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

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ABBREVIATIONS

ADH	-	alcohol-dehydrogenase
ALDH1A2	-	aldehyde dehydrogenase 1 family, member A2
AMPs	-	anti-microbial peptides
APC	-	antigen presenting cell, allophycocyanin
ATRA	-	all-*trans* retinoic-acid
BM	-	bone marrow
CLR	-	C-type lectin receptor
DAMP	-	danger associated molecular pattern
DC(s)	-	dendritic cell(s)
DC-SIGN	-	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
ELISA	-	enzyme-linked immunosorbent assay
ELISPOT	-	enzyme-linked immunoSpot
GM-CSF	-	granulocyte-macrophage colony stimulating factor
HLA	-	human leukocyte antigen
IBD	-	inflammatory bowel disease
IEC	-	intestinal epithelial cell
IFN	-	interferon
ILC	-	innate lymphoid cell
iNKT	-	invariant natural killer T cell
IRF	-	interferon regulatory factor
KO	-	knock-out
LPS	-	lipopolysaccharide
MΦ	-	macrophage
mAb	-	monoclonal Ab
MALT	-	mucosa-associated lymphoid tissues
MAMP	-	microbe-associated molecular pattern
MFI	-	median fluorescence intensity
MHC	-	major histocompatibility complex
MucBP	-	mucus-binding protein
NF-κB	-	nuclear factor-κB
PAMP	-	pathogen-associated molecular pattern
PBL	-	peripheral blood lymphocytes
PBMC	-	peripheral blood mononuclear cells
pDC	-	plasmacytoid DC
PPAR	-	peroxisome proliferator-activated receptor
PRR	-	pattern-recognition receptor
RA	-	retinoic-acid
RALDH	-	retinaldehyde-dehydrogenase
RAR	-	retinoic-acid receptor
RDH	-	retinol-dehydrogenase
RSG	-	rosiglitazone
RXR	-	retinoid X receptor
SCFA	-	short-chain fatty-acid
TLR	-	Toll-like receptor
TNF-α	-	tumor necrosis factor-α

CONTENTS

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. However, it has remained a mystery for a long time how these arms of immune mechanisms are connected to each other. In 1973, Steinman and Cohn discovered a small population of cells in secondary lymphoid organs with tree-like morphology (1) and defined them as dendritic cells (DC). Some years later, Steinman and Inaba demonstrated that DCs are essential to prime and polarize antigen specific immune responses (2, 3).

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 protein (MucBP). 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.

The differentiation program of DCs largely depends on the actual tissue microenvironment and is mediated by master transcription factors involving nuclear hormone receptors. The ligation of these receptors with agonists results in their activation and enables to modify the actual expression level of defined sets of genes playing role in phagocytosis, cell migration, cytokine production and antigen presentation via cooperating with *trans* and *cis* elements of the transcriptional machinery acting in DCs. Diet and the gut microbiota also impacts the local and the immune responses 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-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 a retinoid enriched milieu may affect the differentiation, inflammatory properties and immunogenicity of human primary DCs in the presence of normal gut microbiota members.

2. THEORETICAL BACKGROUND

2.1 Dendritic cells have the potential to polarize adaptive immunity

Dendritic cells represent a relatively rare population of immune cells detected in peripheral tissues however, they play a fundamental role in induction and polarization of immune responses against microbial, tumor and other non-self structures. DCs function as active phagocytes equipped with a wide repertoire of both cell surface and intracellular sensors and continuously collect samples from their microenvironment to alarm the immune system. Depending on the dose and the type of the invading microbes, DCs can rapidly be activated by different sets of pattern-recognition receptors (PRRs) and simultaneously increase the cell surface expression of adhesion molecules, chemokine receptors, co-stimulatory and antigen presenting molecules as well as the production of pro-inflammatory and T cell polarizing cytokines and chemokines. The classical paradigm of DC biology describes that in the presence of microbes, DCs lose their capacity to capture and process antigens and during time their immunogenicity is increased (**Figure 1**) leading to the polarization of T helper (Th) cells and licensing Tc cells for cellular killing. In addition to these events, immature and resting DCs are involved in the maintenance of self-tolerance by deleting autoreactive T-lymphocytes.

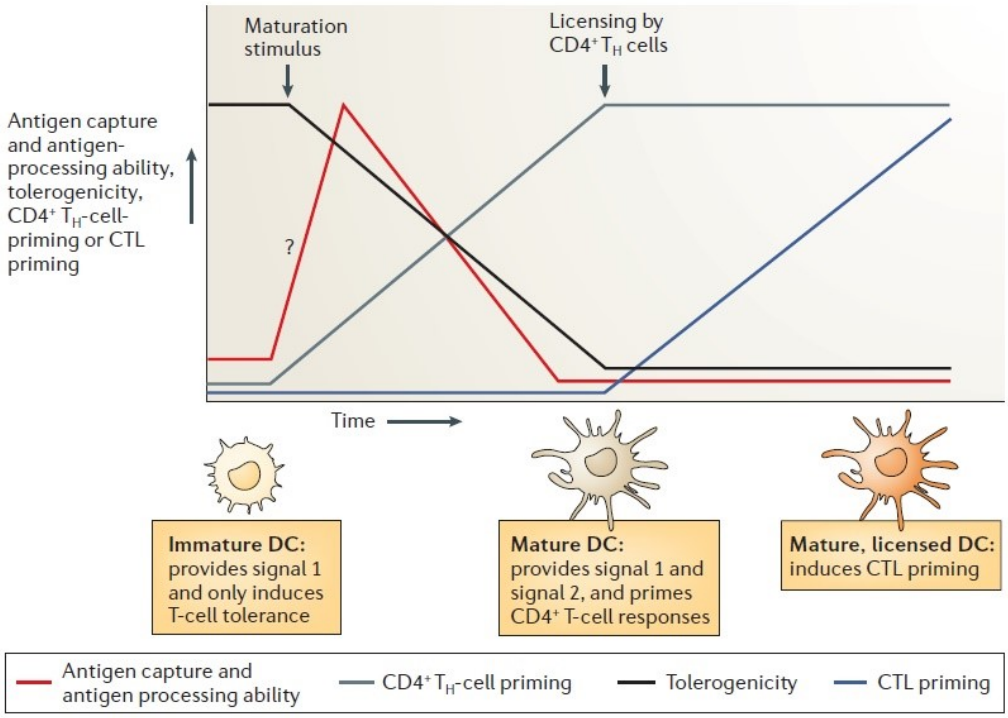


Figure 1. The classical DC maturation paradigm. *e Sousa: Dendritic cells in a mature age. Nat Rev Immunol,* ***6****, 476-483, 2006.*

The very first steps of DC activation and the induction of antigen-specific immune responses are assisted by the phagocytic and macropinocytic potential and the standby physiological activities of monocyte-derived (mo) DCs (4). These events can be further modulated by the unique characteristics of the internalized corpuscular antigens as well as by the cell surface receptor repertoire of a given cell upon engulfment. Activated DCs migrate and reach the T cell zone at the nearby lymphoid tissues and present microbe-derived antigens to T-lymphocytes (5). The specific recognition of the major histocompatibility complex (MHC)-peptide complex and the T cell receptor (TCR) expressed on the T cell surface leads to the formation of the immune synapses followed by the activation of antigen-specific T-lymphocytes. In the presence of cytokines, the activated T cell is further polarized guided by local stimuli, infectious agents or other danger signals (**Figure 2**). It is noteworthy that DCs activate naïve helper and cytotoxic T-lymphocytes (Tc) the most efficiently as compared to other professional antigen presenting cells to drive T cell priming and licensing (5).

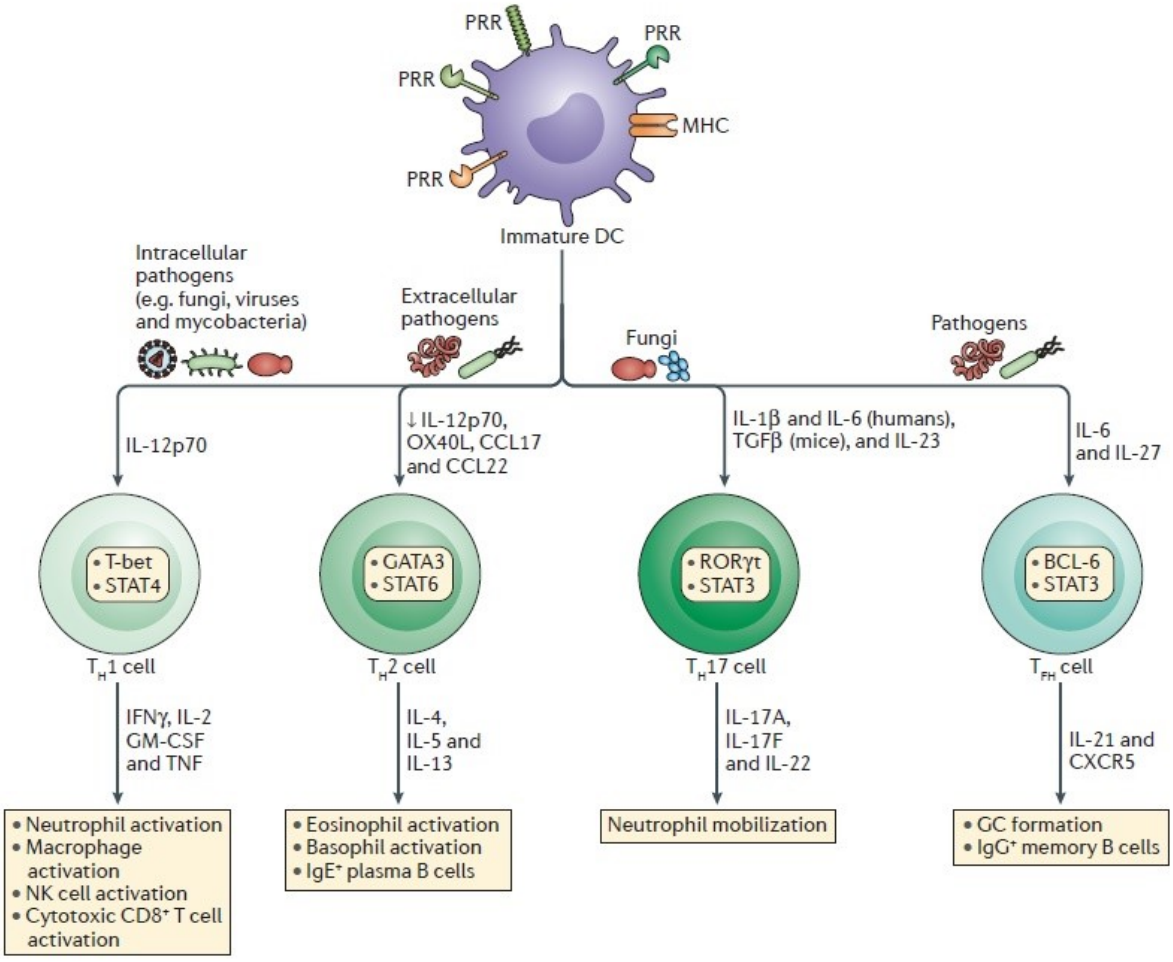


Figure 2. Dendritic cells activate and polarize Th cells depending on the type of the invading pathogen. *Geijtenbeek and Gringhuis, Nat Rev Immunol,* ***16****, 433-448, 2016. DCs*

acquire information from surrounding microbes and translate this information to naïve T cells via antigen presentation, co-stimulation and the production of T cell polarizing cytokines. This information served by DCs induces polarization of naïve T-lymphocytes driven by transcription factors such as T-bet, GATA-3, RORγt and BCL-6. Intracellular pathogens and tumors induce cellular immunity involving the action of Th1, Tc and natural killer (NK) cells, while extracellular and multicellular pathogens induce Th17 and Th2 cell activation, respectively. T follicular helper (Tfh) cells are also induced to help the differentiation of B cells expressing pathogen-specific B cell receptor (BCR).

2.1.1 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 (c)DC, plasmacytoid (p)DC (6) as well as moDCs. All these DCs derive from a $CD34^+$ hematopoietic stem cell (HSC) progenitor in the bone marrow (BM) and differentiate to monocyte/macrophage and DC progenitor (MDP) (**Figure 3**). MDP can differentiate to common dendritic cell progenitor (CDP) or to the PU.1 transcription factor (7) mediated common myeloid progenitor (CMP). CMP have the potential to differentiate to granulocytes, megakaryocytes, mast cells and $CD14^+$, $CD16^+$ or transient $CD14^+CD16^+$ monocytes owing to their capacity to differentiate into effector cells during inflammation (8) and angiogenesis (9).

Human cDCs are subdivided to CD141 $(BDCA\text{-}3)^{high}$ and CD1c $(BDCA\text{-}1)^+$ cells (10). $CD141^{hi}$ cDCs are present in the blood, tonsils and lymph nodes at low levels, as well as in the lung, liver and skin. They secrete high levels of tumor necrosis factor (TNF)-α, CXCL-10, interleukin (IL)-12 and interferon (IFN)λ, and in the presence of poly(I:C) or viral infections (11, 12) they cross-present viral and tumor antigens to Tc lymphocytes efficiently. $CD1c^+$ DCs express SIRPα, CD11b/complement receptor (CR) 3 and CD11c (inactivated C3b receptor 4, CR4) and present in the circulation. Upon stimulation with lipopolysaccharide (LPS), skin associated $CD1c^+$ DCs secrete TNF-α, IL-10 and IL-23, while lung $CD1c^+$ DCs induce Th17 immune response against *Aspergillus fumigatus* infection by releasing IL-23 (13) contributing to the diversity of DC-mediated functional activities. Murine models have also been applied to investigate the complex biology of DC *in vivo* and it was established that human $CD141^{hi}$ DCs resemble the murine $CD8\alpha^+/CD103^+$ DC population, while $CD1c^+$ DCs are equivalent to mouse $CD11b^+$ DCs.

By contrast, $CD14^+$ DCs are unique in humans and are phenotypically similar to blood $CD14^+$ monocytes however, $CD14^+$ DCs express antigen presenting molecules and CD11c at a higher extent. Microarray data suggest that this DC population expresses an

overlapping set of genes with macrophages (MΦ). It is still uncovered whether this cell type can migrate to the lymph nodes as a $CD14^+$ DC without CCR7 expression (14) are less effective to induce naïve T cell activation. However, $CD14^+$ DCs are potent inducers of Tfh cells as well as plasma cells and upon stimulation they produce IL-1ß, IL-6, CXCL-8 and IL-10 cytokines (15, 16). Circulating $CD1c^+CD14^+$ DCs express interferon regulatory factor (IRF) 4 at high levels and are characterized by abundant IL-23 secretion however, this cell population may also be considered as tissue resident $CD1c^+$ DCs (17).

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 (18) can be generated *in vitro* from monocytes, BM-derived cells and embryonic stem cells (19) in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) (17), IL-4, TNF-α (20) and IL-10, transforming growth factor (TGF)-ß or vitamin (Vit)-D, respectively (21). Inflammatory DCs, including skin DCs and moDCs generated in the presence of GM-CSF and IL-4 or TNF-α, express high levels of MHCII, CD11b, CD11c, CD1a and CD206, but differ in the levels of CD1c and CD14 expression. In the course of monocyte to DC differentiation, several genes including the monocyte markers CD14 and CD1d are down regulated, while the genes encoding PRRs (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin/DC-SIGN), co-stimulatory and antigen presenting molecules (CD80, CD86, CD40, CD1a, MHCII) and transcription factors (IRF4, peroxisome proliferator-activated receptor γ/PPARγ) are up regulated (22). In the absence of other blood cells, the *in vitro* differentiated moDCs represent a highly homogeneous population of cells and thus could modify the outcome of the immune response. MoDCs can act as potential regulators of both systemic (3, 4) and mucosal (5, 6) immune responses and also drive a wide array of cell communications at gene, protein expression and cytokine levels. In the presence of lipids, $CD1a^-$ moDCs have been associated with high expression levels of PPARγ, while this effect could be prevented in the absence of lipid ligands showing an interplay between lipid homeostasis and the immune response. Human moDCs are characterized by the cell surface expression of the CD1 glycolipid receptors CD1a or CD1d, which are expressed in a lipid microenvironment-dependent manner (23) and present different lipid/glycolipid antigens to T cells. It was also shown that $CD1a^-$ moDC can be further differentiated to $CD1a^{low}$ and $CD1a^{hi}$ cells and this process could be terminated by inflammatory signals (24).

The circulating $BDCA\text{-}2^+BDCA\text{-}4^+CD123/(IL\text{-}3R)^+$ pDCs are considered as important sensors of viruses because pDCs express nucleic-acid receptors at a higher level

as compared to cDCs or moDCs. Moreover, they act as professional type I and III IFN producing cells (25, 26) and also as effective activators of T cells in the secondary lymphoid organs and also in various tissues (27, 28).

