer T (iNKT) cell activation, RA synthesis are all regulated by PPARγ and overlaps with genes that are regulated by RARα.

1.3 The origin of retinoic-acid

ATRA is a Vit-A (retinol) derivate, which can be supplemented by diet such as carotenoids and plays role in maintaining the gut homeostasis and in other organs. Vit-A is processed to ATRA by DCs, MΦ, mucosal epithelial and stromal cells and in mammals the main source of ATRA synthesis is retinol derived from the blood circulation complexed with RBP4. Retinol is transported to the cytoplasm via STR6 and binds to cellular retinoic acid-binding protein (CRBP) 1 and CRBP2 through a storage mechanism. Retinol oxidation is catalyzed by alcohol-dehydrogenase (ADH) and retinol-dehydrogenases (RDHs) to generate retinaldehyde which is served as a substrate for retinaldehyde-dehydrogenases (RALDHs) to generate RA. RALDHs have 3 isoforms encoded by the aldehyde dehydrogenase 1 family member A (ALDH1A) 1, ALDH1A2 and ALDH1A3 genes, which are expressed in DCs and MΦ differently.

1.4 Dendritic cells acquire signals from CLRs to initiate and polarize T cell responses

DCs express genetically conserved sensor proteins referred to as PRRs, which include AIM2-like receptors (ALRs), C-type lectin receptors (CRLs), NOD-like receptors (NLRs), RIG-I-like helicases (RLHs) and Toll-like receptors (TLRs), all having the capacity to sense danger-, microbe- and pathogen-associated molecular patterns (DAMPs, MAMPs and PAMPs). Different microbes activate different sets of PRRs depending on their extra- or intracellular localization of the given cell.

CLRs sense microbial and/or host carbohydrates including mannose, fucose, sialic-acid and ß-glucan. DAMPs also act as potential CLRs targets exemplified by DNGR1 playing role in DC-mediated cross-presentation by recognizing the extracellular F-actin derived from dead cells. CLRs are expressed by myeloid cells, preferentially by MΦ and DCs, but Dectin-1 is also expressed by B cells and keratinocytes. The recognition of CLRs through high affinity and high avidity interactions provoke phagocytosis and the degradation of microbes and thus modulate cell signaling pathways induced by other PPRs. Host carbohydrates differ from those of bacteria, fungi and parasites and CLRs are able to distinguish pathogens and/or tumor cells thus inducing specific adaptive immune responses by DCs. The CLRs are able to induce Th17 responses against pathogens and maintain intestinal homeostasis by controlling the resident fungal microbiota.

1.5 Dendritic cells orchestrate mucosal immune responses via targeting the gut microbiota

The mucosal DC network involves a highly heterogeneous population of cells of myeloid and BM origin, which in the course of balancing regulatory events moDCs act as potent organizers of adaptive immunity leading to the maintenance of peripheral tolerance against gut resident microbes and harmless food-derived antigens. Intestinal mononuclear phagocytes including DCs and MΦ express mucosa-associated cell surface molecules such as CX_3CR1 or CD103 however, the clear

distinction of DCs from MΦ is still critical. In the *lamina propria*, both $CD103^+$ and CX_3CR1^+ DCs can sample luminal antigens by projecting dendrites through the epithelial cell (EC) layer. The main sources of human intestinal CX_3CR1^+ DCs are circulating monocytes, which lose this marker within 24 hours. In contrast to this event, the chemokine receptor CX_3CR1 remains expressed on the cell surface of intestinal mononuclear phagocytes, and acts directly as an inflammatory and migratory cell population with high phagocytic capacity. In the mucosa, CX_3CR1^+ DCs also act as potent activators of Th17 cells. $CD103^+CD11b^+$ DCs can migrate to the draining lymph nodes in a CCR7-dependent manner and present intestinal antigens to T cells. ATRA-producing $CD103^+$ DCs also induce peripheral regulatory T cell (Treg) differentiation in the presence of commensal bacteria and thus help to ensure gut integrity.

1.5.1 Mucosal immunity constantly produces and maintains effector and regulatory T-lymphocyte populations

The special conditions presented by the mucosa leads to stable effector, regulatory and memory T cell populations specific for the gut microbiota and food-derived antigens. The activation of these T cells is mediated by the professional antigen presenting cells which collect luminal antigens and present them to the naïve T cells in the Peyer's patches and mesenteric lymph nodes. Effector T-lymphocytes such as Th1, Th17 and Tfh cells are abundant in the gut mucosa and produce IFNγ, IL-17, IL-21 and IL-22 cytokines acting on the local innate and adaptive immune cells and also on the gut epithelia via cytokine receptors. To avoid tissue damage during the activity of effector Th cells in the presence of luminal antigens, Treg cells are also activated and produce anti-inflammatory IL-10 and TGF-ß.

Mucosal Treg cells are produced in the thymus and in the periphery including mucosal tissues. Depending on the cytokine milieu and the immunological context, the differentiation of peripheral Treg cells from naïve Th cells can be initiated by the presence of host-derived ATRA and the commensal-derived SCFAs such as butyrate in the gut. Butyrate facilitates histone H3-acetylation in the promoter and conserved non-coding sequence regions of the FoxP3 locus playing role in Treg differentiation. Interestingly, ATRA has dual effects on naïve Th cell differentiation. The differentiation of Treg cells can be induced in the presence of TGF-ß and ATRA-producing $CD103^+$ *lamina propria* DCs on a RAR-dependent manner. This mechanism helps in promoting oral tolerance, a type of peripheral tolerance against gut microbes and food. On the other hand, Vit-A and IL-6 act in concert on DC mediated Th17 cell differentiation and play essential role in antifungal immune responses in the mucosa. In summary, we can conclude that the actual environmental conditions served by the microbial community and diet potentially determine the outcome of luminal antigen-specific Th cell differentiation on a DC and RAR-dependent manner.

1.6 The human microbiota

In the human body the skin and the mucosa serve as the first line of defense, which surfaces are habitat for a complex ecosystem involving various bacteria, Archaea and fungi referred as the normal 'microbiota', which is highly specialized due to the special local environment of the mucosa. Commensals are normally confined to mucosal surfaces, but in contrast to pathogenic bacteria, they do not express virulence factors to penetrate the mucus layer or the epithelium and thus cannot disseminate throughout the body. Bacteriophages and eukaryotic viruses, protozoa and helminths are not considered members of the normal microbiota however, these microbes can also be presented in healthy human individuals as transient microbiota members.

Upon birth the human gastrointestinal (GI) tract becomes colonized by commensal microbes co-evolved with humans in a symbiotic or at least mutualistic manner together with the immune system. The resident, non-pathogenic bacteria occupy the available attachment sites of the skin and the mucosa, produce antimicrobial factors thus can inhibit the colonization of pathogenic bacteria and pathobionts leading to 'colonization resistance'. This type of protection can be further supported by probiotics containing bacteria and/or fungi and by prebiotics containing nutrients for the normal microbiota. Commensals also produce nutrients, saccharides and physiologically active SCFAs playing an important role in the maintenance of gut mucosal integrity. If the normal microbiota becomes suppressed by trauma, chemicals and drugs, the pathogens may grow out and cause disease. Moreover, in the absence of gut microbiota, metabolic and immune failures are developed and increase the risk of obesity, insulin resistance, autoimmunity, chronic inflammatory diseases and neural – behavior defects.

Enterobacteriaceae, a family of *γ-Proteobacteria* is a large group of facultative anaerobic bacteria involving *Morganella morganii* and *Escherichia coli* which are Gram-negative rods and presented mainly in the colon. Both have commensal relationships within the intestinal tract of vertebrates and considered as typical representatives of the normal gut microbiota. *E. coli var. mutabilis* (so-called *Schaedler's E. coli*) is derived originally from conventional mice and is used as a strain among of eight others in Schaedler-flora. As it has previously been described, *E. coli Schaedler* and *M. morganii* exert unique stimulatory effects on the developing immune system and are also able to induce oral tolerance in mice.