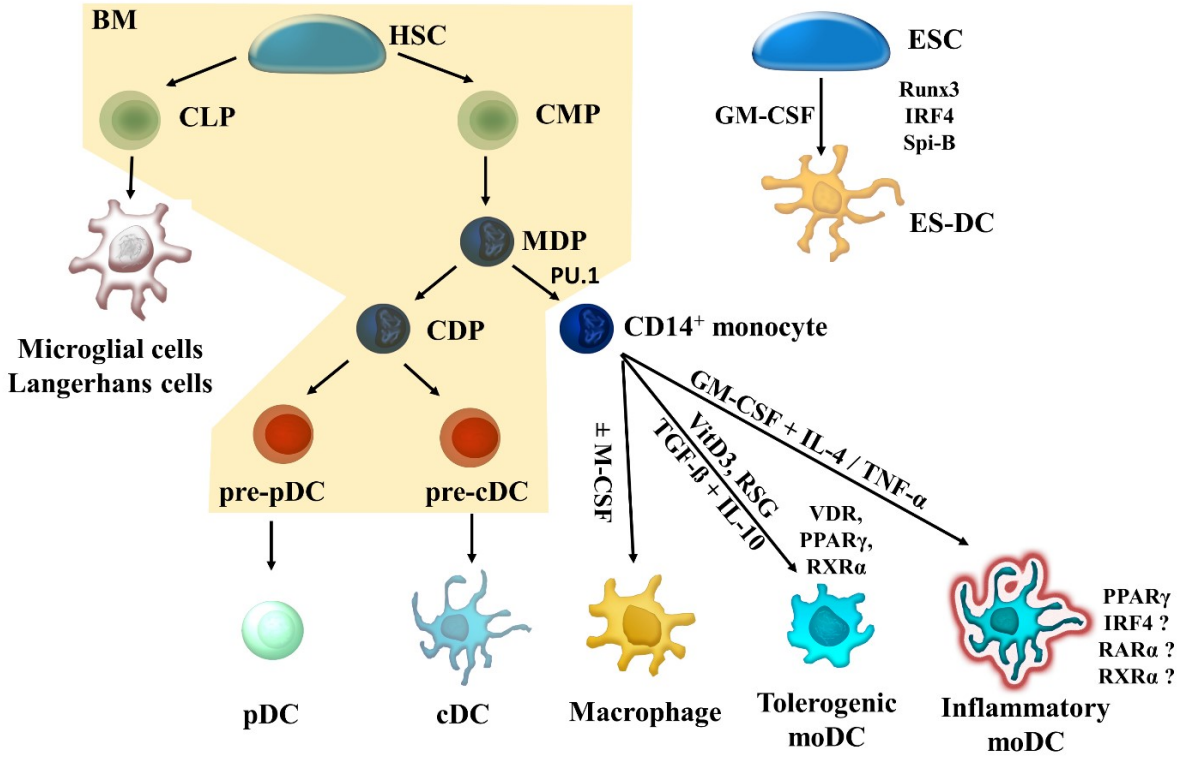


Figure 3. Dendritic cell lineages. *Human DCs are generated from common DC progenitors (CDP) or from monocytes. Depending on the actual environmental conditions, monocytes can differentiate to immature MΦ or DCs. MoDCs, including 'fast' DCs offer a potential tool for analyzing DC-mediated immune responses in the systemic and mucosal immune systems. Pluripotent embryonic stem cells (ES) also serve as a promising source of tolerogenic ES-DC generated in the presence of GM-CSF (19). Cytokines and growth factors drive the differentiation of DCs however, the role of master transcription factors is not fully uncovered in different DC lineages.*

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

As summarized before, human DCs may derive from different origins and differentiate to effector and/or regulatory cells with individual characteristics to drive immune responses. However, DC development remains plastic till its terminal differentiation ensues to acquire a mature phenotype, which is generated in the presence of multiple extracellular signals including cytokines, growth factors, microbes and lipids. During the differentiation of DCs, the correct regulation of genes is essential to support the function of 'master' transcription factors such as the IRF4, IRF8 and Runx3 (19, 29, 30),

which also may contribute to the characterization of various DC subtypes (31, 32). Murine models suggested that BM-derived $CD11b^+$ DCs cultured in the presence of GM-CSF and IL-4 express IRF4 and regulate the cell surface expression of the MHCII protein, while IRF4 increases the antigen presenting capacity of moDCs resulting in potent Th cell priming (30). These proteins may also synergize or interfere with other transcription factors such as NF-κB or AP-1.

In the presence of specific ligands, the nuclear receptors bind to the DNA through their DNA-binding motifs and trans-regulate the expression level of target genes in collaboration with *cis*-elements such as enhancers in the genome. However, it is still uncovered which transcription factors play the crucial role in the development of a given subpopulation of DCs.

Nuclear receptors bind to endogenous or exogenous lipophilic ligands (33) (**Figure 4**) and upon microbial stimuli allow DCs to shape immune responses. These receptors can be divided into four groups: homodimer steroid hormone receptors (thyroid, estrogen and testosterone receptors), RXR heterodimers (PPARs, retinoid X receptors/RXRs, retinoic-acid receptors/RARs, liver X receptors/LXRs, vitamin-D receptor/VDR), dimeric and monomeric orphan receptors. In vertebrates, the vast majority of nuclear receptors share an amino-terminal activation function, a DNA-binding domain and a hinge region and a carboxyl-terminal ligand binding domain. At steady state, nuclear receptors are present typically in the cytosol in an inactive form bound to heat-shock proteins or in the nucleus (RXR heterodimers, orphan receptors) 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 (34).

Retinoic-acid receptors such as RXRs and RARs have isoforms and bind vitamin-A derivatives (35) with different affinities (36). 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 all-*trans* retinoic-acid (ATRA), while RXRs are activated by 9-*cis* RA (37). RARs play a pivotal role in embryogenesis (38), wound healing, keratinization and the development of myeloid cells, the skeletal and the nervous systems (39, 40). RARs also interact with PU.1 and through restore PU.1 functionality (41).

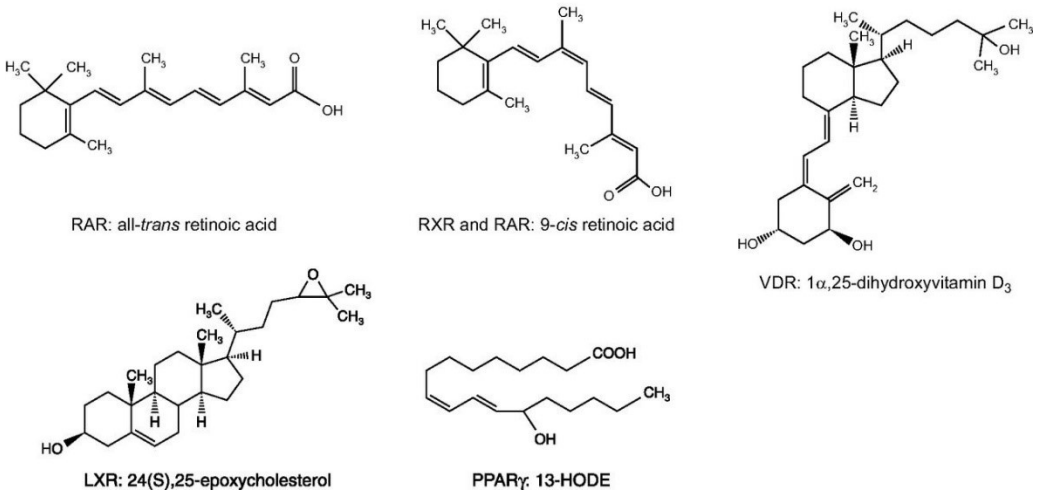


Figure 4. Prototypic ligands of nuclear receptors. *The carotenoid derivate ATRA and 9-cis RA are recognized by RARα and RXRα specifically. Nagy et al Physiol Rev,* ***92****, 739-789, 2012.*

RARα is the dominant isoform in human myeloid cells including moDCs, while RARß and RARγ are expressed at low levels (42, 43). RARα regulates the gene expression of GM-CSF, GM-CSFR and CD1 proteins (44, 45) moreover, RARα also up regulates PPARγ through CEBPε (46). RA can increase or decrease the migratory capacity of DC depending on the production of MMP12 (47) associated with the down regulation of the CCR7 and CXCR4 chemokine receptors (48), respectively.

PPARγ, the other dimerization partner of RXRα is up regulated in the presence of IL-4 and GM-CSF (49) also confirmed by *in vivo* studies (50) and collaborates with retinoid receptors acting as a master transcriptional regulator upon human moDC differentiation and function (29). In the presence of poly-unsaturated fatty-acids, oxidized fatty-acids, phospholipids, prostanoids and rosiglitazone 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 (**Figure 5**). In particular, a set of genes involved in metabolism (transglutaminase 2/TGM2), lipid antigen processing and presentation, invariant natural killer T (iNKT) cell activation (class I and II CD1 proteins, cathepsin D), retinoic-acid (RA) synthesis (RALDH2, RDH10) are all regulated by PPARγ and overlaps with genes that are regulated by RARα (42, 51-53). At *in vivo* conditions PPARγ-agonist reduced the number of TNF producing and inducible nitric-oxide synthase (iNOS) expressing DCs in the lung infected by the highly pathogenic influenza A virus H5N1 showing that PPARγ-dependent DC migration is responsible for the reduced recruitment of inflammatory DC in the airways (54). The ABCG2 gene is highly expressed on the cell surface of DC and is

regulated by PPARγ in several drug-resistant tumor cell lines (55). Collectively, PPARγ is considered as a modulator of lipid metabolism and transport in DCs and is also involved in regulating immune responses both directly and indirectly (56).

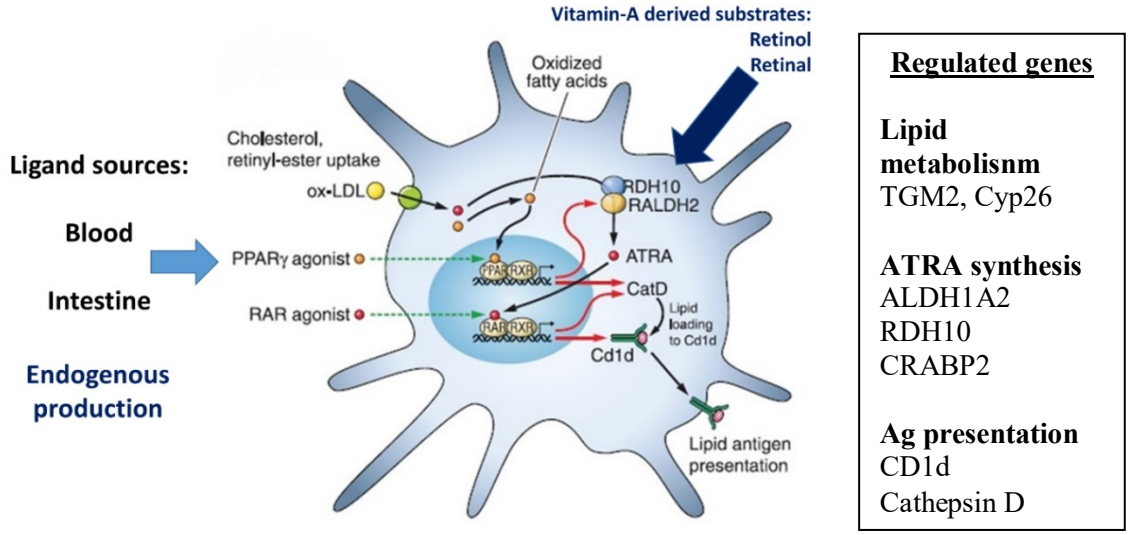


Figure 5. In moDCs PPARγ and RARα drive the expression of genes related to metabolism, endogenous ATRA synthesis and lipid antigen presentation. *Modified from Nagy et al Physiol Rev,* ***92****, 739-789, 2012.*

2.1.3 The origin of retinoic-acid

It has previously been established that the outcome of moDC differentiation and function is dictated by the actual microenvironment of the cell, while the mucosal surface represents a special, highly complex and dynamic milieu containing microbe-derived antigens as well as exo- and endogenous metabolites such as RA playing role in pre-conditioning the differentiating moDCs. 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 (57). ATRA is important for maintaining tolerance against commensals and food-derived antigens as well as to induce pathogen specific immune responses. For example, β-carotene and Vit-A absorption occurs in the intestine and fetal vitamin-A deficiency (VAD) exacerbates rotavirus infection. Moreover, it was also found that in healthy infants the retinol concentrations correlate inversely with the results of the skin prick tests within the range of 5 – 20 years (58). It was also observed that in mildly malnourished children intestinal integrity correlated inversely with serum retinol concentrations.

Vit-A is processed to ATRA by DCs, MΦ, mucosal epithelial and stromal cells (59-62) and in mammals the main source of ATRA synthesis is retinol derived from the blood

circulation complexed with RBP4 (63). Retinol is transported to the cytoplasm via STR6 (64) and binds to CRBP1 and CRBP2 through a storage mechanism (65). Retinol oxidation is catalyzed by alcohol-dehydrogenases (ADHs) and retinol-dehydrogenases (RDHs) to generate retinaldehyde/retinal which is served as a substrate for retinaldehyde-dehydrogenase (RALDH) to generate RA. RALDHs have 3 isoforms encoded by the ALDH1A1, ALDH1A2 and ALDH1A3 genes, which are expressed in DCs and MΦ differently followed by the action of catalytic P450 cytochrome enzymes (Cyp26a1, b1 and c1), which convert RA preferentially to 4-hidroxyl-RA (66) followed by binding to CRABP2 delivered to the nucleus (67).

Beside targeting the highly conserved receptor RARα (68), ATRA also serves as a potential therapeutic drug in anti-cancer settings (69) and in combinations with other therapeutic agents such as GM-CSF (70), which is able to promote myelomonocytic cell differentiation. In contrast to Vit-D, ATRA is a potent inducer of monocytic cell development and serves as a useful target of monomyelocytic leukemia. Interestingly, the combination of ATRA and Vit-D act synergistically during myeloid cell development (71).

ATRA also acts as an immunomodulator of DC activities via inducing the expression of mucosa-associated homing receptors in T-lymphocytes, such as CCR9, and also supports T-cell differentiation. This molecular interplay suggests that effector lymphocytes generated in the draining lymph nodes or in the mucosa-associated lymphoid tissues (MALT) of a particular regional immune system (lung, small bowel) can enter the blood circulation and subsequently home back to the host organ.

2.1.4 Dendritic cells acquire signals from pattern recognition receptors 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 receptor (CLRs), NOD-like receptors (NLRs), RIG-like helicases (RLHs) and Toll-like receptor (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. Cell surface PRRs such as TLR-1, -2, -4 and -6 together with scavenger receptors and CLRs recognize bacterial and fungal cell wall components and facilitate the phagocytosis of bacteria and fungi. Viral nucleic-acids and unmethylated CpG motifs derived from bacterial DNA are recognized by endosomal sensors

such as TLR-3, -7, -8 and -9, while in the cytosol RLHs, NOD2 and NLRX1 are able to recognize viral RNA.

In moDCs, the specific ligation of PRRs turns on cell signaling pathways and the activation of NF-κB and IRF3/7 transcription factors regulating the production of pro-inflammatory cytokines (TNF-α, IL-6, IL-1ß, IL-12), chemokines (CXCL-8, CXCL-10), type I (IFNß, IFNα) and III interferons (IFNλ) in moDCs (72, 73). Interestingly, commensal bacteria are able to augment the expression of intestinal RA synthesis via the activation of PRR families (74). Microbial patterns are also recognized in the cytosol by NLRs, RIG-I or AIM2 receptors and recruit inflammasome components playing role in the cleavage of pro-IL-1ß and pro-IL-18 to generate their physiologically active forms.

CLRs sense microbial and/or host carbohydrates including mannose, fucose, sialic-acid and ß-glucan. DAMPs also act as potential CLR-targets exemplified by DNGR1 (CLEC9) playing role in DC-mediated cross-presentation by recognizing the extracellular F-actin derived from dead cells (75). CLRs are also expressed by myeloid cells, preferentially by MΦ and DCs, but Dectin-1 (CLEC7A) is also expressed by B cells and keratinocytes (76). 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. CLRs also can sense microbes though their carbohydrate fingerprint (77). Host carbohydrates differ from those of bacteria, fungi and parasites thus CLRs are able to distinguish pathogens and/or tumor cells thus inducing specific adaptive immune responses by DCs. Viral envelops may contain altered composition of host's carbohydrates and tumor cells with altered expression of glycosyltransferases exhibiting tumor-specific carbohydrate patterns on the tumor cell surface. The CLR family members, including Dectin-1, Dectin-2 (CLEC6A) and DC-SIGN (CD209) are able to induce Th17 responses against pathogens and maintain intestinal homeostasis by controlling the resident fungal microbiota (78) however, their cell surface expression and ligating potential may vary among different DC and MΦ populations.

DC-SIGN is expressed by sub-epithelial DC subsets including $CD1c^+$ and inflammatory DCs. The ligation of DC-SIGN does not induce cytokine secretion however, this receptor is a potent modulator of signals derived from other PRRs (79, 80). DC-SIGN is also used by viruses, mycobacteria and fungi acting as a co-receptor and plays role in the penetration of the microbe. DC-SIGN recognizes extracellular pathogens such as parasites and *Helicobacter pylori* via fucose containing PAMPs (76, 77). SIGN-R1, resembling human DC-SIGN binds a broad variety of pathogenic microbes and their polysaccharides

including HIV, Ebola and hepatitis C viruses (81), *Mycobacterium tuberculosis* and *M. leprae* (82), *Candida albicans* (83, 84), *Helicobacter pylori* (85) and serves as a target of penetration and infection of the host's cell while the induction of a specific immune response is prevented (86).

PAMPs derived from these pathogens activate Dectin-1, TLR-2 and TLR-4 together with DC-SIGN leading to the enhanced secretion of IL-12 and IL-6 cytokines and mediate Th1 and Th17 type immune responses. Moreover, simultaneous activation of DC-SIGN by fungal species leads to the formation of the RAF1 signalosome localized around the intracellular domains of DC-SIGN in line with subsequent NF-κB phosphorylation and increasing IL-1ß, IL-23 and IL-6 cytokine production. In contrast to these regulations, *Schistosoma mansoni*, *Fasciola hepatica* and *H. pylori* trigger Th2 and Tfh responses by the fucose signalosome at DC-SIGN and the secretion of IL-27. Dectin-2 induces Th17 and Th2 cell responses but it is able to signal in complex with FcRγ via ITAM only. Dectin-2 is expressed on the surface of MΦ and DCs and can be ligated with fungal mannose and mycobacterial ManLam structures. Human Dectin-2 activates exclusively Rel-p50 transcriptional complexes thus unable to induce the secretion of IL-1ß, IL-23 and to polarize activated Th cells by itself. Dectin-1 is the major receptor on MΦ for β-1,3-glucan, a glucose polymer presents in the fungal cell wall (87), whereas Dectin-2 expressing MΦ and various DC subsets recognize high-mannose ligands including *C. albicans* (88), *Malassesia furfur* (89), and *S. mansoni* (90). It also interacts with mycobacterial mannosylated lipoarabinomannan (ManLAM) and can associate with the FcRγ acting as a signaling adaptor.

2.2 The microbiota and its interaction with the immune system

2.2.1 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 (*Firmicutes, Actinobacteria*, *Bacteriodetes*, and *Proteobacteria*), Archaea and fungi (*Aspergillus*, *Candida*, *Cladrosporium*, *Malassezia* and *Saccharomyces*) referred as the normal 'microbiota', which is highly specialized due to the special local environment of the gut and the respiratory mucosa. Commensals are normally confined to mucosal surfaces, but in contrast to pathogenic bacteria, they do not express a high number of virulence factors to penetrate the mucus layer or the epithelium and thus cannot disseminate throughout the

body. The resident microbiota involves a relatively stable group of microbes with re-establishing potential, whereas transient microbiota inhabits the skin and the mucosal tissues transiently i.e. for hours, days or weeks. 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. Interestingly, commensal gut bacteria also may serve as a route for pathogenic microbes to disseminate in the mammalian body. Enteric viruses, such as poliovirus, reoviruses and retroviruses require the presence of intestinal microbes to penetrate and spread (91, 92). For example, the intestinal replication of poliovirus is microbiota but not IFN-α receptor (IFNAR)-dependent, which surface molecule is a receptor for viral penetration. It has been also established that poliovirus binds to N-acetyl-glucosamine (GlcNAc)-containing surface polysaccharides (LPS, peptidoglycan) of bacteria and it was showed that in mice colonized with antibiotic-resistant gut bacteria after antibiotic-treatment had pathogenic poliovirus infection.

Some bacteria, such as *Clostridium difficile* are opportunistic pathogens (pathobionts) and these microbes can invade the host in case the abundance of the mutual bacteria is decreased or by reduced host defenses. These pathogens rarely cause disease in immunocompetent people but can cause serious infection in immunocompromised patients.

The development and the metabolic activity of the human immune system critically depends on the amount and the diversity of the human microbiota acquired from the actual tissue microenvironment (93-95). Upon birth the human gastrointestinal tract becomes colonized by commensal microbes co-evolved with humans in a symbiotic or at least mutualistic manner together with the immune system (96, 97). 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 and for probiotic bacteria. Commensals also produce nutrients such as B vitamins, vitamin-K, saccharides and physiologically active short-chain fatty-acids (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 (96, 98, 99).

The composition and the actual number of the normal microbiota members varies among its body localization. For example, the colon is colonized by the two largest phyla of bacteria i.e. *Firmicutes* (64%, prominent members are *Clostridia* and *Faecalibacteria*) and *Bacteriodetes* (23%, *Bacteriodes* and *Prevotella*) (**Figure 6**).

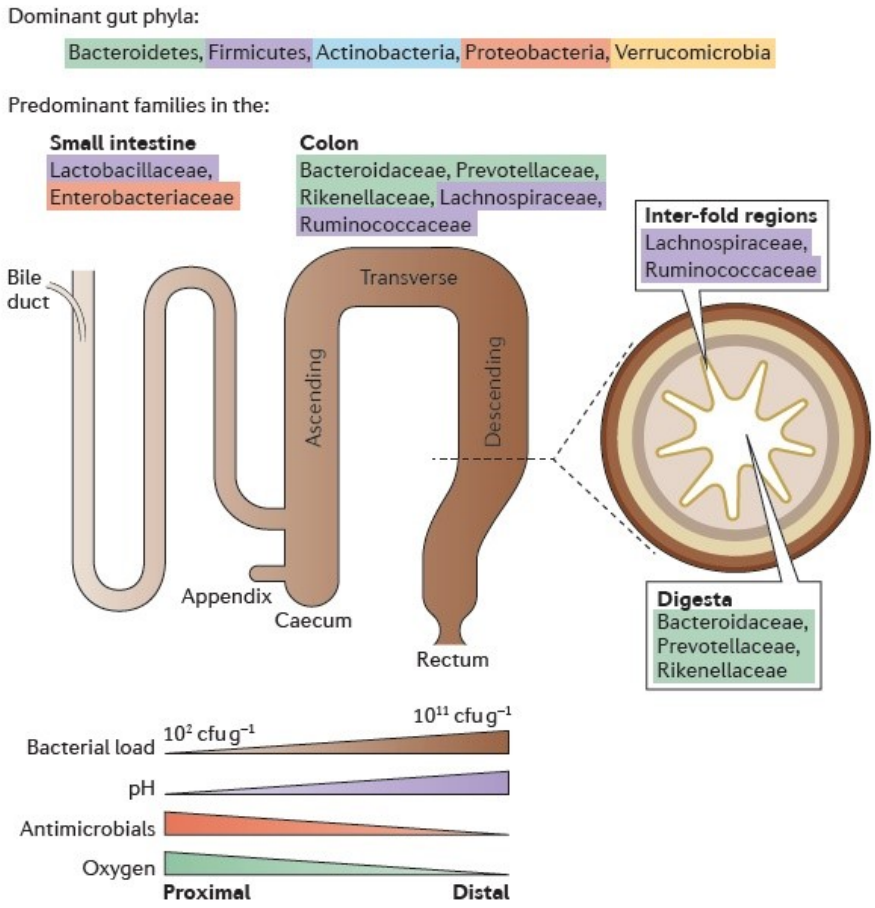


Figure 6. Gut microbiota species and strains inhabit different anatomical parts of the GI tract. *Donaldson et al, Nat Rev Microbiol,* ***14****, 20-32, 2015. Firmicutes (B. subtilis, L. reuteri) and Proteobacteria/Enterobacteriaceae (Escherichia coli, Morganella morganii) used in this study colonize the intestine and are core members of the normal gut microbiota.*

Although several diseases are caused by enteric bacteria including enteropathic *Escherichia coli* (EHEC, EPEC, ETEC, STEC), *Salmonella*, *Shigella*, *Klebsiella* or *Yersinia*, the commensal strains of *Escherichia coli* and other coliforms (*Escherichia*, *Klebsiella* and *Enterobacter*) are localized to the gastrointestinal (GI) tract and may cause disease exclusively by colonizing for example the urigenitary tract.

Enterobacteriaceae, a family of *γ-Proteobacteria* is a large group of facultative anaerobic bacteria involving *Morganella morganii* and *E. 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. These bacteria stimulate germinal center reaction, maintain B and T cell dependent 'natural' immunity and induce RegIIIb, RegIIIc secretion (100). However, it was previously reported that bacterial colonization of the murine colon with *E. coli Schaedler* stimulates intestinal epithelial cells (IECs) and intraepithelial lymphocytes (101) independently, and may play role in the pathogenesis of colitis as demonstrated in adoptive transfer models using SCID mice, which may also operate in patients with inflammatory bowel disease (IBD). Moreover, the translocation of disease-causing *L. salivarius* into the respiratory tract could be prevented in immunoglobulin (Ig)A-KO mice by prior oral inoculation of *E. coli Schaedler*, leading to the secretion of surfactant-associated protein D. Colonizing germ-free BALB/c mice with *E. coli Schaedler* and *M. morganii* resulted in unique characteristics in the total and antigen specific secretory (s)IgA levels showing that *M. morganii* is also able to stimulate the production of bacterial phosphatidylcholine-specific immunoglobulins of various isotypes locally. In addition, *M. morganii* can be covered with sIgA to prevent its clearance from the germ-free host.

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 strain is widely used in veterinary practice due to the active constituents of probiotic Monosporyn^{TM} 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 diseases such as IBD, celiac disease, allergy, metabolic and neurobehavioral diseases. For example in Crohn's disease, the ratio of *Proteobacteria* could be increased (102), while the diversity and the fraction of *Firmicutes* in the gut microbiota is decreased (103). 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 (104, 105). The various effects of probiotic gut bacteria also may prevent infection by pathogens such as the

probiotic *E. coli Nissle* 1917 strain, which is able to inhibit the growth of enteropathogenic *E. coli,* which also may serve as a safe strain in IBD treatment (106-108).

2.2.2 The first encounter of the human body with microbe: License and protection for a lifetime

The first days and weeks of postnatal life play a crucial role in the development of the monocyte - MΦ - DC network upon encountering microbiota members at the very early stage of colonization. It has been proposed that the functional status of monocytes, cDCs, pDCs and moDCs differ in the fetal and the postnatal stage (early and adult) life (109). This observation is explained by the highly restricted environment of the maternal development, while the postnatal immune system and the developing resident microbiota interacts with each other (**Figure 7**). This phenomenon is confirmed by several studies, for example, Langerhans cells reduce their T cell stimulating capacity in the first weeks of life (110) and the production level of TNF-α was less but as much or even more that of IL-12 and IL-6 in response to TLR-2, TLR-4 and TLR-7/8 ligands as compared to adult monocytes. At the first year of life myeloid DCs produce low levels of IL-12 however, IL-23 levels were higher as compared to the adult cells. Interestingly, cytokine production of IL-23, IL-6 and IL-10 declined, while the secretion of TNF-α and IL-1ß increased gradually with age (111, 112). Moreover, cord blood moDCs from infants found to produce low levels of IL-12 in response to LPS and polyinosinic:polycytidylic-acid/poly(I:C) (113, 114). It was also showed that human monocytic cells derived from embryonic or fetal liver progenitors express sets of genes similar to that of anti-inflammatory MΦ.

The colonization of commensal bacteria in the intestine drives the development of Peyer's patches, mucus and antimicrobial peptide secretion, sIgA release and the differentiation of mucosal and systemic T-lymphocytes (115). TLR and NLR ligands derived from the microbiota may diffuse into the blood and modulate peripheral immune responses (97) and thus enhance systemic antiviral immune responses at early life (116). Using cord-blood derived moDCs it was also shown that in the absence of normal gut microbiota the IRF3 and NF-κB mediated gene transcription was reduced upon LPS stimulation.

Effector T-lymphocytes are uniquely represented in the mucosa at healthy state of the body because some microbial species continuously maintain the population of Th1 and Th17 cells in the gut. Using murine models it was also shown that germ-free mice lacking Th17 cells can be restored by the intestinal inoculation of small filamentous bacteria (117). Moreover, several species of *Clostridia* and saccharide-A produced by *Bacteriodetes fragilis*

are able to induce IL-10 producing regulatory T (Treg) cells in the gut. The microbiota also acts on iNKT cells within the neonatal period, because commensals can abolish the number of epithelial iNKT cells thus representing immunological imprinting of early microbial colonization.

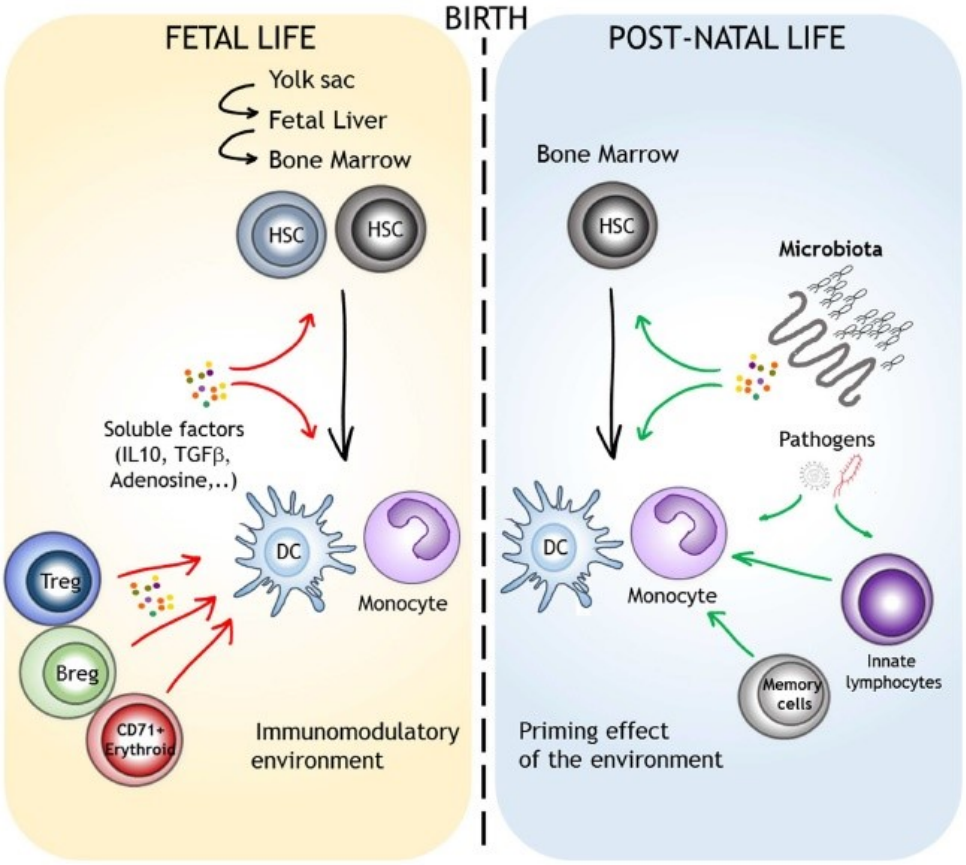


Figure 7. Restricted and environmental factors mediate DC differentiation in fetal and postnatal life. *De Kleer et al., Front Immunol,* ***5****, 1-11, 2014. At the early period of life, the colonization of the GI and the respiratory tract is important to generate adult-like responsiveness to PRR ligands orchestrated by DCs.*

Evidence exists for communication between the three key factors of the GI tract including diet, hosts factors (immune system, genetics, glycosylation patterns) and the microbiota. Diet plays a pivotal role on the functional activities of the immune system exemplified by Vit-A and -D supplementation, and it has also been shown that the immune system can also affect nutrient uptake. Diet determines the composition and the metabolic capacity of commensal bacteria, and this is connected to nutrient absorption. Thus, the microbiota not only processes food and drugs in the gut, but also generates metabolic products with beneficial, neutral or harmful effects on the host. The healthy composition of the diet in childhood is essential for developing a healthy microbiota as childhood

undernutrition is directly linked to the failure of gut microbial maturation. The pathogenesis of childhood undernutrition derived from disrupted microbiota development can be prevented by breastfeeding during the first six month of postnatal life as recommended by the WHO/UNICEF (94). The mother's milk contains several probiotic bacteria including *L. reuteri* and prebiotics which are necessary to generate a healthy gut microbiota.

2.2.3 Dendritic cells orchestrate mucosal immune responses *via* targeting the gut microbiota

The main role of the mucosal immune system is to induce strong effector immune responses to eliminate the obligate pathogens and/or the invading pathobionts and to maintain tolerance against antigens derived from the normal microbiota members and food components. Mucosal tissues are the hallmark of various immunologically relevant cell types, such as mononuclear phagocytes including resident DCs, moDCs and MΦ. The mucosal DC network involves a highly heterogeneous population of cells of myeloid and BM origin (118), which in the course of balancing regulatory events moDCs (119) act as potent organizers of adaptive immunity leading to the maintenance of peripheral tolerance against gut resident microbes and harmless food-derived antigens. At steady state the intestinal *lamina propria* contains functionally different DC subsets contributing to maintain gut homeostasis. DCs are continuously exposed to foreign antigens which can directly be translated to T cells supporting tolerance or triggering inflammation. DC-primed T-lymphocytes together with monocyte precursors play an important role in the maintenance of tolerance against gut commensal bacteria (118). DCs have a pivotal role in the initiation of adaptive immune responses and can directly contact and internalize intestinal bacteria (120). Furthermore, DCs can be conditioned by IECs thus control their inflammatory potential (121). Most importantly, DCs have the potential to distinguish different members of the gut microbiota and drive the activation and differentiation of naïve T-lymphocytes into effector Th1, Th17 or Treg cells (77). The nature of the T cell polarizing signals is largely determined by the type of the microbial products, the nature and strength of the inflammatory signals encountered in peripheral tissues during the immature phase (76, 122) (**Figure 8**).

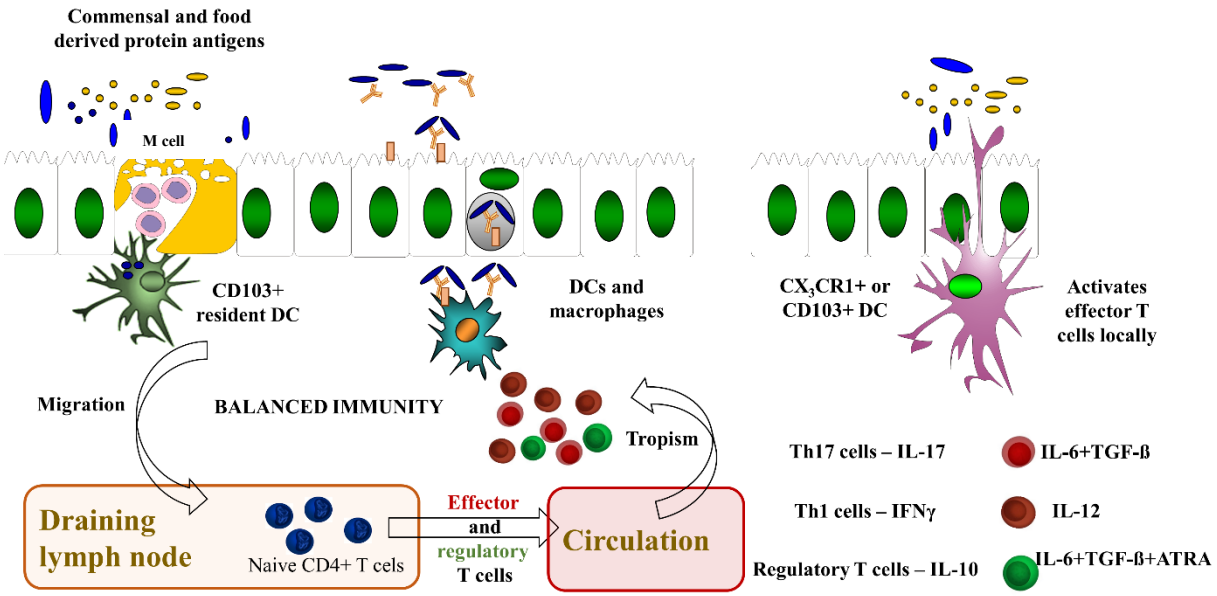


Figure 8. Dendritic cells regulate mucosal immunity targeting commensal bacteria and food-derived antigens in the intestine. *Commensal bacteria by itself cannot induce systemic immune responses similar to the oral tolerance induced by protein antigens, but may provoke primary systemic immune response in the blood or in other internal non-mucosal sites of the body. Tolerance against commensal and harmless food derived proteins is initiated by antigen presenting cells such as DCs which migrate between the lamina propria and mesenteric lymph nodes or Payer's patches, but the immunological memory and immune tolerance is maintained by adaptive mucosal immunity.*

Intestinal mononuclear phagocytes including DCs and MΦ express mucosa-associated cell surface molecules such as CX_3CR1 or CD103 (123, 124) however, the clear distinction of DCs from MΦ is still critical (125, 126). In the *lamina propria*, both $CD103^+$ and CX_3CR1^+ DCs can sample luminal antigens by projecting dendrites through the epithelial cell layer. The main sources of human intestinal CX_3CR1^+ DCs are circulating monocytes, which loose this marker within 24 hours (127). 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 (120, 127-129). 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 Treg differentiation in the presence of commensal bacteria and thus help to ensure gut integrity (130). Moreover, ATRA-producing DCs can 'imprint' T and B cells to express gut-homing proteins such as α4β7 integrin.