In this study two probiotic gut bacteria were tested, namely the strain of *Bacillus subtilis* 090 and *L. reuteri* both belonging to *Firmicutes*. *B. subtilis* bacteria are an obligate/facultative aerobic Gram-positive spore-forming rod bacterium from soil and the GI tract of humans and ruminants. *B. subtilis* 090 bacterial strain is widely used in veterinary practice due to the active constituents of probiotic Monosporyn™ developed at the Uzhhorod National University.

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

1.7 The normal gut microbiota member *L. reuteri* co-evolved with humans

In vertebrates, *L. reuteri* bacteria are common inhabitants of the GI tract and display remarkable host adaptation. *L. reuteri* has beneficial strain-specific properties relevant to human health by the exclusion and inhibition of growth of intestinal pathogens, maintenance of gut barrier integrity, and modulation of the host immune system at both local and systemic levels. *L. reuteri* strains have a highly stable genome thus are often used as probiotic bacteria except strain with genes encoding antibiotic-resistance. *L. reuteri* strains can produce SCFAs and reuterin, a metabolite which has antibacterial and antifungal effects. Moreover, the produced lactic-acid also enhances the inhibition of pathogen invasion.

Specific probiotic strains of *L. reuteri* have been shown to suppress intestinal inflammation in a trinitrobenzene sulfonic-acid-induced mouse colitis model through the down regulation of genes encoding IL-6 and IL-1ß cytokines in the colon. *L. reuteri* 100-23 was found to stimulate Treg development and also the oral administration of *L. reuteri* I5007, where immunomodulation was also reported in piglets resulting in increased TGF-ß and decreased IFNγ gene expressions in mesenteric lymph nodes. In humans, *L. reuteri* ATCC 55730 was shown to colonize temporarily in the stomach and the small intestine of healthy subjects inducing increased Th cell numbers in the ileum. However, modulation of cytokine production by *L. reuteri* appears to be strain-dependent as demonstrated *in vitro*. It was also showed that *L. reuteri* strains derived from human-associated clades differed in their ability to modulate human cytokine production by myeloid cells. The bacterial molecule(s) responsible for down regulating TNF-α in APCs have not been identified so far, but appear to be strain-specific. Recently, it was suggested that the cell-surface proteins may play a role in regulating the immunomodulatory properties of lactobacilli.

1.8 Bacterial mucus binding adhesins

The human intestine is covered by a mucus layer which allows the penetration of microbiota members and live as commensals or symbionts. Adhesion to host tissues is a required first step of bacterial colonization mediated by multiple factors including bacterial flagella, fimbriae, pili, blood group antigen adhesin and cell surface adhesion proteins. Typical adhesins, such as pili and fimbriae as well as other cell surface proteins of enteropathogens have extensively been studied. *L. reuteri* expresses surface MucBPs in a strain dependent manner and promotes the evolution of *L. reuteri* species primarily as gut organisms. The most well characterized MucBP is the extracellular 353-kDa MUB protein derived from the *L. reuteri* ATCC 53608 variant. These adhesion proteins not only contribute to adhesion and probiotic effects, but also correlate with the expression of host-clade MucBPs. *L. reuteri* adhesins include MucBPs such as MUB and CmbA, and serine-rich-repeat proteins. Upon recognition by LP DCs is still uncovered, therefore our primary aim was to analyze the means how different MucBPs can modify *L. reuteri* specific immune responses and the molecular interaction inducing these effects in DCs.

2. AIMS OF THE STUDY

Aim 1. Analyzing the impact of MucBPs expressed by the common microbiota member *Lactobacillus reuteri* on moDC-mediated inflammation and effector Th cell responses

As discussed before, MucBPs allow the penetration of *L. reuteri* to gut epithelia and also may play role in regulating the immunomodulatory properties of gut-colonizing *L. reuteri*. However, the detailed molecular mechanisms by which *L. reuteri* may interact with moDCs to modulate immune responses and promote mucosal homeostasis are not well understood. The goal of this study was to:

- measure the uptake of wild type and MucBP-mutant *L. reuteri* strains by moDCs;
- follow up the inflammatory and Th polarizing potential of *L. reuteri* strains in moDCs lacking MucBP as compared to the wild type strains;
- identify the inflammatory potential of purified MucBP in moDCs;
- characterize the role of C-type lectin receptors in moDC – *L. reuteri* interactions.

Aim 2. Study the effects of the vitamin-A derivate ATRA on moDC differentiation and T cell polarizing capacity in response to gut commensal bacterial species

We hypothesized that human monocytes migrating from the blood to the intestinal *lamina propria* have access to the special microenvironments conditioned by growth factors, metabolites, exogenous and endogenous ATRA and thus take part in the coordination of immune responses raised against the targeted gut commensal species. To characterize moDCs-mediated immune responses against individual normal and probiotic gut bacteria, we aimed to:

- characterize the phenotype of moDCs differentiated in the presence of GM-CSF and IL-4 with or without of the physiologically relevant dose of ATRA;
- monitor the phagocytic, inflammatory and T cell polarizing capacity of moDCs in the presence of the commensal *E. coli Schaedler, M. morganii* and the *probiotic B. subtilis*;
- uncovering possible molecular targets of ATRA during moDC differentiation.

3. MATERIALS AND METHODS

3.1 Bacterial strains

In the first part of this study, *L. reuteri* ATCC PTA 6475 and *L. reuteri* ATCC PTA 6475 lar_0958-KO mutant (6475-KO) bacteria and *L. reuteri* ATCC 53608 and 1063N (MUB-KO) isolates were used. The *L. reuteri* strains were kindly provided by Dr. Nathalie Juge (Institute of Food Research, Norwich, United Kingdom). In the second part of this study, the experiments were performed with the commensal bacteria as follows: *E. coli var. mutabilis (Schaedler)* (O83:K24:H31), *M. morganii* and *B. subtilis* 090 which strains were provided by Nadiya Boyko (Uzhhorod National University, Uzhhorod, Ukraine).

3.2 Reagents

Anti-human Dectin-2 IgG was from InvivoGen, 31400 Toulouse, France. The anti-DC-SIGN/CD209 mAb (clone 120507) was from Abcam, Cambridge, UK. The ATRA, the selective RARα antagonist BMS-195614 (BMS614) and the anti-hß-actin mAb were from Sigma-Aldrich, Schnelldorf, Germany. The anti-hIRF4 antibody was from Cell Signaling Technology, Inc. (Trask Lane, Danvers, MA, USA).

3.3 Human moDC cultures

Leukocyte-enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service of the University of Debrecen, Faculty of Medicine (Hungary) and from the Regional and Institutional Research Ethical Committee of the University of Debrecen (DEOEC RKEB/IKEB 3855-2013). Written, informed consent was obtained from blood donors prior to blood donation followed by processing and storing according to the directives of the European Union. Peripheral blood mononuclear cells (PBMCs) were separated by standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation and anti-CD14 microbeads, according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). After separation on a VarioMACS magnet, 96–99% of the cells were shown to be $CD14^+$ monocytes, as measured by flow cytometry. Isolated monocytes were cultured for 2 days in 12-well tissue culture plates at a density of 5.0×10^5 cells/ml in serum-free AIM-V Gibco's medium (Thermo Fischer Scientific, Waltham, MA, USA), supplemented with 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium) and 100 ng/ml IL-4 (PeproTech EC, London, UK). The cells were differentiated in the

presence or absence of 1 nM ATRA followed by a 75 min incubation period with or without 1 µM BMS614-specific RARα-antagonist at 37°C atmosphere containing 5% CO_2.

3.4 Bacterial growth for moDC activation

Selected gut commensal bacteria were grown in 2% Lysogeny Broth (LB) medium (Serva Electrophoresis GmbH, Heidelberg, Germany) for overnight at 37°C. *L. reuteri* strains were grown in Difco™ de Man, Rogosa & Sharpe (MRS) broth medium for 18 hours (BD BioSiences, Franklin Lakes, NJ USA). Bacterial suspensions were washed with 25 ml sterile phosphate-buffered saline (PBS) three times and OD_{600nm} was measured by spectrophotometry converted to cell/ml following $OD_{600nm} \times 2.5 \times 10^8$ colony forming units (CFU)/ml. Human moDC cultures were activated with lipopolysaccharide (LPS) (250 ng/ml ultrapure LPS, InvivoGen, San Diego, CA, USA) and with live commensal bacteria at a non-toxic ratio of 1 : 0.4 followed by co-culturing the cells for another 1.5 or 24 hours.

3.5 Phagocytosis assay

Live bacteria were centrifuged at 1000 x g for 5 min and washed three times in 25 ml PBS. Bacterial cell suspensions were heat inactivated at 65°C by heating for 45 min and were re-suspended in 0.25 M carbonate-bicarbonate buffer (pH 9.0). The heat-killed bacterial cell suspensions (900 µl) were stained with 100 µl fluorescein-isothiocyanate (FITC) dissolved in dimethyl-sulfoxide (DMSO) at 5 mg/ml and were rotated overnight in dark at 4°C. FITC-labeled bacteria were washed three times with cold PBS and were co-incubated with moDCs for three hours at 37°C or 4°C at a moDC : bacteria ratio of 1 : 20. MoDCs positive for FITC-labeled bacteria were analyzed by flow cytometry.

3.6 Flow cytometry

Phenotyping of resting and activated moDCs was performed by flow cytometry using anti-human CD1d-phycoerythrin (PE), CD103-FITC, HLA-DQ-FITC, PD-L1/CD274-PE (BD Biosciences, Franklin Lakes, NJ, USA), CD1a-allophycocyanin (APC), CD40-FITC (BioLegend, San Diego, CA, USA), CX_3CR1-PE, CD80-FITC, CD83-FITC, CD86-PE, DC-SIGN-FITC, CCR7-PE, CD14-PE (R&D Systems, Minneapolis, MN, USA), isotype-matched control antibodies and mouse or goat serum to prevent unspecific bindings. The ratio of Treg cells was measured by flow cytometry using anti-human CD25-PE (BD Pharmingen), CD4-FITC (BioLegend), FoxP3-APC (R&D Systems) and anti-IL-10-AlexaFluor488 (BioLegend). The viability of moDCs was determined by using 2 µg/ml 7-AAD dye (LKT Laboratories Inc., St. Paul, MN, USA) followed by a 24 h activation period with live bacteria or LPS. Fluorescence intensities were measured by

FACSCalibur (BD Biosciences), data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA).

3.7 RNA isolation, cDNA synthesis and real time quantitative PCR (RT-qPCR)

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

3.8 Measurement of cytokine concentration

Culture supernatants of moDCs were harvested 24 h after moDC activation, and the concentration of TNF-α, IL-1ß, IL-6, IL-10, IL-12(p70), IL-23(p19) cytokines and chemokine CXCL-8 was measured using OptEIA kits (BD Biosciences) following the manufacturer's instructions.

3.9 Stimulation of moDCs to measure T-lymphocyte polarization

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

staining of cells. Naïve $CD4^+$ T-lymphocytes were isolated by the Naïve $CD4^+$ T Cell Isolation Kit II, human (Miltenyi Biotec).

3.10 Stimulation of moDCs to measure iNKT cell expansion

Two-day moDCs were co-incubated with live bacteria, LPS or 100 ng/ml α-galactosyl-ceramide (KRN7000, Funakoshi, Tokyo, Japan) for 24 h in AIM-V medium. Activated and resting moDCs were washed and co-cultured with PBL for five days in AIM-V medium at a moDC : T cell ratio of 1 : 10 in 24-well plates in AIM-V medium. On day five, cells were labeled with anti-human CD3-PECy5, TCR Vα24-FITC, TCR Vß11-PE monoclonal antibodies (Beckman Coulter, Brea, CA, USA) and the double-positive iNKT population was monitored by flow cytometry using FACSCalibur.

3.11 Western blotting

Cells were lysed in Laemmli buffer, and the protein extracts were tested by antibody specific for IRF4 diluted to 1:1000; secondary antibodies were used at 1:10 000. Anti-rabbit antibody, conjugated to HRP (GE Healthcare Life Sciences, Little Chalfont Buckinghamshire, UK), was used as a secondary antibody. The SuperSignal ECL system was used for probing target proteins (Thermo Fischer Scientific). After the membranes had been probed for the target protein, they were stripped and re-probed for ß-actin.

3.12 *In vitro* detection of CLR-mediated inflammatory responses

To assess the role of CLRs in the induction of moDC-mediated inflammatory cytokine release, purified endotoxin-free MUB was coated to sterile high protein-affinity OptEIA ELISA 96-well plates (BD Biosciences) at 38.5 µg/ml concentration followed by overnight incubation at 4°C. After repeated washing steps with sterile PBS, the cells were co-incubated with moDCs in the presence or absence of 5 µg/ml anti-Dectin-2 monoclonal antibody or with an anti-DC-SIGN antibody for 1 h on ice. After the washing steps with 5 ml fresh AIM-V medium $2x10^5$ moDCs were cultured in microwell plastic plates for 24 h coated or uncoated with MUB. The concentration of TNF-α and IL-6 cytokines was measured in the culture supernatants using OptEIA kits.

In another set of experiments, moDCs were first co-incubated with blocking antibodies specific for Dectin-2 or DC-SIGN and after the washing steps with fresh AIM-V medium, the cells were co-cultured with *L. reuteri* ATCC 53608 or 1063N strains for 1.5 h at 37°C at a moDC : bacteria ratio of 1:4. The supernatants were removed by centrifugation at 1000 rpm for 5 min and the cells were washed repeatedly with 5 ml PBS at 4°C (5 times) followed by centrifugation at 1000 rpm. In a final step, moDCs were co-cultured with autologous T-lymphocytes in AIMV medium for 4 days at a

moDC : T-cell ratio of 1 : 20. The secretion of IFNγ was analyzed by ELISPOT assay. T-lymphocytes co-cultured with either resting moDCs, bacteria or culture media served as negative controls.

3.13 Statistical analysis

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

4. RESULTS

4.1 *The role of Lactobacillus reuteri-derived mucus binding adhesins in the modulation of moDC-mediated inflammatory immune responses*

4.1.1 Mucus adhesins facilitate the phagocytosis of *L. reuteri* strains in moDCs

The *L. reuteri* strains ATCC PTA 6475 and ATCC 53608 (1063) express the mucus-binding proteins CmbA and MUB, respectively on their cell surface. It is well established that the phagocytic process of bacteria can modulate the outcome of immune responses. To assess the impact of mucus adhesins on the internalization of *L. reuteri* strains by moDCs, we used the *L. reuteri* strains 6475-KO and 1063N deficient for CmbA and MUB, respectively as compared to the wild-type strains. When the FITC-labeled strains were incubated with moDCs at 37ºC for 1.5 h, the 1063N MUB-mutant was internalized ten-fold less efficiently than the *L. reuteri* 1063 wild-type strain, and the engulfment of PTA 6475 was two-fold more efficient than that of the 6475-KO CmbA mutant strain. Moreover, there were significant differences between the mutant strains with ten-fold decreased level of internalized 1063N mutant as compared to the 6475-KO strain. At 4ºC, the phagocytic process was significantly inhibited but the ratio of the adherent bacteria was higher in the wild type strains as compared to the mutants. Taken together, these results demonstrate that the cell-surface expressed mucus adhesins have the potential to promote the internalization process by moDCs.