Mucosal DCs also support the differentiation of naïve B cells to develop IgA secreting plasma cells or memory B cells by the production of soluble NO, IL-10 and ATRA

and an indirect manner by priming of antigen-specific Th cells. IgA class switching can be enhanced by DC-derived NO which up regulates TGF-β receptor on B cells. T cell-dependent B cell differentiation generates high-affinity IgA antibodies, which target preferentially protein components of the bacterial cell wall derived from viruses and toxins.

2.2.4 Organization of the mucosal immune system

Based on functional and anatomical considerations, the human immune system can be subdivided into the systemic and the ancient mucosal immunity. It has been suggested that secondary lymph nodes and tissues of the vertebrate immune system are later specializations of the mucosal immunity. This theory is further supported by the fact that the central lymphoid organs derived from the embryonic intestine.

Due to the precise compartmentalization and signal regulation at mucosal surfaces, chronic inflammatory responses are avoided in the presence of the microbiota. Tissues of the mucosal immune system involve the lymphoid organs, which are associated with the gastrointestinal, respiratory and urogenital tracts. The mucosal lymphoid organs of the nose, mouth, skin, stomach, intestine, colon, vagina and lungs together belong to the MALT, which will be discussed in the context of gut-related mucosal immunity.

In the human GI tract multiple cell types have an intimate contact with a huge load of microbes. In this milieu common and specialized epithelial cells, MΦ, DCs and lymphocytes act in concert to maintain the delicate balance of the host's immune system and the actual microbiota composition. At mucosal surfaces, the effector cells are localized in three compartments: the mucus layer, the epithelium and the *lamina propria*. By immunological terms, these tissues represent distinct immunological compartments, where the total number of immune cells within the mucosal tissues probably exceeds that of most other parts of the body (**Figure 9**).

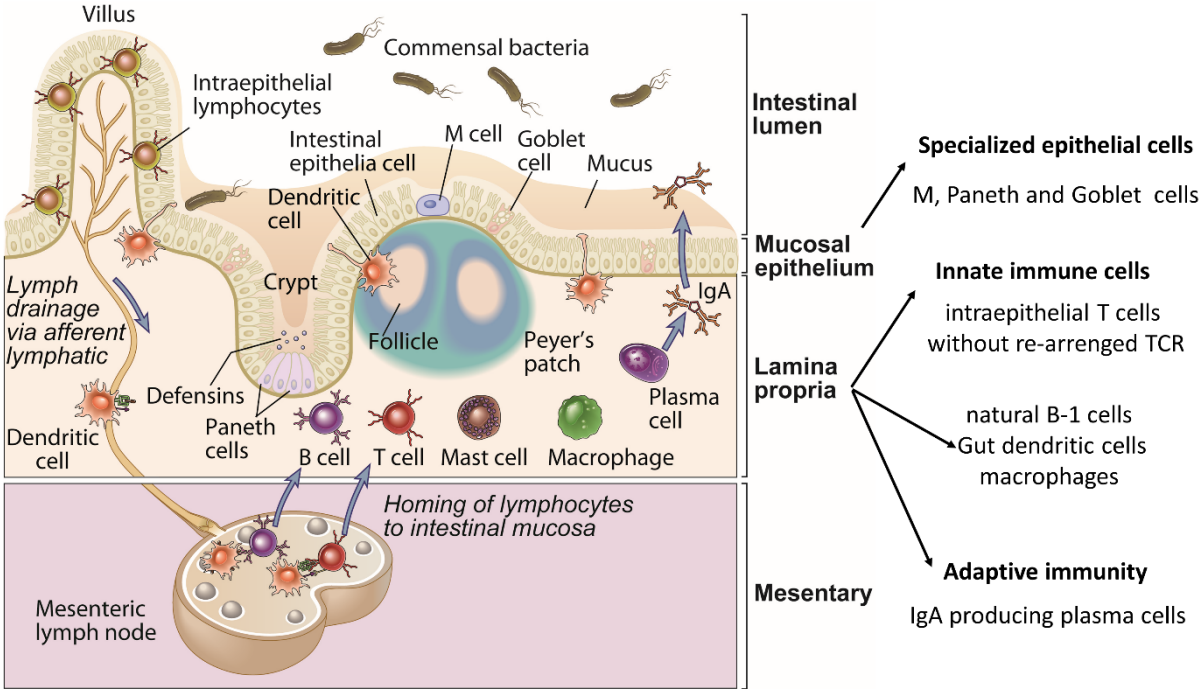


Figure 9. Some cell types are abundant in the intestinal mucosa. *Modified from Abbas: Cellular and Molecular Immunology, 8th edition, 2015.*

The first line of defense of the mucosa is protected by the mucus layer localized above the epithelia, which provides a biochemically complex compartment containing commensals, food derivatives, electrolytes, lipids and anti-microbial peptides (AMPs) as well as Ig, preferentially IgG and sIgA types. In the crypts of the intestinal villi, Goblet cells produce *O*-glycosylated proteins continuously referred as mucins of several isoforms (MUC2, MUC5, MUC6, MUC19 and MUC7). Mucins form a viscous physical barrier between the lumen and the epithelia, which prevents microbes to reach an intimate contact with the cells of the GI tract (131, 132). The small intestinal mucus is preferentially made up by gel-forming MUC2, while the surface of the stomach contains mainly MUC5, which is extensively *O*-glycosylated. The diversity of oligosaccharides provides a vast library of potential recognition sites for both the commensal and the pathogenic organisms (133). The type of mucin *O*-glycans varies along the GI tract and may contribute to the selection of microbial communities within the gut, because bacteria express cell surface adhesins compatible with host tissue-specific glycans. The highest thickness of the mucus layer is formed in the colon and rectum. The outer and inner layer of the mucus contains different numbers of colonizing microbiota species, because the inner layer is tightly attached to the epithelial surface (**Figure 10**).

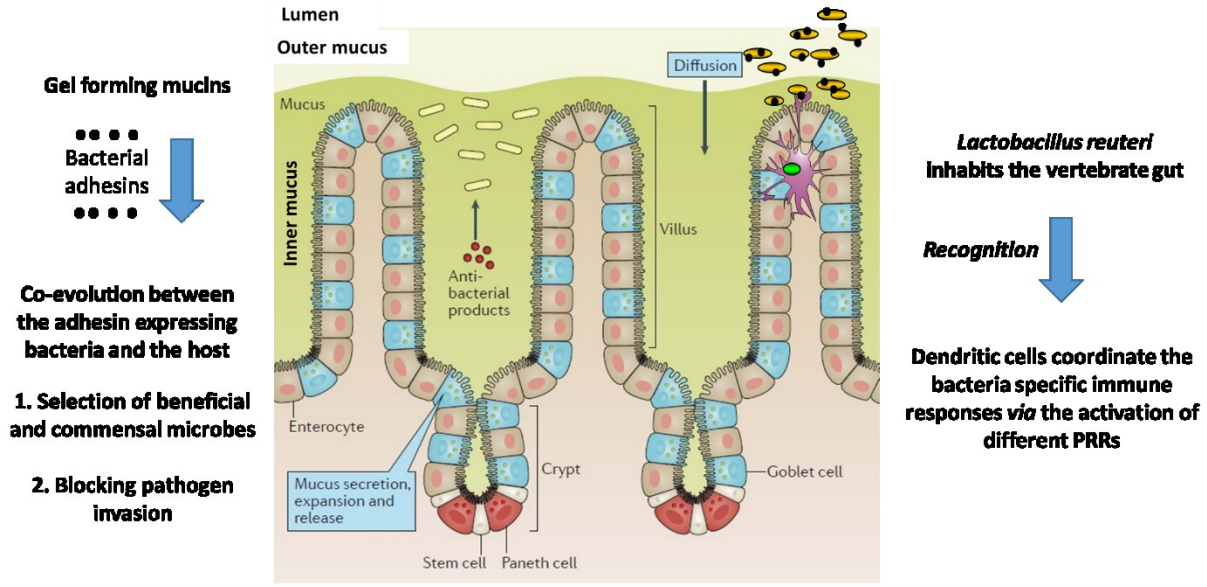


Figure 10. The functional role of mucus-binding adhesins. *Modified from Johansson and Hansson, Nat Rev Immunol,* ***16****, 639-649, 2016. The mucus layer serves as a surface for both beneficial and pathogenic microbes, which penetrate and compete with each other. Commensal bacteria, such as L. reuteri strains express specialized structures including adhesins which support the adherence to the mucus and epithelia. Bacterial adhesins expressed on commensal and beneficial microbes facilitate their colonization and inhibit the evasion of pathogenic microbes. Moreover, these MucBPs may have a role in inducing immune responses by DC.*

The secretory function of Goblet cells is mediated by environmental factors such as cytokines and chemokines produced by myeloid cells and by microbiota-specific effector T helper cells. These commensals also produce SCFAs such as butyrate, which increase the mucus and antimicrobial peptide secreting capacity of Goblet and Paneth cells (134).

Paneth cells exhibit dual functions by secreting AMPs, namely defensins and enzymes such as lysozyme and LPS-hydrolyze playing role in inhibiting the invasion of pathogenic bacteria. Paneth cells also have the capacity to produce Wnt signal molecules to maintain the stem cell population from which the Goblet cells and the epithelial cells derive. Intestinal mucus also contains the RegIIIγ system, a member of the CLR family, which recognizes peptidoglycan of Gram-positive bacteria, the vascular and antimicrobial factor angiogenin 4, lipocalin and calprotectin. All these factors remove essential metals (iron, zink, calcium) of bacteria. SIgA limits bacterial adhesion to the epithelia by forming bacteria containing clumps and thus can prevent increased inflammation. IgA also prevents the transmission of microbes to another host.

Under the mucus layer, intestinal epithelial cells lining the small and large bowel represent an integral part of the innate immune system of the GI. It is involved in pathogen induced responses, support tolerance induction to commensal organisms and assists antigen sampling during the adaptive immune responses in the gut. Microfold (M) cells, Goblet cells and Paneth cells are terminally specialized epithelial cells with different functions and play role in luminal antigen transport targeting to the *lamina propria* in the gut and in the respiratory tract. The epithelial barrier contains mainly lymphocytes, which cell population in the small intestine consists of intraepithelial $CD8^+$ and γδ T cells. These T cells do not express antigen-specific receptors, but after non-specific recognition of microbes they produce cytokines, which stimulate the epithelium, DCs and MΦ and recruit antigen specific memory and effector T cells.

The *lamina propria* is more heterogeneous than the epithelium containing large numbers of Th and Tc cells, iNKT and ILCs accompanied with plasma cells, MΦ, DCs, eosinophils and mast cells to maintain gut and respiratory homeostasis. This special tissue is conditioned by cytokines, growth factors including GM-CSF, metabolites such as ATRA and the gut lumen derived antigens. Local APCs differentiate and are activated in such a microenvironment and induce mucosal adaptive immune responses in Peyer's patches and mesenteric lymph nodes.

2.2.5 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 lumen-derived antigens, Treg cells are also activated and produce anti-inflammatory IL-10 and TGF-ß (135).

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 (136, 137). 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 (130). 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 (138). Moreover, nanomolar concentration of ATRA also promotes Th17 differentiation in splenic DC – T cell co-cultures (139). 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.

2.2.6 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. It has diversified into separate phylogenetic clades reflecting host origin (140) with genomic differences exhibiting niche characteristics in the host GI tract (141). *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 (141). *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 which has antibacterial and antifungal effects moreover, the produced lactic-acid also enhances the inhibition of pathogen invasion.

L. reuteri with potent immunomodulatory properties contribute to mucosal tolerance in the vertebrate GI tract. Specific probiotic strains of *L. reuteri* have recently been shown to suppress intestinal inflammation in a trinitrobenzene sulfonic-acid (TNBS)-induced mouse colitis model through the down regulation of genes encoding IL-6 and IL-1ß cytokines in the colon (142). *L. reuteri* 100-23 was found to stimulate Treg development (143) 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 (144). 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 (145). However, modulation of cytokine production by *L. reuteri* appears to be strain-dependent as demonstrated *in vitro*. For example, anti-inflammatory *L. reuteri* strains ATCC PTA 6475 and ATCC PTA 5289, but not the immuno-stimulatory *L. reuteri* strains ATCC 55730 and CF48–3A, suppressed TNF-α production induced by bacterial LPS-activated monocytic cells (146, 147). Moreover, a recent study showed that *L. reuteri* strains derived from human-associated clades differed in their ability to modulate human cytokine production (TNF-α, MCP-1, IL-1ß, IL-5, IL-7, IL-12 and IL-13) by myeloid cells (148). The bacterial molecule(s) responsible for down regulating TNF-α in antigen-presenting cells (APCs) have not been identified so far, but appear to be strain-specific (146, 147). Recently, it was suggested that the cell-surface proteins may play a role in regulating the immunomodulatory properties of lactobacilli (149-153). However, the detailed molecular mechanisms by which *L. reuteri* bacteria may interact with DCs to modulate immune responses and promote mucosal homeostasis are not well understood.

2.2.6.1 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 (133). Typical adhesins, such as pili and fimbriae as well as other cell surface proteins of enteropathogens have extensively been studied. *L. reuteri* expresses MucBPs in a strain dependent manner and promotes the evolution of *L. reuteri* species primarily as gut organisms (**Figure 11A**). However, the systems responsible for intestinal adhesion of gut commensal and probiotic bacteria to mucus are poorly understood.

MucBPs are cell surface proteins containing a signal peptide and a LPxTG anchoring motif at the C terminus including leucine (L), proline (P), X (representing any amino-acid substitution), threonine (T) and glycine (G) moieties covalently attached to the bacterial cell wall (154) and are characterized by peptide sequence repeats (**Figure 11B**). The most well characterized MucBP is the extracellular 353-kDa MUB protein derived from the *L. reuteri* 53608 variant consisting 2 types of related amino-acid repeats (Mub1 and Mub2), six copies of the type 1 repeat (Mub1) and eight copies of the type 2 repeat (155). It has also been established that MUB has Ig binding activity for the repeat structure of the MUB protein (156). Some domains of lactobacilli-derived MucBPs are also found in *Listeria monocytogenes*, an intracellular food-borne pathogen (157). Moreover, Lar_0958 mucus-

binding adhesin derived from human symbiont *L. reuteri* showed structural homology with *Listeria* internalins.

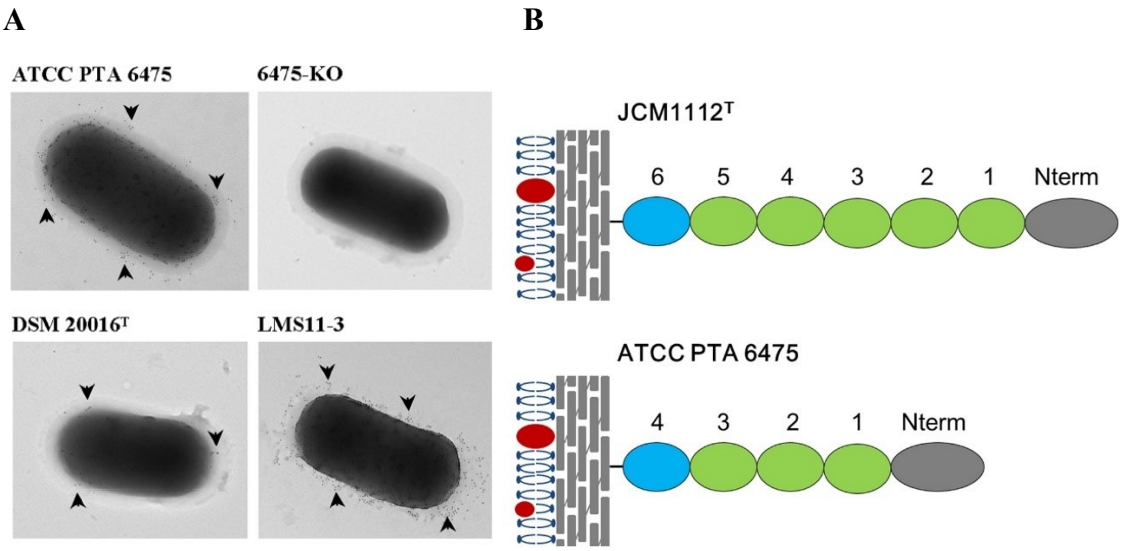


Figure 11. Lactobacillus reuteri strains express mucus-binding adhesins. *Etzold et al., Molecular Microbiology,* ***92****, 543-556, 2014. (****A****) L. reuteri PTA 6475, DSM 20016T and LSM11-3 strains express Lar_0958 (CmbA) MucBP as shown by anti-Lar_0958 immunogold labelling. MUB also shows similar expression pattern on L. reuteri ATCC 53608 bacteria (158). (****B****) L. reuteri JCM1112 and PTA 6475 strains use homologue repeat domains (green) to bind and penetrate to the mucus. Less homologous domains of Lar_0958 were also observed (blue).*

These adhesion proteins not only contribute to adhesion and probiotic effects, but also correlate with the expression of host-clade MucBPs (154, 158). *L. reuteri* adhesins include MucBPs such as MUB (154, 158, 159) and CmbA (154, 160), and serine-rich-repeat proteins (161, 162). Upon recognition by *lamina propria* 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.

3. 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.

4. MATERIALS AND METHODS

4.1 Bacterial strains

In the first part of this study, *L. reuteri* ATCC PTA 6475 and *L. reuteri* ATCC PTA 6475 lar_0958/CmbA-KO mutant (6475-KO) bacteria (154), and *L. reuteri* ATCC 53608 and 1063N MUB-KO isolates (158) were used. The *L. reuteri* strains were kindly provided by 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, member of the original Schaedler's flora), *M. morganii* and *B. subtilis 090*. *M. morganii* was provided by Nadiya Boyko (Uzhhorod National University, Uzhhorod, Ukraine).

4.2 Reagents

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

4.3 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. PBMCs were separated by standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from peripheral blood mononuclear cells (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 x 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.

4.4 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 (MRS, 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 $OD_{600nm} \times 2.5 \times 10^8$ CFU/ml. Human moDC cultures were activated with TLR4, the specific ligand of bacterial 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.