4.1.2 Mucus adhesins modulate moDC-mediated immune responses to *L. reuteri* strains

The moDC activating potential of *L. reuteri* ATCC PTA 6475 and ATCC 53608, as well as their mutant strains, was further analyzed by measuring the cell surface expression of CD83, the co-stimulatory molecules CD80 and CD86, and the MHCII-protein human leukocyte antigen (HLA)-DR by flow cytometry. We showed that the cell surface expression of CD83 was induced by all *L. reuteri* strains however, in the presence of the 1063N mutant, CD83 expression was reduced significantly, suggesting a potential role of MUB in moDC activation. Co-culturing moDCs with *L. reuteri* strains for 24 h also induced cell surface expression of the co-stimulatory molecules CD80 and CD86 and HLA-DR. However, the co-stimulatory potential was reduced exclusively by 1063N. The level of moDC-derived pro-inflammatory cytokines and that of the T-lymphocyte polarizing cytokines was monitored upon incubation with wild-type and mutant *L. reuteri* strains. *L. reuteri* ATCC PTA 6475 induced TNF-α secretion more efficiently than ATCC 53608. The 6475-KO and 1063N mutants induced lower levels of TNF-α production than their wild-type counterparts. The *L. reuteri* 1063N mutant was further associated with lower IL-1ß, IL-6 and anti-inflammatory IL-10 production as compared to the wild-type ATCC 53608 strain. Interestingly, both *L. reuteri* wild-type

ATCC PTA 6475 and mutant 6475-KO cells induced the secretion of immunoregulatory IL-10 in moDCs to a similar extent, whereas in presence of 6475-KO the production of the inflammatory cytokines (TNF-α, IL-1ß, IL-6) was reduced. In addition, all *L. reuteri* strains tested in these experiments provoked potent IL-12 and IL-23 cytokine responses as compared to the LPS control. These results demonstrated that mucus adhesins expressed by *L. reuteri* strains have the potential to enhance pro-inflammatory cytokine production in moDCs.

Next, we addressed the question whether moDC-mediated T cell responses targeting *L. reuteri* mucus adhesin-expressing strains were able to orchestrate T-lymphocyte polarization. In this context, moDCs were first exposed to *L. reuteri* strains or to LPS, followed by co-culturing the cells with autologous T-lymphocytes for 4 days. The secretion of IFNγ and the IL-17 cytokine was monitored at a single cell level by using ELISPOT assays with IFNγ and IL-17 specific monoclonal antibody-coated plates. MoDCs activated by the ATCC PTA 6475 or ATCC 53608 wild-type strains induced IFNγ secretion by T-lymphocytes and resulted in increased IL-17 production, as compared to the immature moDC : T cell co-cultures. In contrast to this finding, the *L. reuteri* 6475-KO and 1063N mutant strains induced IFNγ producing Th1 cell activation, but were unable to trigger IL-17 polarized immune responses. Taken together, these results demonstrate that in moDCs mucus adhesins are able to up-regulate immune responses against *L. reuteri* strains.

4.1.3 The immunomodulatory properties of MUB are mediated by DC-SIGN and Dectin-2 interactions in moDC

To gain further insights into the enhanced inflammatory response induced by bacterial MUB in moDCs, we analyzed the role of selected CLRs including Dectin-2 and DC-SIGN in MUB – moDC interaction. We found that immobilized MUB activates the secretion of pro-inflammatory cytokine TNF-α and IL-6 and this effect could be prevented efficiently in moDCs by neutralizing the signal transducer function of Dectin-2 and DC-SIGN receptors by blocking antibodies against DC-SIGN or Dectin-2, while IL-1ß and IL-12 cytokine production could not be detected.

Interestingly, we also found that the uptake of *L. reuteri* was not eliminated in moDCs in the presence of DC-SIGN or Dectin-2 antibodies. To confirm the involvement of Dectin-2 and DC-SIGN in the activation of moDC-mediated T-cell differentiation, blocking antibodies targeting these cell surface CLRs were used in the presence of *L. reuteri* ATCC 53608 and 1063N. The Th1 polarizing capacity of moDCs induced by the wild-type strain was reduced in the presence of anti- Dectin-2 or anti-DC-SIGN antibodies as compared to CLR-unblocked moDCs. As expected, the Th1 polarizing capacity of the mutant strain 1063N was not affected by blocking DC-SIGN and Dectin-2 on moDCs. These results clearly demonstrated that the interaction of Dectin-2 and DC-SIGN with MUB

presented by *L. reuteri* ATCC 53608 is crucial to acquire Th1-cell differentiation upon bacterial stimulation of moDCs.

4.2 *Characterization of gut commensal bacteria based on their effects on dendritic cell-induced immune responses in the presence or absence of retinoids*

In this study, we observed that unique microbial features represented by the normal microbiota member *L. reuteri* bacteria expressing MucBPs impact the outcome of moDC-mediated inflammatory and regulatory immune responses. We further characterized how other core members of the gut microbiota are able to polarize the immune response by moDCs. The primary goal of this study was to characterize a selected set of the normal gut microbiota including *E. coli Schaedler*, *M. morganii* from *Proteobacteria* and probiotic *B. subtilis* 090 from *Firmicutes,* all with individual immunogenic and/or modulatory potential during moDC maturation and T-lymphocyte polarization. Moreover, we also examined the impact of ATRA, a metabolite produced in the intestine upon commensal-induced immunity, which may act on moDC differentiation and functions.

4.2.1 The expression profile of master transcription factors and the cell surface expression of CD1 glycoprotein receptors differ in human moDCs

We found that in the presence of 1 nM ATRA monocytes generated in the presence of GM-CSF and IL-4 induced the differentiation of monocytes to moDCs within 2 days accompanied by the increasing expression levels of genes encoding the nuclear hormone receptor RXRα as well as its dimerization partners RARα and PPARγ in line with the ALDH1A2 gene playing role in the regulation of RA production in moDCs. In the absence of ATRA, the CD1d gene was expressed in moDCs at low levels, but the CD1d gene transcripts and the cell surface expression of the translated protein was up regulated, while in ATRA-conditioned moDCs the cell surface expression of CD1a decreased. Moreover, on day 2 and 3 the differentiation of moDCs could be re-programmed to induce CD1d while inhibited CD1a expression, respectively. Importantly, the cell surface expression of the DC-SIGN remained constant at these conditions, while ATRA maintained the expression level of CD14 suggesting a decelerated differentiation phase of moDCs.

DCs can also be classified according to the expression levels of the transcription factors guiding both DC differentiation and re-programming. In this human *in vitro* model system, we also found that moDCs express CD11b independent on the presence of ATRA. Interestingly, ATRA was able to down modulate the gene expression levels of IRF4, while up regulated the cell surface expression of CD103. Collectively, these results demonstrate that nanomolar concentration of ATRA has the potential to modify the moDC differentiation program in a coordinated manner leading to increased mRNA levels of PPARγ, retinoid-receptors, ALDH1A2 and CD1d, while the expression of CD1a

and IRF4 remained inhibited. Based on these finding, we were able to identify two separate moDC subsets exhibiting distinct phenotypic characteristics based on the expression patterns of CD1 and CD103 proteins and transcription factors. The ATRA-primed $CD1a^{-}CD103^{+}CD1d^{+}$ cells represent the $RAR\alpha^{hi}IRF4^{lo}$ subpopulation and in contrast to this combination, the $CD1a^{+/-}CD103^{-}CD1d^{-}$ cells are identified as a resting $RAR\alpha^{lo}IRF4^{hi}$ cell population.