4.5 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 65ºC 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 4ºC. FITC-labeled bacteria were washed three times with cold PBS and were co-incubated with moDCs for three hours at 37ºC or 4ºC at a moDC : bacteria ratio of 1 : 20. MoDCs positive for FITC-labelled bacteria were analyzed by flow cytometry.

4.6 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-PE (BD Biosciences, Franklin Lakes, NJ, USA), CD1a-allophycocyanin (APC), CD40-FITC (BioLegend, San Diego, CA, USA), CX_3CR1-PE, CD80-FITC, CD83-FITC, CD86-PE, DC-

SIGN-FITC, CCR7-PE, CD14-PE (R&D Systems, Minneapolis, MN, USA), B7RP1 (ICOSL)-PE (EBiosciences), 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-aminoactinomycin-D (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).

4.7 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).

4.8 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.

4.9 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 (PBLs) 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 (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).

4.10 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 (GalCer) (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.

4.11 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 horseradish-peroxidase (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.

4.12 *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 enzyme-linked immunosorbent assay (ELISA) 96-well plates (BD Biosciences) at 38.5 µg/ml concentration followed by overnight incubation at 4ºC. 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.

4.13 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 $P < 0.05$. Significance was indicated as *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.0001$.

5. RESULTS

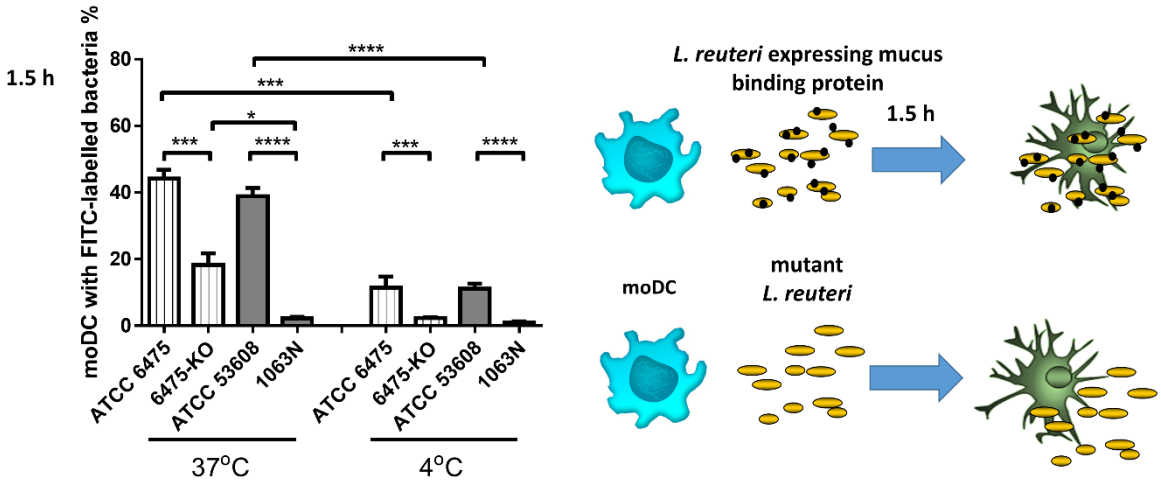
5.1 *The role of Lactobacillus reuteri-derived mucus binding adhesins in the modulation of moDC-mediated inflammatory immune responses*

5.1.1 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 (154, 160) 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 (**Figures 12A, B**). Moreover, there were significant differences (p=0.0142) 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.

A



B

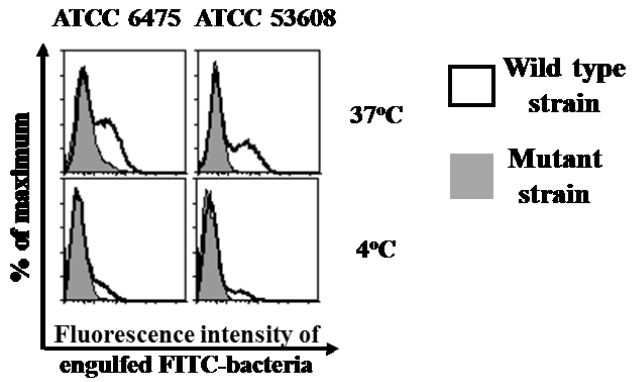


Figure 12. The phagocytic capacity of moDCs co-cultured with L. reuteri is strain dependent. *Human moDCs were differentiated in the presence of GM-CSF, IL-4 for two days. On day 2, moDCs were co-cultured with heat-inactivated bacteria at 37°C or at 4°C (**A** and **B**) for 1.5 h at a moDC:bacteria ratio of 1:20. Bacterial uptake was measured by flow cytometry. The number of moDC carrying phagocytosed FITC-labeled bacteria was calculated from 3 independent experiments +SD. Histogram overlays show one of three independent experiments. Statistical analysis was performed by the Student's t-test.*

5.1.2 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 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 **(Figures 13A, D**). Co-culturing moDCs with *L. reuteri* strains for 24 h also induced cell surface expression of the co-stimulatory molecules CD80 and CD86 (**Figures 13B**) and HLA-DR (**Figure 13C**). However, the co-stimulatory potential was reduced exclusively by 1063N.

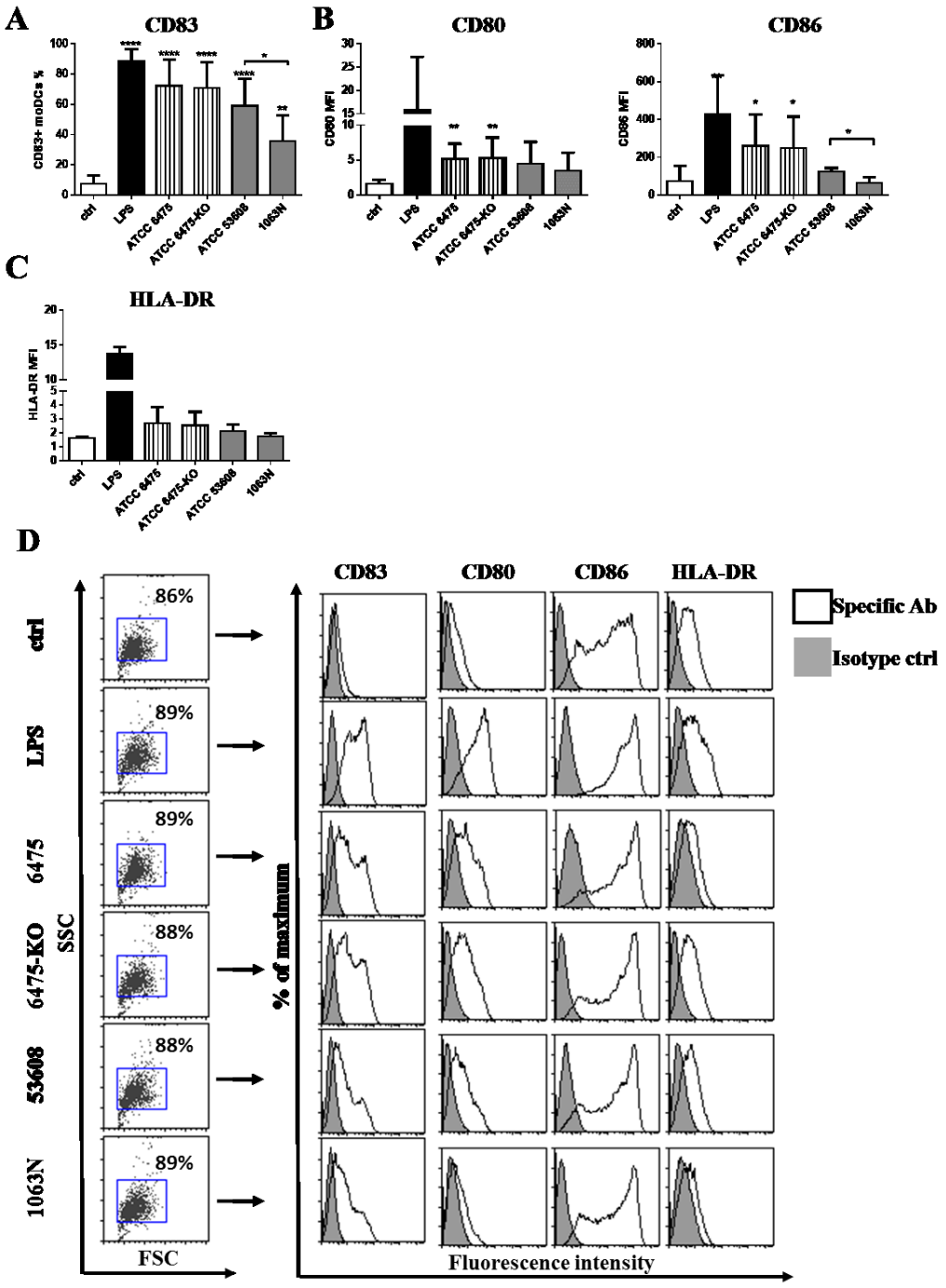


Figure 13. L. reuteri strains modulate the expression of cell surface activation markers in moDC. *MoDCs were co-incubated with live L. reuteri strains or LPS as a control, for 24 h. Expression of moDC-associated activation marker CD83 (**A**), the co-stimulatory molecules CD80 and CD86 (**B**) and the MHCII-protein HLA-DR (**C**) were measured by flow cytometry. Mean values were calculated from 5 independent experiments +SD. Histogram overlays show one of five independent experiment (**D**). Statistical analysis was performed by the Student's unpaired two-tailed t-test.*

The level of moDC-derived pro-inflammatory cytokines (TNF-α, IL-1ß, IL-6) and that of the T-lymphocyte polarizing cytokines (IL-10, IL-12, IL-23) 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 (**Figure 14A**). 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 (**Figure 14B**). 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 (**Figure 14C**). These results demonstrated that mucus adhesins expressed by *L. reuteri* strains have the potential to enhance pro-inflammatory cytokine production in moDCs.

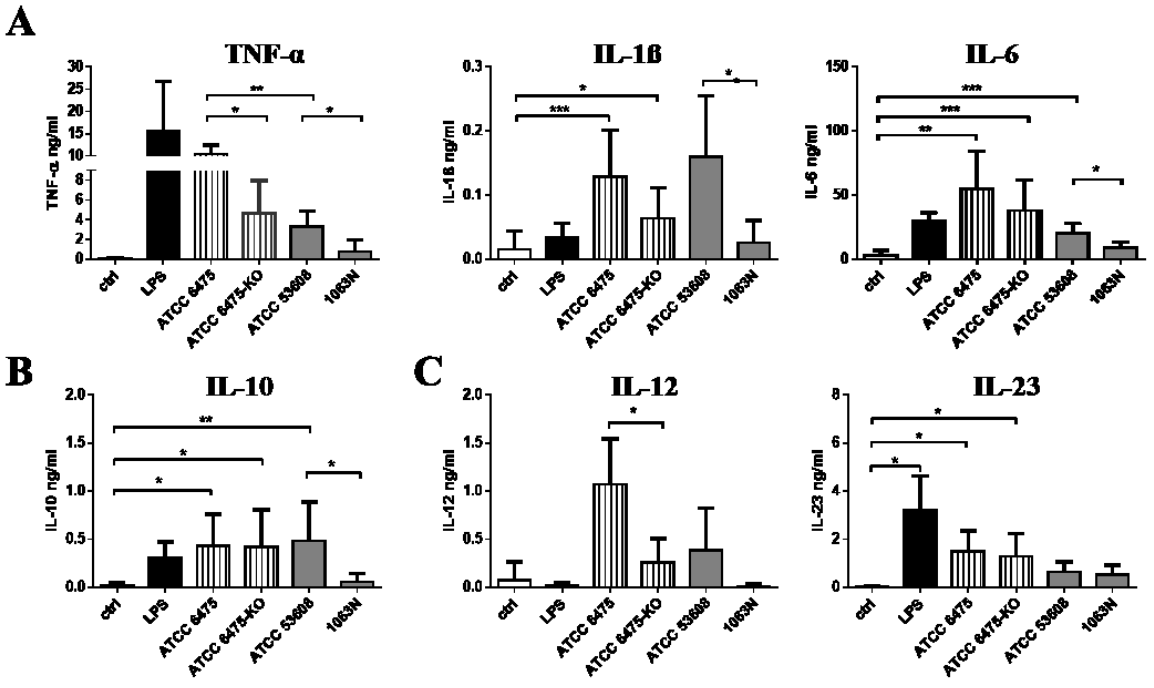


Figure 14. In moDCs the secretion of inflammatory and regulatory cytokines is modified in a L. reuteri strain-dependent manner. *MoDCs were co-incubated with L. reuteri strains or with LPS as control for 24 h. The concentration of the TNF-α, IL-1ß, IL-6, (**A**) IL-10 (**B**), IL-12, IL-23 (**C**) cytokines was measured by ELISA in 5 independent experiments. Mean values +SD are shown.*

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 used as a control, 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 (**Figure 15A**) and resulted in increased IL-17 production (**Figure 15B**), 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.

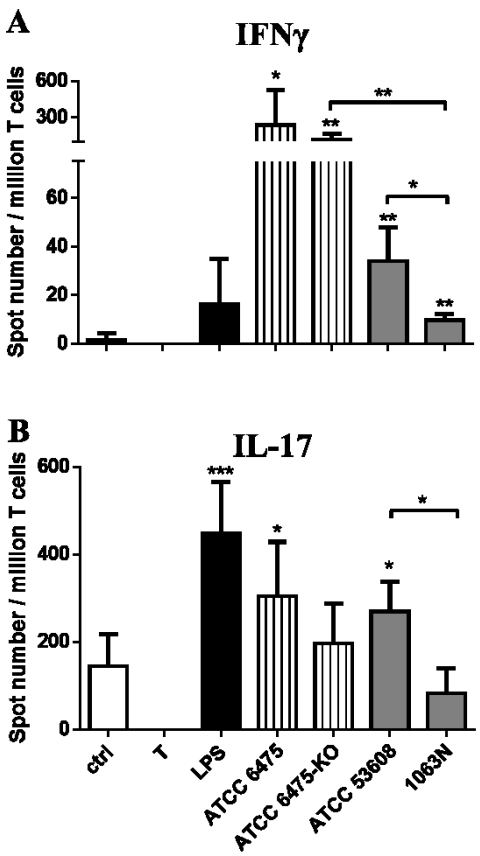


Figure 15. L. reuteri mucus adhesins modulate moDC-mediated T-lymphocyte polarization. *The T cell activating and polarizing capacity of moDCs was monitored by activating moDCs with L. reuteri strains or with LPS followed by co-culturing with T cells. Freshly isolated PBLs were obtained and co-cultured with autologous moDCs for 4 days. The mean values of spot numbers were calculated from five independent experiments + SD. The number of cytokine producing T-lymphocytes induced by LPS or moDCs exposed to L. reuteri strains was measured by ELISPOT assays for IFNγ (**A**) and IL-17 (**B**). Non-stimulated T cells (T) are served as negative controls.*

5.1.3 Purified mucus-binding protein (MUB) triggers CD83 expression and provokes Th1 polarized immune responses in moDCs

To further characterize the immunomodulatory potential of *L. reuteri* strains, native MUB was purified from *L. reuteri* ATCC 53608 spent medium, resulting in fractions containing either a MUB:glycolipid mixture, or separate MUB and glycolipid fractions together with CHAPS employment (data not shown**)**. When we tested the inflammatory nature of purified, endotoxin-free MUB as compared to LPS, and the glycolipid fraction in

the course of the moDC-regulated immune response, the increasing concentrations of purified MUB induced CD83 expression on the moDC cell surface (**Figures 16A, C**). The cell surface expression of the co-stimulatory molecule CD80 was also enhanced independent of MUB concentration, although this effect did not reach statistical significance (**Figures 16B, C**). The level of moDC-secreted pro-inflammatory cytokines, including TNF-α, IL-1ß, IL-6, IL-12 and the anti-inflammatory cytokine IL-10 depended on the concentration of MUB, except in the case of IL-6. When moDCs were cultured in the presence of 0.5 or 1 µg/ml MUB, the production of IL-10 cytokine, known to have regulatory potential, was found to be in the logarithmic range of 0.01 – 0.1 ng/ml, while the concentration of the pro-inflammatory cytokine IL-12 varied within 0.1 to 1 ng/ml range (**Figure 16D**). Furthermore, moDC cultures activated by LPS or MUB induced Th1 polarized immune responses associated with increased IFNγ production (**Figure 16E**) in line with the increased concentration of IL-12.