4.2.2 Stimulation of $RAR\alpha^{lo}IRF4^{hi}$moDCs by non-pathogenic commensal bacteria polarize T-lymphocytes differently as compared to $RAR\alpha^{hi}IRF4^{lo}$ cells

In this experimental setting moDCs were activated by live *E. coli Schaedler* or *M. morganii* both of them being capable to increase the number of IFNγ producing T-lymphocytes. In contrast, the Th17 response could be activated by all of the tested species. In addition, ATRA-conditioned moDCs exhibited a completely different T-lymphocyte stimulatory potential as compared to moDCs manipulated in the absence of ATRA. In this case, the number of IFNγ secreting T cells was decreased, while that of the Th17 cells remained undetectable in the moDC – T cell co-cultures. Taken the individual features of commensal bacteria, the $RAR\alpha^{lo}IRF4^{hi}$ moDCs could be activated by both *E. coli Schaedler* and *M. morganii* leading to the differentiation of $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cells, while the $RAR\alpha^{hi}IRF4^{los}$ reduced this effect. To confirm this unexpected observation, we validated the existence of the Treg population by detecting the level of the IL-10 cytokine derived from $CD4^{+}CD25^{+}FoxP3^{+}$ T-lymphocytes co-cultured with moDCs upon the prior activation by commensal bacteria. Based on these results we were able to identify two moDC populations, which respond to gut commensal species differently, but in a strain and ATRA dependent manner. To get further insight how microbiota species guide immune responses of distinct characteristics, we sought to analyze the impact of selected bacterial strains driving the differentiation and functional activities of moDCs by using various experimental approaches.

4.2.3 Commensal bacteria modulate the cell surface expression of CD1, CX_3CR1 and CD103 proteins in an ATRA-dependent manner

To test how gut microbiota strains may act on human moDC differentiation at *in vitro* culture conditions mimicking the intestinal milieu, the cells were exposed to stimulatory signals such as LPS and selected live commensal bacteria. At this experimental setting, exclusively *E. coli Schaedler* was capable to reduce the ratio of $CD1a^{+}$ moDCs indicating the potential of this commensal bacterium to reduce CD1a expression selectively, but it had no effect on CD1d expression, even though the viability of moDCs remained intact as compared to the immature cells. Interestingly, *B. subtilis* exerted an opposing effect on the cell surface expression pattern of CD1 proteins, and LPS reduced the levels of both CD1d and CD1a in moDCs, while *M. morganii* had no effect on the cell surface

expression level of CD1 proteins. These results indicated that lipid antigen presentation by moDCs via CD1a and CD1d proteins is regulated by both ATRA and the gut microbiota in a species-specific manner. Using the *in vitro* system, we also established that live commensal bacteria were able to up-regulate the cell surface expression of CX_3CR1 within 24 h, but had no effect on CD103 expression. Moreover, ATRA-conditioned moDCs down regulated the cell surface expression of CD103, but stimulation by commensal bacteria upregulated the CX_3CR1 receptor. These data altogether confirmed that in the presence of live commensal bacteria, ATRA drives the differentiation of moDCs leading to either synergistic or inhibitory directions, thus modulating the cell surface expression pattern of CD1 and that of the gut-tropic proteins.

4.2.4 The phagocytic capacity of moDCs depends on the individual characteristics of the tested bacteria and on actual environmental cues

To assess the phagocytic potential of the previously identified moDC populations, we established an *in vitro* phagocytosis assay in which the FITC-labeled heat-inactivated bacteria were exposed to 37°C for 3 hours, or were kept at 4°C as control. As expected, the engulfment of commensal bacteria could be enhanced significantly and was found to be mediated by the $RAR\alpha^{hi}IRF4^{lo}$ moDC population. When the moDCs were co-incubated with FITC-labeled bacteria at 4°C, background fluorescence intensities varied remarkably indicating differences in the individual functional characteristics of the tested commensal bacteria upon penetrating through the moDC membrane. These results altogether confirmed that in the presence of gut-derived microbial stimuli ATRA supports the differentiation of phagocytic $CD1a^{-}CD1d^{+}$ moDCs, while the expression of the gut-tropic protein CD103 is partially down modulated.

4.2.5 Activation of $RAR\alpha^{hi}IRF4^{lo}$ moDCs by commensal bacteria provokes exacerbated inflammation as compared to $RAR\alpha^{lo}IRF4^{hi}$ moDCs

Exposure of moDCs to live commensal bacteria such as *E. coli Schaedler*, *B. subtilis* or LPS for 24 h was found to increase the cell surface expression of CD83, while ATRA could down modulate this response significantly. The cell surface expression of the chemokine receptor CCR7, playing an essential role in driving DC migration to reach the secondary lymphoid organs, could also be induced in the presence of LPS or *E. coli Schaedler*, but the expression level of CCR7 remained inhibited in ATRA-treated moDC. In line with these results showing the potential of microbial components to generate mature moDCs, we detected the species-specific production of inflammatory cytokines including TNF-α, IL-1ß, IL-6 and CXCL-8 chemokine. Furthermore, *B. subtilis* was found to induce negligible pro-inflammatory cytokine production as compared to Gram-negative *E. coli Schaedler*, but the effects of *B. subtilis* could be boosted significantly upon ATRA treatment confirmed by the

increased secretion of TNF-α, IL-1ß and IL-6. We also observed that *M. morganii* induced the expression of a similar panel of moDC-derived inflammatory cytokines as compared to that of *E. coli Schaedler*. These results collectively indicate that *E. coli Schaedler* and *B. subtilis* harbor individual moDC-provoking potential, while ATRA can boost the production of pro-inflammatory mediators. In contrast to this finding, the expression level of CCR7 becomes down modulated presumably associated with its decreased migratory potential guided by the RARα^{hi}IRF4^{lo} moDC population. Based on these results we conclude that *E. coli Schaedler* acts as a potent inducer of inflammatory responses in moDCs accompanied by the production of TNF-α, IL-1ß and IL-6, while *B. subtilis* is less efficient to trigger TNF-α and/or IL-1ß secretion.

4.2.6 *E. coli Schaedler* and *B. subtilis* increase the T-lymphocyte stimulatory and polarizing capacity of moDCs but ATRA interferes with this effect

When moDCs were exposed to LPS or to commensal bacteria, the cell surface expression of the MHCII and the co-stimulatory molecules was increased. In such an experimental system, the secretion of IL-10 was independent on ATRA in case of moDC activation by bacteria. More importantly, the secretion level of the Th1 polarizing cytokine IL-12 was decreased, while that of the IL-23 cytokine was enhanced significantly in the RARα^{hi}IRF4^{lo} moDC population. Interestingly, *B. subtilis* was unable to induce IL-23 secretion and the level of IL-12 also remained lower than the effect provoked by moDCs in the presence of the Gram-negative commensal bacterium *E. coli Schaedler*. Considering that the differentiation of T-lymphocytes is regulated by both co-stimulatory and inhibitory signals, the cell surface expression of known co-stimulators of T-lymphocytes were also monitored. These data altogether suggest that both LPS and gut associated commensal bacteria can induce the cell surface expression of T cell co-stimulatory and inhibitory molecules on the moDC cell surface in a strain-dependent manner, while ATRA-activated moDCs exhibit impaired cell surface expression of MHCII, co-stimulatory and inhibitory cell surface proteins.

4.2.7 Limited microbe-specific effector responses induced by RARα^{hi}IRF4^{lo} moDCs are associated with augmented inflammation that can be rescued by selective inhibition of RARα

Taken the fact that the differentiation of moDCs can be modified in the presence of 1 nM ATRA, we also confirmed that the blockade of RARα signaling by a specific antagonist resulted in the prevention of CD1d and CD103 expression, while in the presence of ATRA the cell surface expression of CD1a remained similar as control cells. The chemical antagonist of RARα i.e. BMS614 was unable to increase the cell surface expression level of CD1a on the cell surface showing that a minimal concentration of endogenous ATRA is presented by moDCs. The specific inhibition of RXRα did not show any differences between the phenotype of moDC populations.

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

Considering that the IRF4 transcription factor plays a pivotal role in setting the degree of DC-mediated antigen presentation, in a final experimental setting we described for the first time in human moDCs that the protein level of IRF4 could be up regulated by live commensal bacteria and this effect could be decreased in a RARα-dependent manner. As we expected, the decreased effector T-lymphocyte polarizing capacity of moDCs could be recovered by the selective blockade of RARα leading to strong Th1 and Th17 responses against the selected microbiota strains. Based on these results we propose that the differentiation program of moDC initiated by GM-CSF and IL-4 can readily be modulated by ATRA, and this effect is associated specifically to the RARα nuclear receptor. In line with the results showing that ATRA is able to down modulate the gene expression of IRF4 in both resting and ATRA-conditioned activated moDCs in the presence of commensal bacteria, the cell surface expression of MHCII molecules is decreased in a RARα dependent manner.