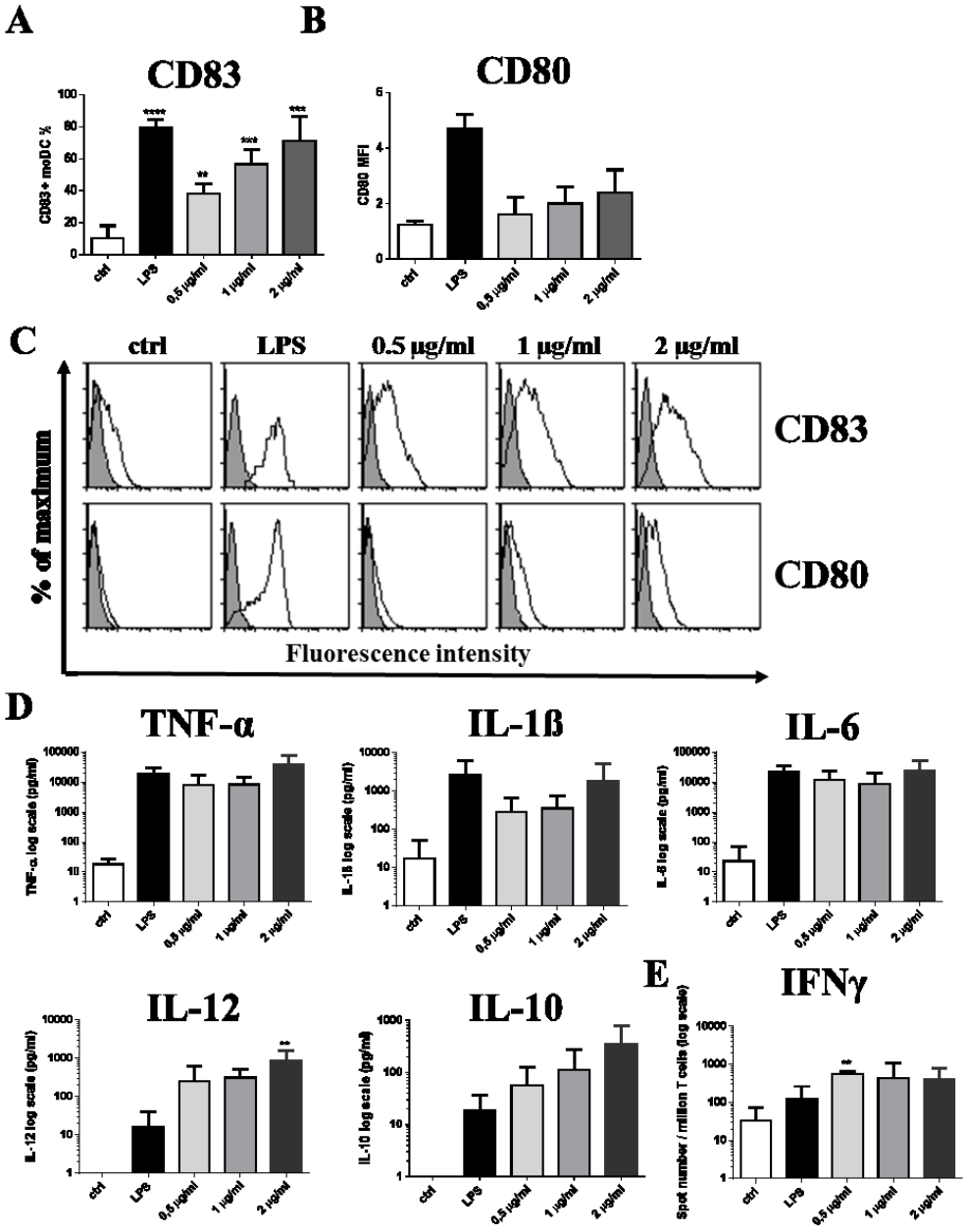


Figure 16. Purified MUB protein triggers CD83 expression and induces Th1 polarized immune responses. *Increasing concentrations of purified MUB was used to activate 2-day moDC cultures. LPS was used as a positive control. Cells were activated for 24 h and the cell surface expression of CD83 (**A**) and the co-stimulatory molecule CD80 (**B**) was measured by flow cytometry. Median-fluorescence intensity (MFI) and the ratio of CD83 positive cells were calculated from three independent experiments +SD. Histogram overlays are shown for one independent experiment (**C**). Production of inflammatory cytokines TNF-α, IL-1ß, IL-6, IL-12 and the regulatory cytokine IL-10 in the supernatants of moDCs was detected after 24 h by ELISA. Mean values of cytokine concentrations were calculated from 3 independent experiments +SD (**D**). Freshly isolated PBLs were co-cultured with autologous moDCs for 4 days. The number of cytokine-producing PBLs in response to LPS as control or MUB-exposed human moDCs was measured by the IFNγ ELISPOT assay. Mean values of spot numbers were calculated from 3 independent experiments +SD (**E**).*

The extracted lipid fraction also induced CD83 and CD86 expression on the moDC cell surface to a similar extent as induced by the lipid containing MUB fraction (**Figure 17A**). However, pure MUB lacking glycolipids was associated with the reduced secretion of IL-12 and was unable to induce IL-23 production by moDCs **(Figure 17B**). Furthermore, the lipid fraction was unable to induce IL-12 or IL-23 production suggesting that the immunogenicity of MUB can be enhanced by the lipid component of this fraction.

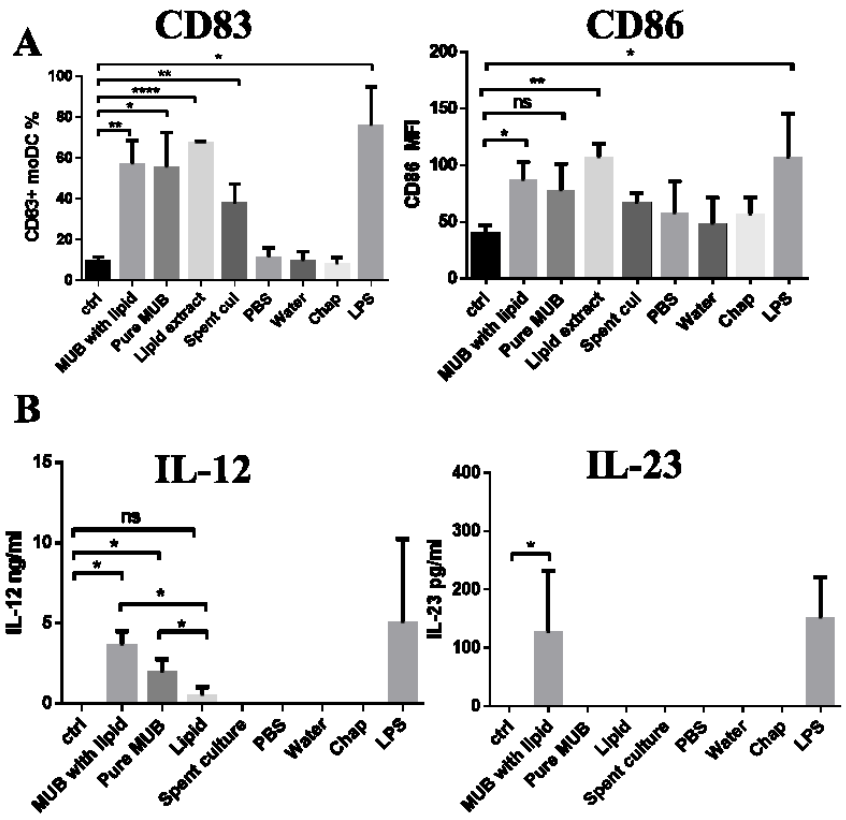


Figure 17. Immunomodulatory properties of MUB-associated glycolipids. *Purified MUB protein, lipid containing MUB and lipid extract was tested for their moDC-activating capacity in a 2-day moDC culture. 250 ng/ml LPS served as positive control, spent culture media, PBS, destilled water and CHAP were used as negative controls. Cells were activated for 24 h and the cell surface expression of CD83 and the co-stimulatory molecule CD80 was measured by flow cytometry (**A**). MFI and the ratio of CD83 positive cells was calculated from three independent experiments +SD. Production of the T cell polarizing cytokines IL-12 and IL-23 was detected after 24 h from the supernatants of moDCs by ELISA (**B**). Mean values of cytokine concentrations were calculated from three independent experiments +SD. NS=non significant.*

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

To get insight into the mechanisms mediating the interaction of MUB with moDCs, we first tested the binding of MUB to reporter cells expressing specific CLRs on the cell surface. In collaboration with Dr. Nathalie Juge and her colleagues it was detected that MUB bound to mDectin-2 and SIGN-R1 reporter cells in a dose-dependent manner significantly. However, using the same approach, no binding was detected between MUB and murine Dectin-1. The binding of MUB to DC-SIGN was further investigated by force spectroscopy (data not shown).

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 (**Figure 18**) 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 (data not shown).

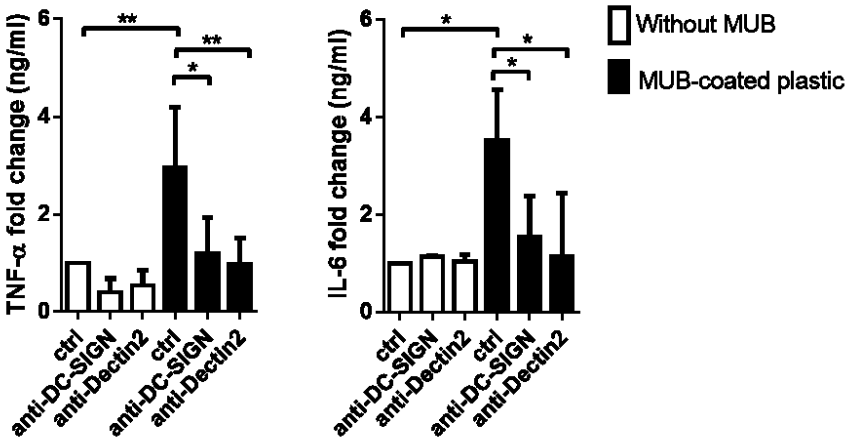


Figure 18. The molecular background of bacterial MUB adhesin – moDC interaction causing inflammation *MUB immobilized on a microtiter plate was incubated with 2-day moDC cultures treated with anti-Dectin-2 or anti-DC-SIGN antibodies. Unlabeled (ctrl) moDCs incubated on MUB-coated and uncoated wells served as negative and positive controls, respectively. The production of pro-inflammatory TNF-α and IL-6 cytokines was measured by ELISA after 24 h. Mean values of cytokine concentrations was calculated from 5 independent experiments +SD.*

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 (data not shown). To confirm

the involvement of Dectin-2 and DC-SIGN in the activation of moDC-mediated T-cell differentiation, neutralizing antibodies targeting these cell surface CLRs were used in the presence of *L. reuteri* ATCC 53608 and 1063N (**Figure 19**). 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.

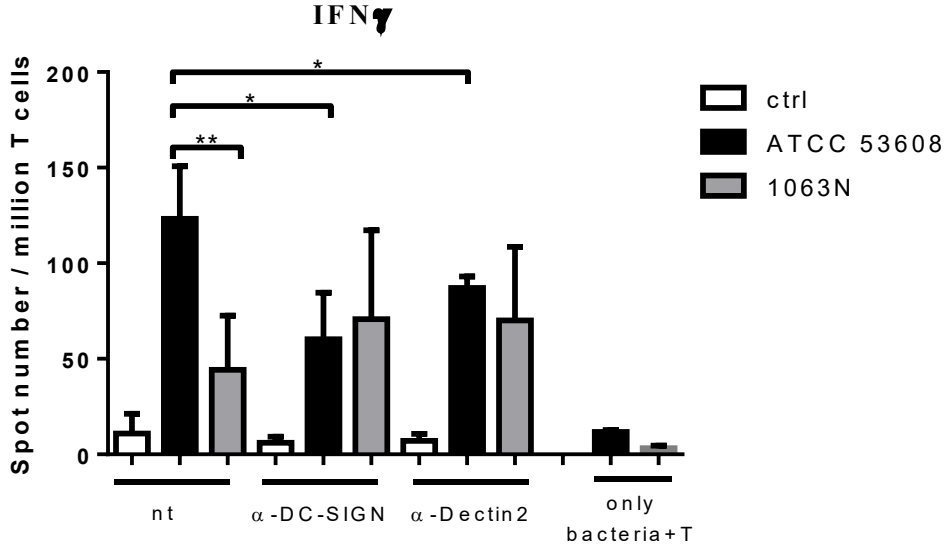


Figure 19. The molecular background of bacterial MUB adhesin – moDC interaction triggering Th1 polarization. *MoDCs treated with anti-Dectin-2 or anti-DC-SIGN antibodies were co-cultured with live L. reuteri ATCC 53608 and 1063N strains for 1.5 h at 37°C, respectively. Freshly isolated PBLs were co-cultured with autologous moDCs for 4 days. The number of cytokine producing T cells was measured by IFNγ ELISPOT assay. Mean values of spot numbers were calculated from 5 independent experiments +SD.*

5.2 *Characterization of gut commensal bacteria based on their effects on dendritic cell-induced immune responses*

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 from Vit-A in the intestine upon commensal-induced immunity, which may act on moDC differentiation and functions.

5.2.1 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 (**Figure 20A**) 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 (**Figure 20B**). Moreover, on day 2 and 3 the differentiation of moDCs could be re-programmed to induce CD1d while inhibited CD1a expression, respectively (data not shown). Importantly, the cell surface expression of the DC-SIGN remained constant at these conditions (**Figure 20C**), while ATRA maintained the expression level of CD14 (**Figure 20D**) suggesting a decelerated differentiation phase of moDCs.

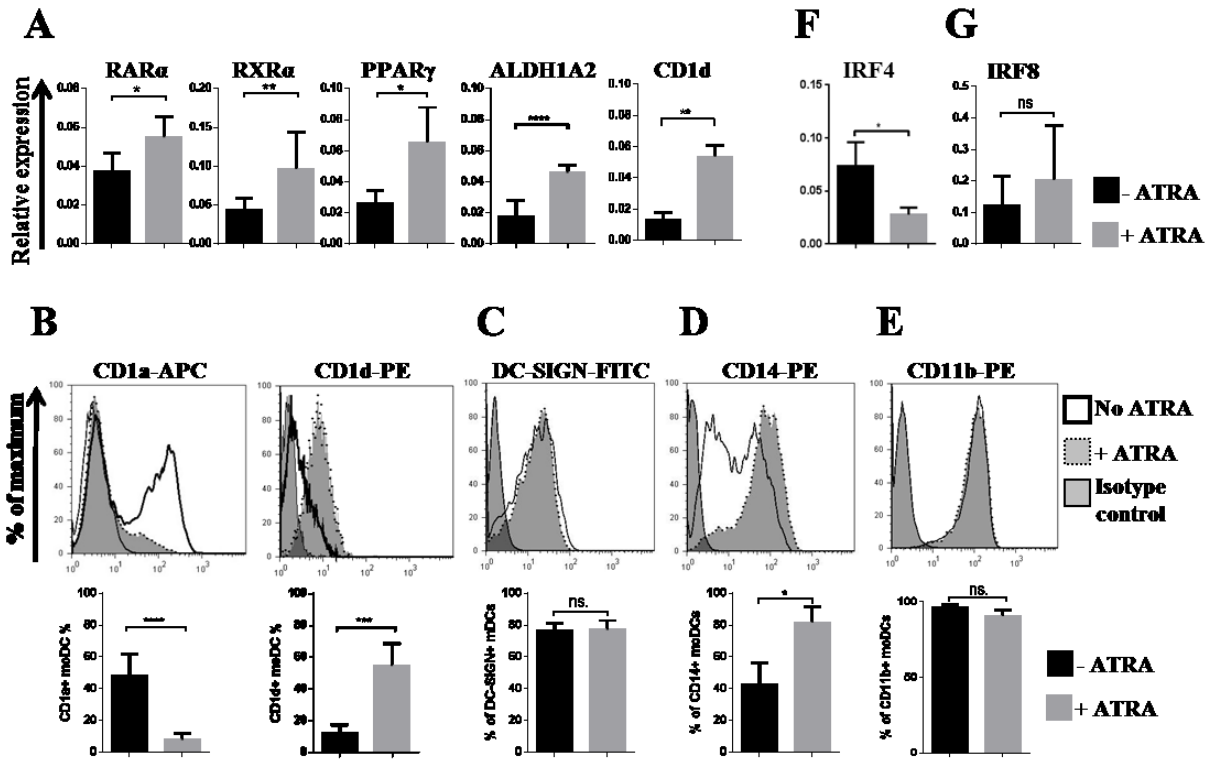


Figure 20. The effects of ATRA on human moDC differentiation. *Two-day moDCs were differentiated in the absence or presence of 1 nM ATRA. The relative gene expression levels of RARα, RXRα, PPARγ, ALDH1A2, CD1d (**A**), IRF4 (**F**), IRF8 (**G**) was measured by quantitative real time PCR and the cell surface expression level of CD1a, CD1d (**B**), DC-SIGN (**C**), CD14 (**D**) and CD11b (**E**) was measured by flow cytometry. Mean values of relative mRNA levels and the ratio of moDCs positive for the measured cell surface proteins were calculated from five independent experiments +SD. Student's unpaired two-tailed t-test was used in the statistical analysis with significance defined as * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001.*

DCs can also be classified according to the expression levels of the transcription factors guiding both DC differentiation and re-programming (18, 163). In this human *in vitro* model system, we also found that moDCs express CD11b independent on the presence of ATRA (**Figure 20E**). Interestingly, ATRA was able to down modulate the gene expression levels of IRF4 (**Figure 20F**), while up regulated the cell surface expression of CD103 (**Figure 21F**). Importantly, the relative mRNA level of IRF8, responsible for regulating CD103 protein expression in DCs (30) remained unaffected by ATRA (**Figure 20G**). 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.

5.2.2 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

Beside the novel finding showing that the outcome of the inflammatory response of moDCs to engulfed commensal bacteria is determined by the unique characteristics of the tested microbes (4), we were able to follow up the immunomodulatory properties of a given microbe though monitoring the activation state and the direction of cell polarization of moDC-mediated autologous T 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 (**Figure 21A**). In contrast, the Th17 response could be activated by all of the tested species (**Figure 21B**). 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 (**Figures 21C, E**). 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 (**Figure 21D**). CD1d expressing moDCs are able to induce iNKT cell expansion through the ligation of invariant TCR with CD1d - α-GalCer complexes (50). However, we could not identify significant expansion of iNKT cells in moDC – T cell co-cultures with prior commensal stimuli suggesting that the tested commensals or the applied dose of bacteria do not serve ligands for the lipid receptor CD1d (**Figure 21F**).

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.

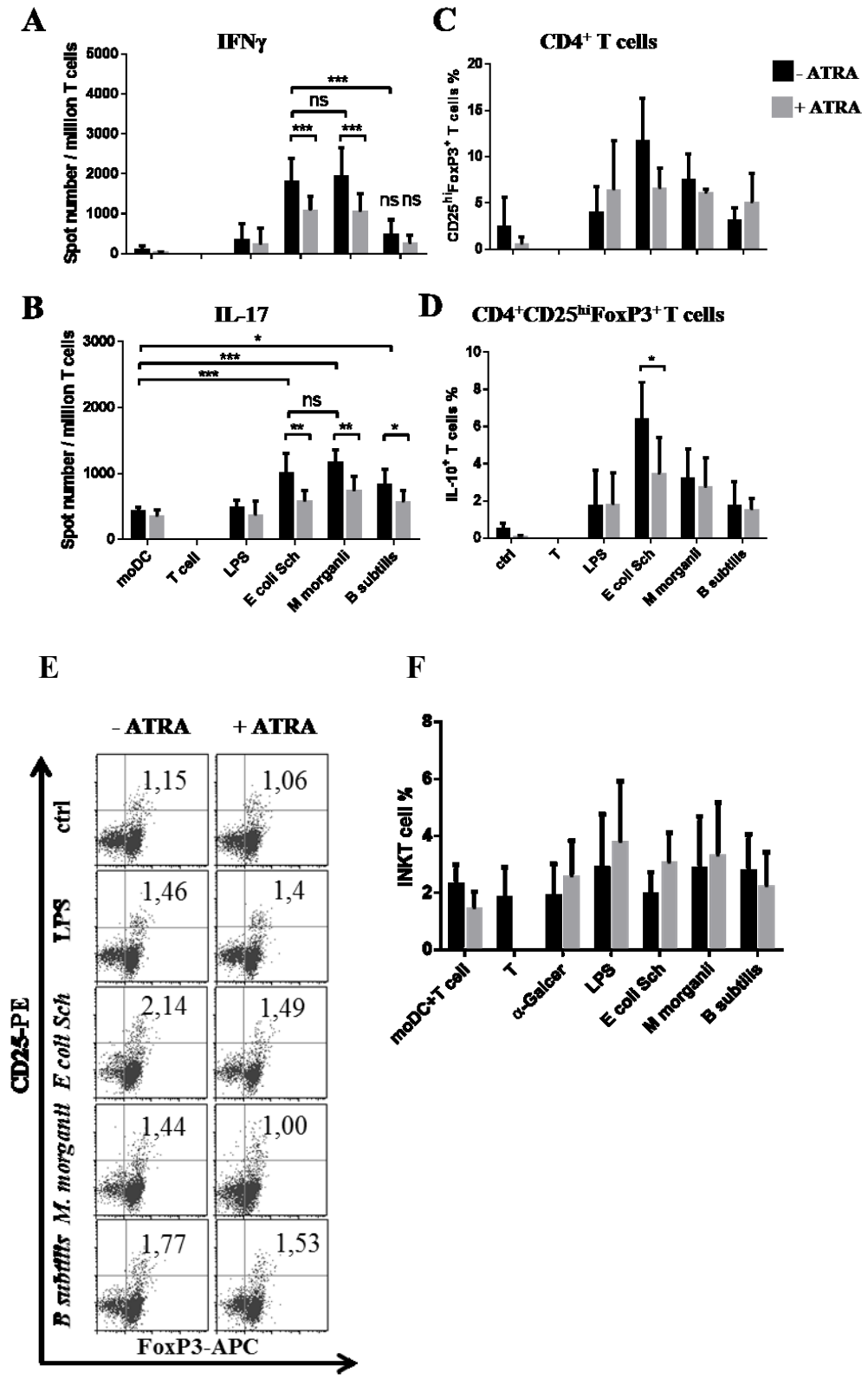


Figure 21. Monitoring moDC-mediated T-lymphocyte polarization induced by commensal stimuli. *The T cell polarizing capacity of moDCs was monitored in moDC stimulated with*

*E. coli Schaedler, M. morganii and B. subtilis or LPS followed by co-culturing the cells with freshly isolated autologous T cells for 4 days. The number of cytokine producing T-lymphocytes induced by LPS or by moDCs exposed to commensal bacteria was measured by IFNγ (**A**) and IL-17 (**B**) ELISPOT assays. The mean value of spot numbers was calculated from five independent experiments +SD. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. To detect the number of Treg cells, resting and stimulated moDCs were co-cultured with PBL for six days. The ratio of $CD25^{hi}FoxP3^{+}$ Th cells (**C, E**) and the IL-10 producing Treg cells (**D**) were analyzed by flow cytometry, respectively. Dot-plots show one out of five independent experiments. The mean value of Treg cell numbers was calculated from 5 independent experiments +SD. To detect the number of iNKT cells (**F**), moDCs were stimulated with live bacteria or with LPS followed by co-incubation with autologous PBL for five days, and the moDC cultures were incubated with the CD1d-ligand α-GalCer served as a positive control. The ratio of CD3+ cells expressing Vα24Vβ11 TCR was analyzed by flow cytometry. The mean values of iNKT cell numbers was calculated from three independent experiments +SD. In the statistical analysis, ANOVA followed by Bonferroni's multiple comparison tests were used.*

5.2.3 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 (**Figure 22A**). 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 (**Figures 22B, C**), but had no effect on CD103 expression in $RAR^{lo}IRF4^{hi}$ moDCs (**Figures 22D, E**). Moreover,

ATRA-conditioned moDCs down regulated the cell surface expression of CD103 while up regulated the CX_3CR1 receptor by the stimulation with commensal bacteria. 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, even though the viability of moDCs remained intact as compared to the immature cells (**Figure 22H**).

5.2.4 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 (**Figures 22F, G**). 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. It was also observed that in the absence of ATRA the gated $CD1a^{-}$ and $CD1a^{+}$ moDC fractions engulfed the tested bacteria with similar activities as the $CD1a^{+}$ cells (data not shown). Consequently, the MFI values within the gated moDC populations of the FITC-labeled bacteria remained similar demonstrating that the efficacy of moDC-mediated phagocytosis depends on both the unique features and the species of the engulfed bacteria, and this effector mechanism can be further enhanced by ATRA.

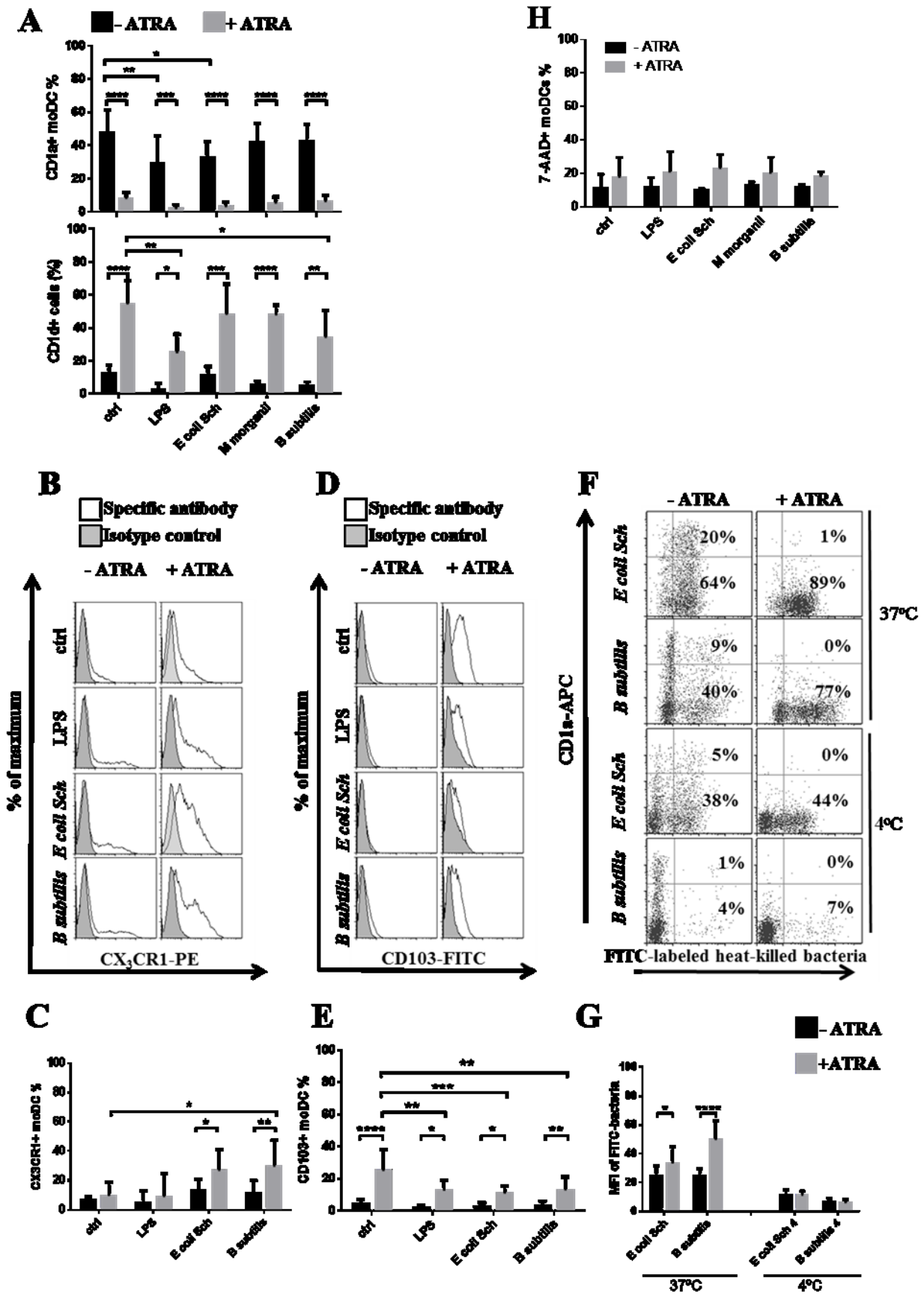


Figure 22. ATRA shifts the cell surface expression pattern of CD1, gut-related receptors and the phagocytic capacity of moDCs in an ATRA and commensal strain dependent

manner. *Human moDCs were differentiated in the presence of GM-CSF and IL-4 with or without 1 nM ATRA for two days. The surface expression level of CD1a and CD1d was measured on resting cells and moDCs activated with live commensal bacteria for 24 h (**A**) by flow cytometry. Histogram overlays show results derived from one representative donor of ten. The cell surface expression level of the mucosa-related CX_3CR1 (**B, C**) and CD103 (**D, E**) was measured by flow cytometry followed by a 24 h activation period with live commensal bacteria or LPS served as a positive control. Mean values showing the ratio of moDCs positive for the measured surface protein were calculated from five independent experiments +SD. To monitor the phagocytic capacity of moDCs, on day 2 moDCs were co-cultured with heat-inactivated and FITC-labeled bacteria at 37°C or at 4°C for three hours at a moDC : bacteria ratio of 1 : 20. (**F, G**) Dot plots show one of four independent experiments. The ratio of moDC positive for heat-inactivated and FITC-labeled bacteria was measured by flow cytometry. The number of moDCs carrying FITC-labeled bacteria was calculated from four independent experiments +SD. (**H**) On day 2, moDCs were co-incubated with live commensal bacteria for 24 hours followed by labelling the cells with 7-AAD dye. Mean values of moDCs positive for 7-AAD staining were calculated from 5 independent experiments +SD. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001.*

5.2.5 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 (**Figure 23A**). 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 (**Figure 23B**). 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 (**Figure 23C**). 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* (data not shown).

These results collectively indicate that *E. coli Schaedler* and *B. subtilis* harbor individual moDC-provoking potential, while ATRA can boost the production of pro-inflammatory mediators. In contrast to this finding, the expression level of CCR7 becomes

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.

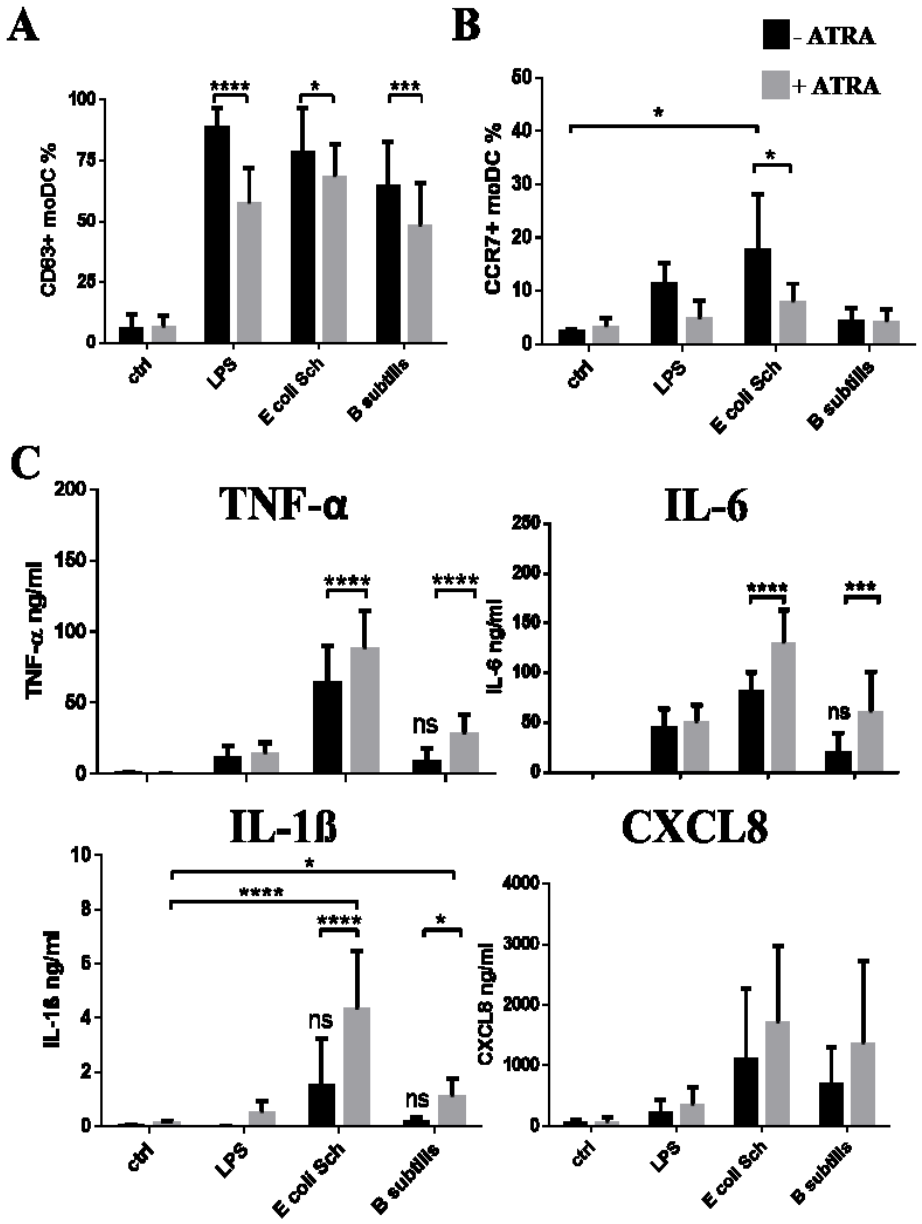


Figure 23. Characteristics of the inflammatory and migratory potential of moDC populations induced by commensal bacteria. *Two-day moDCs were co-incubated with live commensal strains or with 250 ng/ml LPS used as control for 24 hours. Expression of the moDC-associated activation marker CD83 (**A**) and CCR7 (**B**) was measured by flow cytometry. Mean values were calculated from 5 – 7 independent experiments +SD. The*

*concentration of TNF-α, IL-1ß, IL-6 pro-inflammatory cytokines and the chemokine CXCL-8 (**C**) was measured by ELISA followed by a 24-hour activation of moDC in five independent experiments. Mean values +SD are shown. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001.*

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

The first signal for Th cell activation derives from the interaction of the TCR with antigen presenting proteins such as HLA-DQ and HLA-DR loaded with peptide and inducible by LPS or by the selected microbiota strains (**Figure 24A**). When moDCs were exposed to LPS or to commensal bacteria, the cell surface expression of the CD80 and CD86 co-stimulatory molecules was increased (**Figure 24B**). 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 (**Figure 24C**). Interestingly, *B. subtilis* was unable to induce IL-23 secretion and the level of IL-12 also remained lower than the effect provoked by moDCs in the presence of the Gram-negative commensal bacterium *E. coli Schaedler*.

Considering that the differentiation of T-lymphocytes is regulated by both co-stimulatory and inhibitory signals, the cell surface expression of known co-stimulators of T-lymphocytes were also monitored. The results revealed that the cell surface expression of the co-stimulatory molecule CD40 could be induced by LPS and also by the two commensal strains, and this effect could be slightly enhanced in RARα^{hi}IRF4^{low} moDCs upon activation by *E. coli Schaedler* (**Figure 24D**). The induction of the effector T cell inhibitor PD-L1 could also be achieved if moDCs were stimulated by *E. coli Schaedler,* in contrast to *B. subtilis* or LPS without such effects **(Figure 24E)**. 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.

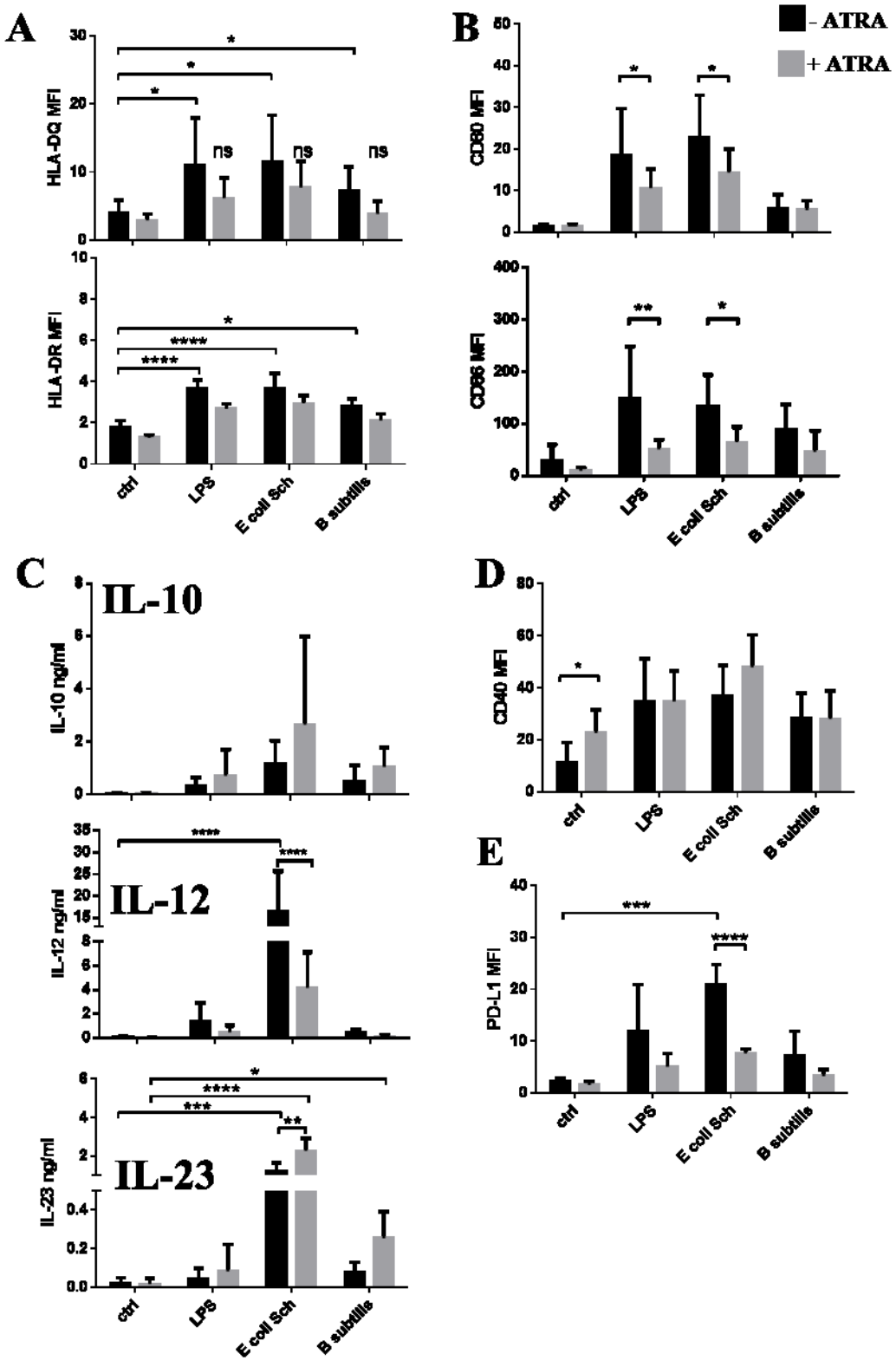


Figure 24. The T-lymphocyte activating and polarizing capacity of moDCs activated by selected commensal bacteria. *Two-day moDCs were co-incubated with live commensal strains or with 250 ng/ml LPS used as control for 24 hours. The expression levels of HLA-DQ and HLA-DR (**A**), the co-stimulatory proteins CD80, CD86 (**B**), CD40 (**D**) and the inhibitory molecule PD-L1 (**E**) was measured by flow cytometry. Mean values of median fluorescence intensities (MFI) were calculated from 5 – 7 independent experiments +SD.*

*The concentration of IL-12, IL-23 and IL-10 cytokines was measured by ELISA followed by a 24-hour activation of moDC and was tested in seven independent experiments (**C**). Mean values +SD are shown. ANOVA followed by Bonferroni's multiple comparison tests was used in the statistical analysis with significance defined as * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. NS= non sigficant as compared to the control moDC.*

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

In a next step, we addressed the question how T-lymphocyte stimulation and maturation may modulate moDC responses in the presence of ATRA or commensal bacteria. Taken the fact that 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 (**Figures 25A, B**). 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.