5. DISCUSSION

In this study, we focused to the underlying mechanisms involved in the recognition and processing of different species of normal gut commensal bacteria and to their ability to activate and polarize Th cells. Considering that the human commensal microbiota is personalized and exhibits high heterogeneity, it also contributes to the development of protective immune responses against pathogens that modulate the type and composition of gut resident effector Th and Treg cells. It is also well established that pathogenic microbes and pathobionts, including fungal and bacterial species, are able to induce different types of immune responses, which are modulated by both external and internal signals. However, the means how non-pathogenic gut commensal microbial species contribute to the coordination and fine tuning of immune responses in moDCs are not completely uncovered. In line with this, the primary goal of this study was to characterize a selected set of the normal gut microbiota including *L. reuteri*, *E. coli Schaedler*, *M. morganii* and *B. subtilis* all with individual immunogenic and/or modulatory potential during moDC maturation and T-lymphocyte polarization.

Probiotic lactobacilli, as part of the healthy microbiota, have been reported to exhibit several beneficial features that involve protective effects against pathogenic microbes in the gut and regulate mammalian cytokine production together with intestinal inflammation in various experimental model systems. However, it is also obvious that the immunomodulatory effects of probiotic strains such as *L. reuteri* are strain-dependent and at *in vitro* conditions support the development of unique DC activation patterns. For example, the *L. reuteri* strains ATCC PTA 6475 and ATCC PTA 5289 were reported to suppress the production of TNF-α in LPS-activated monocytic cells, whereas the *L. reuteri* strains ATCC 55730 and CF48–3A were shown to support immunostimulatory effects. Accordingly, the down regulation of pro-inflammatory cytokines such as TNF-α by *L. reuteri* ATCC PTA 6475 was also observed in primary monocyte-derived MΦ derived from children with Crohn's disease. It has been also showed that the *L. reuteri* DSM 12246 strain was a poor inducer of IL-12, TNF-α, and IL-6 in BM-derived murine DCs. It is likely that the total bacterial cell surface, soluble factors and/or exo-polysaccharides also contribute to the development of different types of immune responses induced by the *L. reuteri* strains. However, the specific mechanisms by which bacterial molecules could modulate cytokine expression in APCs remains to be identified.

The results altogether indicate that host-strain specific adhesins contribute to the immunomodulatory effects of the *L. reuteri* PTA 6475 and ATCC 53608 strains via mediating i) increased adherence and phagocytic activity in moDCs, ii) enhanced CD83 expression, iii) induced secretion of pro-inflammatory cytokines and that of the T-lymphocyte polarizing cytokines and iv) Th1-polarized immune responses characterized by IFNγ secretion and increased IL-17 cytokine

production. Both wild-type PTA 6475 and ATCC 53608 strains showed increased bacterial internalization by moDCs and elevated expression of the moDC activation markers as compared to the mucus adhesin mutant strains. However, strain-specific differences were also observed in terms of cytokine production; TNF-α was induced preferentially by moDCs activated in the CmbA expressing strain PTA 6475, while the MUB-expressing ATCC 53608 strain induced the secretion of IL-1ß and IL-6. Collectively, some strains exhibit anti- or pro-inflammatory effects, which is relevant to the fact that different microbial stimuli may affect the functional output of the cells differently. These results showed that, the immunogenicity of the *L. reuteri* strain could be increased by the MucBPs, and the mutant were characterized with a more tolerogenic immune response represented by decreased Th1 and Th17 immune responses, which in moDCs also involves the activation of the CLR-induced signaling pathways.

CLRs represent a subset of PRRs expressed by a broad spectrum of cells and recognize a diverse range of endogenous and exogenous ligands including fungi, bacteria, parasites and DAMPs driving both innate and adaptive immunity. We demonstrated that purified MUB, either in solution or immobilized, induced the secretion of inflammatory cytokines in moDCs. Furthermore, secretion of TNF-α and IL-6, but not IL-1ß or IL-12, was reduced upon the neutralization of Dectin-2 and DC-SIGN-mediated inflammatory signaling pathways. These results suggest that the enhanced level of Th1 mediated immune response provoked by the wild-type *L. reuteri* ATCC 53680 strain depends on the intimate interaction between MUB and cell surface CLRs in moDCs.

DC-SIGN signaling in DCs can induce tolerance acting as an important mechanism in the maintenance of homeostasis, which can be elicited by certain *Lactobacillus* strains including *L. rhamnosus*, *L. acidophilus*, *L. reuteri* and *L. casei*. Interestingly, *Lactobacillus* cell surface proteins, such as *L. rhamnosus* proteinaceous pili and *L. acidophilus* S-layer proteins have recently been implicated in the interplay of *Lactobacillus sp.* with DC-SIGN. The purified SpaCBA pili of *L. rhamnosus* GG was shown to interact directly with DC-SIGN in a carbohydrate-dependent manner, which and induces the expression of IL-6, IL-10 and IL-12 in DCs. The induction of these cytokines was partially dependent on DC-SIGN, and in agreement with the reported impact of pili on TLR-2 signaling, might also be modulated by DC-SIGN, as well as the ability of *L. rhamnosus* GG wild-type and mutant strains to modulate some pro- and anti-inflammatory cytokines. Our results also indicated that upon ligation to MUB, only TNF-α and IL-6 were dependent on both Dectin-2 and DC-SIGN indicating that other PRRs are also involved in this process.

As far as we know, Dectin-2 has not been implicated so far in *Lactobacillus* immunomodulation. However, previous studies on fungal pathogens showed that the activation of Dectin-2, but not Dectin-1 led to the secretion of IL-23 and IL-1ß supporting the induced polarization of Th17 cells. The *L. reuteri* 1063N mutant was less effective in inducing IL-1ß secretion, but no difference was

detected between the secretion levels of IL-23 induced by the wild-type and the mutant *L. reuteri* strains. Importantly, the prevention of molecular interactions between Dectin-2 and immobilized MUB reduced the production of IL-6, but not IL-1ß levels in moDCs indicating that IL-6 may serve as a pivotal factor in guiding *L. reuteri* ATCC 53608 induced Th17 polarization.

It has also been established that both the *L. reuteri* ATCC PTA 6475 and the ATCC 53608 strains were able to induce the secretion of cytokines and the up regulation of co-stimulatory molecules in moDCs, which in turn results in Th1 and Th17 polarization of T cells. In this context, we propose two interpretations of these data relevant to the *in vivo* situation. 1) It is possible that under homeostatic conditions, limited level of inflammation is sufficient to develop immunological memory and tolerance induction against non-pathogenic/commensal gut bacteria, which could be mediated by the limited number of bacteria and/or mucus adhesins and are able to get through the mucus layer to ensure CLR-mediated interaction with DCs as shown here. 2) Another possibility might be that at homeostatic conditions live bacteria in the mucus are kept away from the epithelial surface, and remain unable to trigger any pro-inflammatory response due to limited contact with DCs. In case the barrier function and the equilibrium of the intestinal mucosa becomes damaged and the bacteria have access to DC via interacting adhesins and CLRs, thus triggering pro-inflammatory responses. This would mediate a signal to the host indicating a defective barrier function.

Taken together, these data provide novel insights into the ongoing interactive mechanisms by which *L. reuteri* strains exhibit immunomodulatory properties via the direct interaction of *L. reuteri* host-specific adhesins and CLRs on the DC cell surface. The MucBPs expressed on *L. reuteri* bacteria may contribute to the maintenance of symbiotic relationships with the host by acting as a natural adjuvant, thus provoking antigen-specific adaptive immune responses in moDCs through the development of effector and memory T-lymphocytes with sufficient stimulatory potential.

It was also demonstrated that during the very early phase of moDC differentiation, the cells remain programmable at physiologically relevant doses of environmental cues such as in the presence of ATRA. Importantly, these events can be prevented by the selective ligation of RARα acting through its natural antagonist resulting in a moDC phenotype similar to that of the 'gold standard' of moDCs, differentiated by GM-CSF and IL-4. In a retinoid rich milieu moDCs shift the cell surface expression pattern of CD1 proteins, while in resting moDCs the expression level of CD103 remains inducible supporting the development of a mucosa-related phenotype.

We first characterized and compared the expression levels of the contributing transcription factors including IRF4, PPARγ and RARα in moDCs. Our results revealed that IRF4^{hi} moDCs act as immunogenic cells able to provoke commensal bacterium-specific Th1 and Th17 polarized immune responses. However, this pattern could be changed in case the T cells were primed with microbiota-stimulated RARα^{hi}IRF4^{lo} moDCs supporting the notion that these cells remain highly inflammatory,

lose their potential to activate autologous Th cells and also lack molecular interactions, which may play role in preventing effector T cell responses induced by commensal bacteria. This observation is further supported by previous studies showing that the increased expression level and activity of PPARγ is associated with CD1d expression and the development of tolerogenic moDCs. These data suggest that the immunogenic, tolerogenic and inflammatory capacity of DCs are critically dependent on the actual microenvironment of DC differentiation and can be linked to the activation level of IRF4. It was found in BM-DCs the Irf4-deficiency leads to impaired Treg generation moreover, the relative mRNA level of genes encoding PD-L2, RALDH2 and MHCII molecules were down modulated upon LPS stimuli while the expression level of TNF-α and IL12 genes were up regulated.

In the presence of heat-killed *E. coli Schaedler* and *B. subtilis* bacteria, the phagocytic capacity of moDCs could be facilitated by ATRA, similar to a previous work showing increased PPARγ activity in moDCs upon internalizing corpuscular antigens more efficiently than moDCs with low PPARγ activity. In line with this we also demonstrated that the stimulation of moDC with selected commensal bacteria resulted in moDCs expressing CX_3CR1 supported by ATRA and exhibiting a phenotype similar to that of the $CD11b^+CX_3CR1^+CD103^-$ mononuclear mucosal phagocytes of myeloid origin. Considering that the up regulated level of inflammatory cytokines play central role in the maintenance and/or disruption of mucosal integrity, exemplified by secreted IL-23 of both DC and MΦ origin. The increased level of secreted IL-23 could directly be associated with chronic inflammatory diseases including IBD. However, in the *lamina propria* the presence of microbiota provides signals for both CX_3CR1^+ inflammatory cells and $CD11b^+CD103^+$ DCs to produce IL-23 and induce IL-22 secretion by ILCs thus playing a critical role in promoting mucosal healing in colitis. Pro-inflammatory *lamina propria*-derived TNF-α can also exacerbate colitis through CX_3CR1^+ DCs indicating that this DC subset also plays role in the maintenance of balanced inflammatory and/or standby conditions upon gut homeostasis.

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

Our results clearly demonstrated that in resting moDCs ATRA is able to up regulate the relative mRNA levels of RARα previously confirmed also by others. In addition, we can exclude the effects of other RAR isoforms such as RARß, as it is not expressed and the expression of RARß could not

be induced in moDCs in the presence of ATRA. It has also been established that the effects of ATRA on the differentiation and the microbiota-induced stimulation of moDCs could be prevented by the selective inhibition of RARα, a transcription factor playing role in regulating moDC differentiation and guiding mucosal immune responses. It has also been found that the gut microbiota has an impact on retinoid signaling-mediated immune homeostasis transmitted by microbial metabolites such as SCFAs. Furthermore, retinoid supplementation through diet also acts on the composition of the gut microbiota and on energy metabolism of the host. For example, Vit-A deficiency causes perturbations in the gut microbiota by reducing the ratio of *Firmicutes* and *Proteobacteria* on a Myd88 and TRIF-dependent manner. It has previously been demonstrated that RA is associated to inflammatory MΦ, as patients with Crohn's disease exhibit an increased capacity to generate RALDH-derived RA, which is associated with $CD14^+$ MΦ derived from the intestinal mucosa, thus maintaining an inflammatory phenotype mediated by RARα. This group also showed that clinical samples derived from Crohn's disease patients involve both $CD103^+$ and $CD103^-$ DCs with elevated expression levels of the ALDH1A2 gene, which is undetectable in RA producing MΦ. Retinoids involving ATRA also improves the antitumor immunity in microbiota-induced colorectal cancer, as it increases the efficacy of tumor-specific Tc cells by increasing RARα-mediated MHCI expression in tumor cells.

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

6. SUMMARY

Enormous diversity of commensal bacteria determines individual functions acting on the development and functional activities of the immune system. These involve specialized DC subsets including moDCs, expression of unique PRR combinations coupled to evolutionally conserved signaling pathways, transcriptional regulation, induction of co-stimulatory molecules, secretion of cytokines and chemokines. These mechanisms can be followed up by measuring the functional activities of moDCs, which are strongly dependent on the cell cycle and the inflammatory signaling pathways during the recognition of microbial ligands. The aim of this study was to set up a sensitive in vitro model system to analyze the role of microbial and host factors playing role in moDC-mediated immune responses to normal gut microbiota members such as commensal bacteria.

Lactobacillus reuteri strains are considered as beneficial members of the gut microbiota, which were adapted evolutionally to the vertebrate gut by expressing mucus-binding proteins (MucBPs). It was an unexpected finding that intact L. reuteri lactic-acid bacteria were able to provoke potent inflammatory T cell responses, which could be enhanced by MucBPs such as CmbA and MUB proteins. Another important aspect of these results was that the inhibition of interactions between C-type lectin receptors and MUB results in decreased pro-inflammatory cytokine secretion and Th1 polarization. MucBPs expressed on L. reuteri bacteria may be implicated to contribute in the maintenance of a symbiotic relationship with the host by acting as a natural adjuvant, which is able to provoke antigen-specific immune responses in moDCs through the development of effector and memory T-lymphocytes with sufficient stimulatory potential.

It was also observed that the physiologically relevant dose of the vitamin-A derivative ATRA drives the differentiation of CD1a-CD1d+CD103+/RARαhiIRF4lo moDCs on a RARα-dependent manner, while in the absence of ATRA monocytes differentiate to CD1amedCD1d-CD103-/RARαloIRF4hi moDCs. In the presence of gut commensals, namely E. coli Schaedler, M. morganii and the probiotic B. subtilis, inflammatory responses can be induced showing that immunogenic RARαloIRF4hi moDCs polarize Th1 and Th17 cells effectively on a strain dependent manner, while RARαhiIRF4lo moDCs display a non-migratory, but tolerogenic and inflammatory subtype of DCs. Collectively, moDC-mediated adaptive immune responses targeting the gut commensal bacteria can be enhanced by natural adjuvants such as MucBPs, but in a retinoid-rich milieu it is associated with the inhibition of RARα.

7. PUBLICATIONS

UNIVERSITY OF DEBRECEN
UNIVERSITY AND NATIONAL LIBRARY

Registry number: DEENK/96/2017.PL
Subject: PhD Publikációs Lista

Candidate: Krisztián Bene
Neptun ID: NP2LRX
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
MTMT ID: 10055079

List of publications related to the dissertation

1. **Bene, K.**, Varga, Z., Petrov, V. O., Boyko, N. V., Rajnavölgyi, É.: Gut Microbiota Species Can Provoke both Inflammatory and Tolerogenic Immune Responses in Human Dendritic Cells Mediated by Retinoic Acid Receptor Alpha Ligation.
   Front. Immunol. 8 (427), 1-17, 2017.
   DOI: http://dx.doi.org/10.3389/fimmu.2017.00427
   IF: 5.695 (2015)
2. **Bene, K.**, Kavanaugh, D. W., Leclaire, C., Gunning, A. P., MacKenzie, D. A., Wittmann, A., Young, I. D., Kawasaki, N., Rajnavölgyi, É., Juge, N.: Lactobacillus reuteri Surface Mucus Adhesins Upregulate Inflammatory Responses Through Interactions With Innate C-Type Lectin Receptors.
   Front. Microbiol. 8, 321, 2017.
   DOI: http://dx.doi.org/10.3389/fmicb.2017.00321
   IF: 4.165 (2015)

Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

List of other publications

3. Pethő, Z., Balajthy, A., Bartók, Á., **Bene, K.**, Somodi, S., Szilágyi, O., Rajnavölgyi, É., Panyi, G., Varga, Z.: The anti-proliferative effect of cation channel blockers in T lymphocytes depends on the strength of mitogenic stimulation.
Immunol. Lett. 171, 60-69, 2016.
DOI: http://dx.doi.org/10.1016/j.imlet.2016.02.003
IF: 2.483 (2015)

4. Szabó, A., **Bene, K.**, Gogolák, P., Réthi, B., Lányi, Á., Jankovics, I., Dezső, B., Rajnavölgyi, É.: RLR-mediated production of interferon-beta by a human dendritic cell subset and its role in virus-specific immunity.
J. Leukoc. Biol. 92 (1), 159-169, 2012.
DOI: http://dx.doi.org/10.1189/jlb.0711360
IF: 4.568

Total IF of journals (all publications): 16,911
Total IF of journals (publications related to the dissertation): 9,86

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

26 April, 2017

ORAL PRESENTATIONS

Krisztián Bene, Davon Kavanaugh, Nathalie Juge, Éva Rajnavölgyi: The human dendritic cell-mediated inflammatory response is initiated by the molecular interaction between mucus adhesins and C-type lectin receptors. *10th Molecular, Cell and Immune Biology Winter Symposium, Debrecen, 07-08. January 2017.*

Krisztián Bene, Varga, Zs., Boyko, N., Rajnavölgyi, E.: The human dendritic cell-mediated inflammation and T-lymphocyte polarization are modulated via the specific ligation of retinoic-receptor alpha. *3rd Meeting of Middle-European Societies for Immunology and Allergology, Budapest, 01-03. December 2016.*

Krisztián Bene, Boyko, N., Rajnavolgyi, E.: Human intestinal dendritic cells dictate inflammation and T-cell polarization. *42th Symposium of Hungarian Society for Immunology, Pécs, Hungary, 15-18. October, 2013.*

Krisztián Bene, Boyko, N., Rajnavolgyi, E.: Regulation of inflammatory responses in human intestinal monocyte-derived dendritic cells. *IL-1 mediated inflammation and diabetes, Nijmegen, The Netherlands, 10-11. October 2013.*

Krisztián Bene, Gregus, A., Debreceni, Z., Boyko, N., Rajnavolgyi, E.: The response of human monocyte-derived dendritic cells to selected microbes in the presence of retinoic acid. *TORNADO Meeting, Stockholm, Sweden, 31. August-02. September 2012.*

POSTER PRESENTATIONS

Krisztián Bene, Szábit Al-Taani, Éva Rajnavolgyi: The outcome of dendritic cell mediated antiviral immune responses is affected by short-chain fatty acid butyrate. *MIT Vándorgyűlés, Velence, 2016. október 19-21.*

Krisztián Bene, Davon Kavanaugh, Sabrina Etzold, Nathalie Juge, Éva Rajnavölgyi: Mucus-binding proteins expressed by *Lactobacilli* initiate human dendritic cell-mediated inflammatory response and autologous T-cell polarization *in vitro*. *The 2nd Microbiome R&D and Business Collaboration Forum: Europe, London. May 7-8. 2015.*

Krisztián Bene, Boyko, N., Rajnavolgyi, E.: Regulation of inflammatory responses to commensal bacteria by human monocyte-derived dendritic cells. *13th International Symposium on Dendritic Cells, Tours, France, 14-18. September 2014.*

Krisztián Bene, Nadiya Boyko, Éva Rajnavolgyi: Dendritic cell-mediated T-cell polarization in the gut. *11th International Conference on Innate Immunity, Olympia, Greece, 01-06. June 2014*.

Krisztián Bene, Nadiya Boyko, Éva Rajnavolgyi: Human monocyte-derived dendritic cell-mediated inflammation in the gut microenvironment. *Semmelweis Symposium, Budapest, Hungary, 07-09. November 2013.*

Krisztián Bene, Márta Tóth, Nadiya Boyko, Éva Rajnavölgyi: Characterization of commensal bacteria based on their effects on human dendritic cell-induced T-lymphocyte polarization. *15th International Congress of Immunology, Milan, Italy, 22-27. August 2013.*

Krisztián Bene, Nadiya Boyko, Éva Rajnavolgyi: Development and regulation of inflammatory responses in human intestinal monocyte-derived dendritic cells. *8th ENII Summer School, Alghero, Italy, 27. May- 03. June 2013.*

Krisztián Bene, Andrea Gregus, Zsuzsanna Debreceni, Nadiya Boyko, Éva Rajnavolgyi: The effects of commensal bacteria on human dendritic cell differentiation and activation. *International Scientific Conference on Probiotics and Prebiotics, Kosice, Slovakia, 12-14. June 2012.*

8. KEYWORDS

Dendritic cell, inflammation, T-lymphocyte, microbiota, RARα, all-trans retinoic-acid, C-type lectins receptors, mucus binding adhesins, *Lactobacillus reuteri*, *Schaedler's Escherichia coli*, *Morganella morganii*, *Bacillus subtilis*.

9. ACKNOWLEDGEMENTS

Now, I would like to thank those people who accompanied me during this important and exciting period of my life.

First of all, I would like to say thank to my supervisor, Dr. Éva Rajnavölgyi who always supported me throughout my bachelor, master and pre-doctoral studies. She always encouraged me to achieve my ideas and also helped me to perform our common work in a scientific manner. I am really thankful that she let me to attend several international conferences where I could develop my knowledge of the fields of biology and medical sciences and take part of great scientific discussions. Moreover, she also taught me to represent, improve and manage myself on a scientific manner.

I would like to say a special thank to Dr. Attila Szabó who introduced me into the precise work in the laboratory and to thank for his selfless support and encouragement.

I am also very grateful to Dr. Tamás Bíró, who always supported me to finalize my pre-doctoral studies at the Department of Immunology and provided several useful critics.

I would like thank *all* of my dear colleagues at the Department of Immunology including the secretarial office and the laboratory assistance who always helped me in a precise and professional manner throughout my research. I am also really thankful to my smart students, Zsófia Varga (Biology BSc, Molecular Biol. MSc), Szábit Al-Taani (General Medicine) and Zsuzsa Szemere (Biol. BSc) for contributing our common works.

I am also grateful for the inspiring discussions about scientific and 'real-life' problems to Zsófia Agod, Dr. Tünde Fekete, Anett Mázló, Dr. Kitti Pázmándi, and Márta Tóth. This acknowledgement would not be complete without giving thanks to my friends who were always interested in my scientific progress and were with me during this journey. I also say thank to my dear colleague and friend Dr. Krisztina Szabó who encouraged me all the time.

Last, but not least, I must acknowledge with deep and tremendous thanks my family, specially my mother, Zsuzsa and my sister, Szabina for their love, patience and for supporting me during my gradual and pre-doctoral studies.

This research was supported by the TORNADO FP7-KBBE-2007-2A "*Molecular targets open for regulation by the gut flora: New avenues for improved diet to optimize European health*," and in the framework of TAMOP 4.2.4.A/2-11-1-2012-0001 and TAMOP 4.2.2.A-11/1/KONV-2012-0023. The work was also supported by the GINOP-2.3.2-15-2016-00050 project. The project is co-financed by the European Union, the State of Hungary, co-financed by the European Social Fund and the European Regional Development Fund.