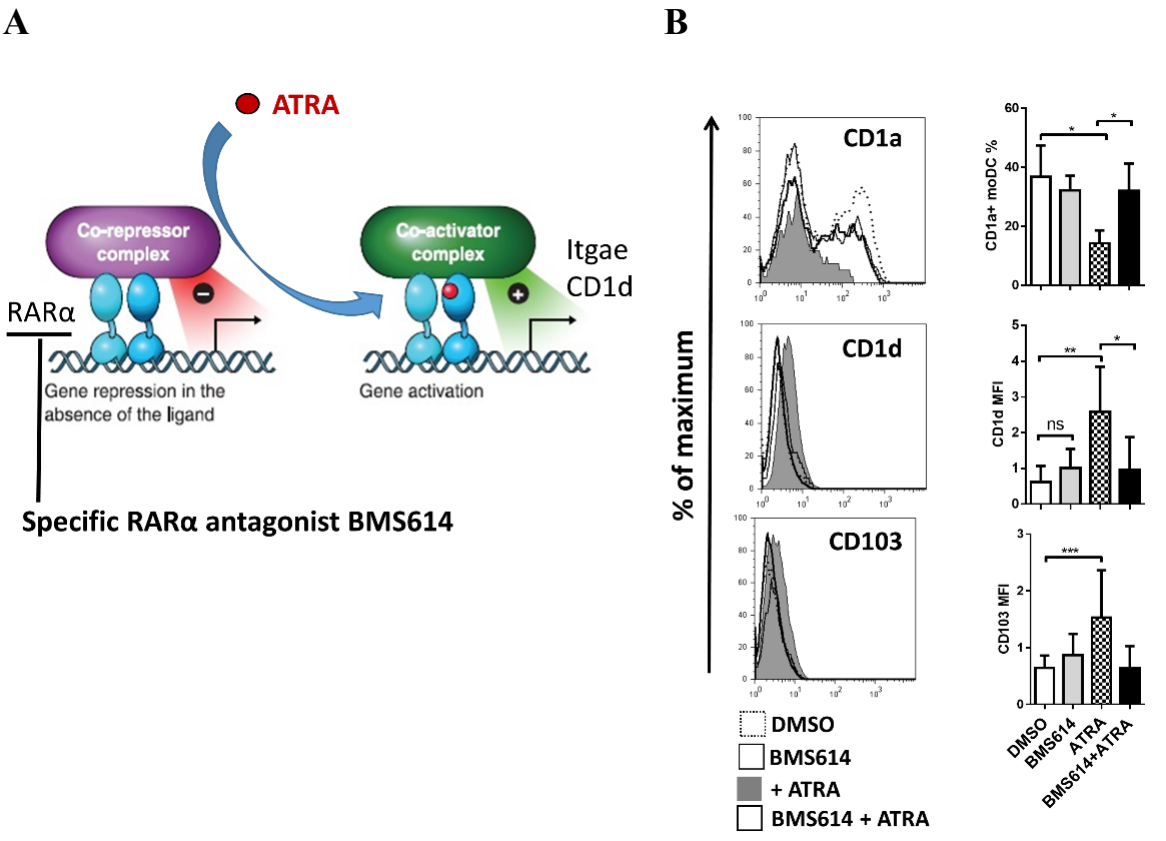


Figure 25. The selective inhibition of RARα prevents the ATRA-induced signature of microbiota-generated immune responses mediated by moDCs. *(A) Freshly isolated monocytes were treated with or without 1 µM BMS-614 natural RARα-antagonist. After 75*

*minutes of incubation, cells were differentiated in the presence or absence of natural RARα-agonist ATRA for two days. (**B**) To analyze how ATRA acts on the moDC-mediated immune response against microbiota species the cells were treated with the RARα antagonist BMS614 by prior to treat the cell culture medium with ATRA. The cell surface expressions of CD1 proteins and mucosa-related CD103 encoded by the ITGAE gene were measured by flow cytometry in two-day moDCs.*

In a further step, we also demonstrated that the enhanced secretion of the pro-inflammatory cytokines (**Figure 26A**) and IL-23 (**Figure 26B**) induced by commensal bacteria could be ameliorated by the prior blockade of RARα. Moreover, the reduced antigen presenting capacity of the ATRA-conditioned moDCs could be restored by the inhibition of RARα (**Figure 26C**).

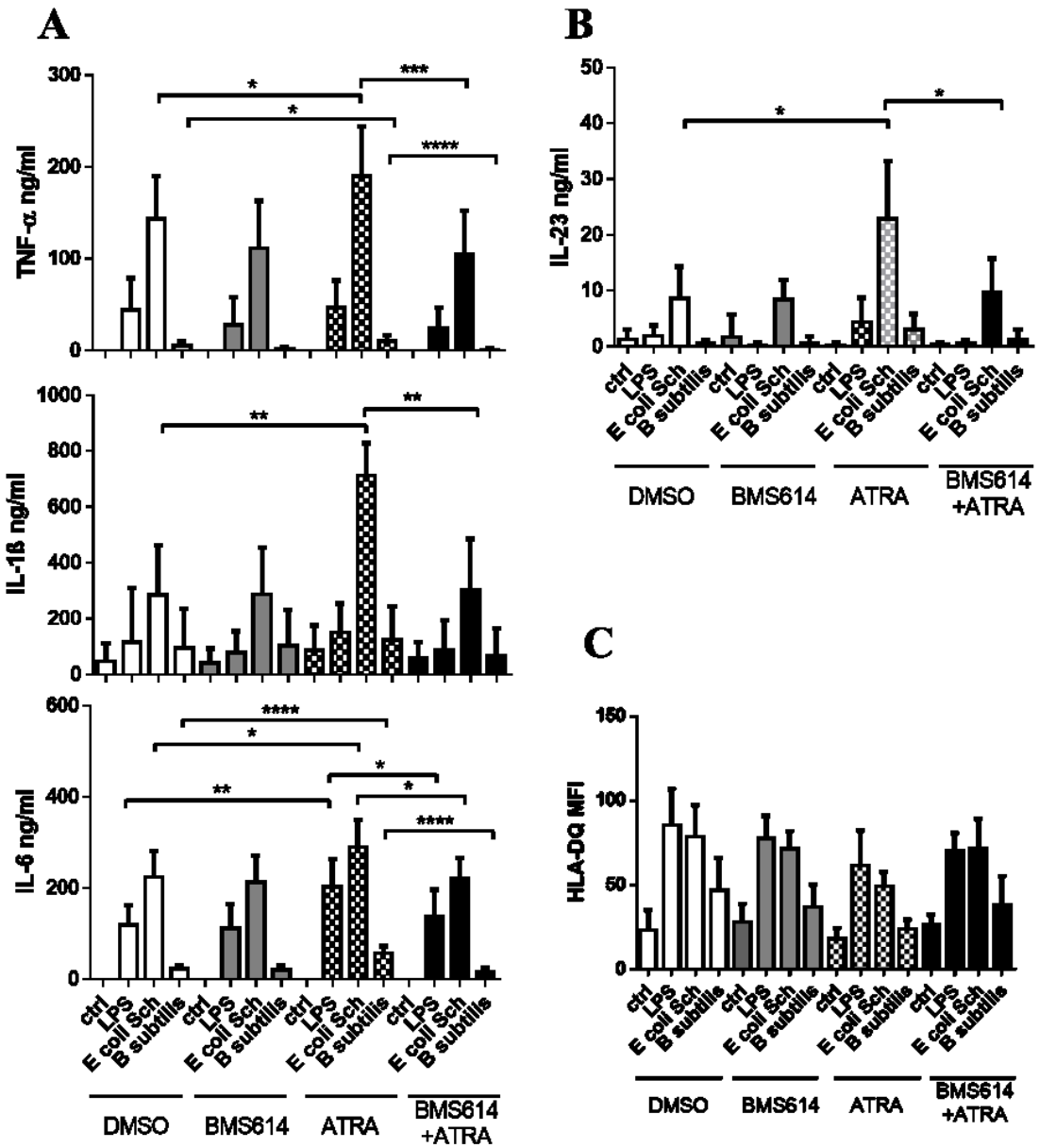


Figure 26. *The concentration of TNF-α, IL-6, IL-1ß (**A**) and IL-23 (**B**) was measured by ELISA followed by a 24-hour activation of moDC performed in seven independent experiments. Mean values +SD are shown. The cell surface expression level of HLA-DQ was measured by flow cytometry followed by a 24 h incubation period with live commensal bacteria. (**C**) Mean values of cells positive for the measured cell surface molecules were calculated from the results of seven independent donors +SD.*

Considering that the IRF4 transcription factor plays a pivotal role in setting the degree of DC-mediated antigen presentation (30), 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 (**Figure 27A**). 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 (**Figure 27B**) 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.

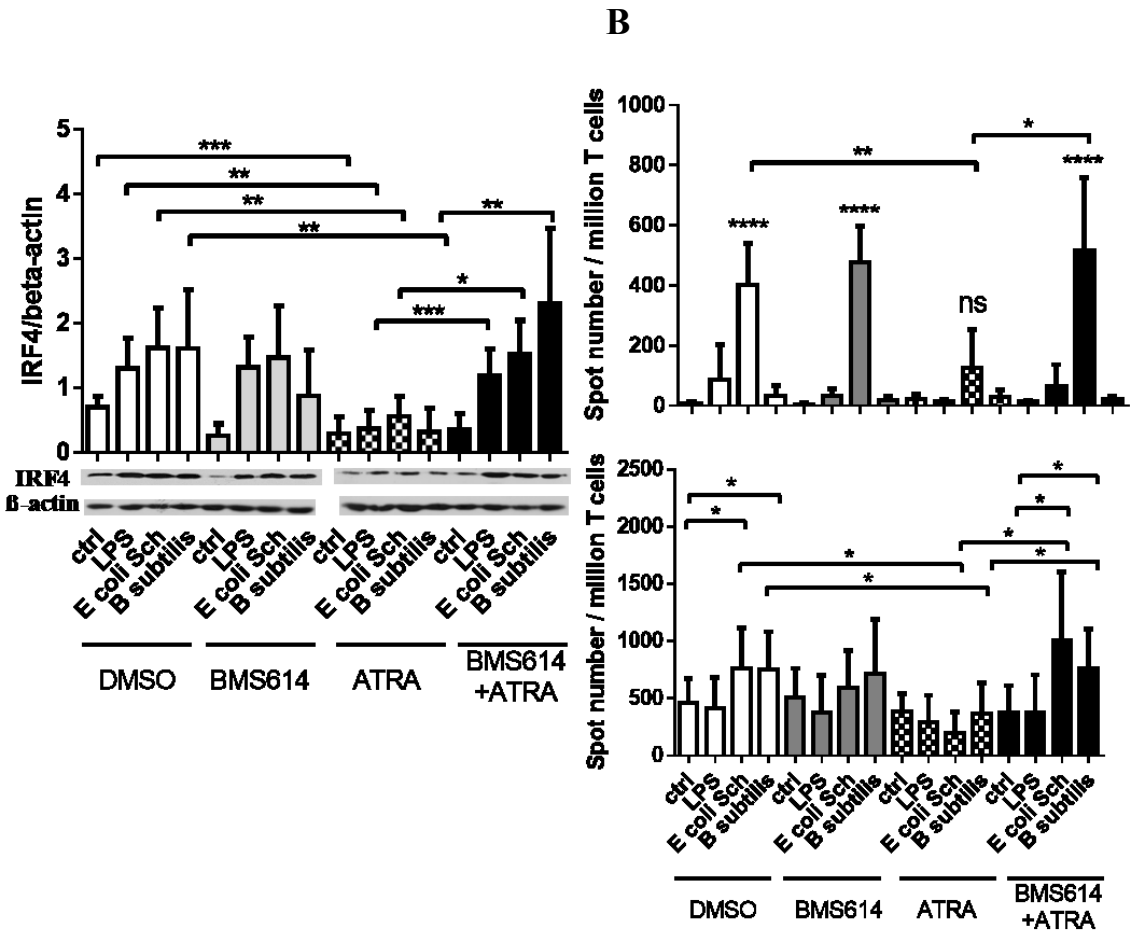


Figure 27. *(**A**) Two days moDCs were activated by live commensal bacteria for 24 hours and the relative expression levels of IRF4 protein was measured by Western-blotting. Bar graphs show IRF4/ß-actin ratios measured after 24 h of stimulation. Mean values of protein densities were calculated from five independent experiments +SD. The T cell polarizing capacity of moDCs was monitored in moDCs activated with the selected commensal strains or with LPS followed by co-culturing the cells with autologous T cells. Freshly isolated PBLs were co-cultured with autologous moDCs for 4 days. The number of cytokine producing T-lymphocytes, induced by LPS or moDCs exposed to E. coli Schaedler and B. subtilis were measured by IFNγ and IL-17 (**B**) ELISPOT assays. The mean value of spot numbers was calculated from five independent experiments +SD. Statistical analysis was performed by the Student's unpaired two-tailed t-test.*

6. 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 helper T-lymphocytes. Considering that the human commensal microbiota is personalized (164) 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 and Treg cells (117, 164, 165). It is also well established that pathogenic microbes and pathobionts, including fungal and bacterial species, are able to induce different types of immune responses (166, 167), 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. Based on our concept, the outcome of moDC differentiation is able to accommodate to unique cellular microenvironments (51, 168) and remains remarkably plastic until terminal differentiation of moDCs ensues. 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. To approach this goal designed *in vitro* conditions were created to analyze the canonical pathways leading to the ATRA-modulated expression of the contributing master transcription factors in line with the impact of individual commensal bacteria exerted on moDC-mediated inflammation and T-lymphocyte priming.

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 (146, 147). 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 (169). Christensen et al. 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 (170). Moreover, a recent study showed that *L. reuteri* strains derived from human-associated clades differed in the ability to modulate human cytokine production in stimulated myeloid cells (148). Although not fully discovered, 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. For example, the soluble factors of the *L. reuteri* CRL1098 strain was able to reduce TNF-α production by human PBMCs (171) and in mouse models (172). Furthermore, human moDCs generated in the presence of the soluble factors of *L. reuteri* DSM 17938 can down modulate LPS-induced IL-6, IL-10 and IL-23 secretion, while the of the regulatory cytokine TGF-ß remained unaffected (173). EPS isolated from the *L. reuteri* strain DSM 17938 and L26 Biocenol™ was recently shown to up regulate the mRNA level of IL-1β, TNF-α, IL-6 (174) and NF-κB. One of the putative *L. reuteri* surface proteins appeared to be of importance in the stimulation of THP-1 cells and also in the activation of NF-κB in U937-3xkB-LUC cells by *L. reuteri* strains (153). 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 (TNF-α, IL-1ß, IL-6) and that of the T-lymphocyte polarizing cytokines IL-12 and IL-23, 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. The observed responses were also shown to be dependent on the actual experimental settings depending on whether *L. reuteri* has been measured following *in vitro* inflammatory stimulation of monocytes by LPS-stimulation or in chemically-induced colitis models. All these factors may also modulate the phagocytic capacity of human monocytes and consequently the type and amount of cytokine production.

It is also worth noting that the *L. reuteri* PTA 6475 strain is generally considered as anti-inflammatory due its reported ability to inhibit or down regulate pro-inflammatory cytokine production in stimulated myeloid cells (147) including TNF-α, MCP-1, IL-1β, and IL-12, consistent with the known ability of *L. reuteri* strain ATCC 6475 to suppress LPS-induced intestinal inflammation (175). These results showed that, in the absence of stimulatory signals by LPS-induced PRRs, 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 pathway.

CLRs represent a subset of PRRs expressed by a broad spectrum of cells (176) and recognize a diverse range of endogenous and exogenous ligands including fungi, bacteria, parasites and DAMPs driving both innate and adaptive immunity (177, 178). The mechanism by which MUB exerts immunomodulatory effects was further investigated using a cell reporter assay specific for different CLRs showing that purified MUB from *L. reuteri* ATCC 53608 was recognized by mDectin-2 and SIGN-R1 but not by mDectin-1. We clearly 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 inflammatory 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. The direct interaction of MUB with DC-SIGN was further confirmed by force spectroscopy. Surprisingly, the uptake of *L. reuteri* ATCC 53608 in moDCs was not affected upon the selective blocking of DC-SIGN or Dectin-2, but played a crucial role in moDC-mediating Th1 type immune responses against *L. reuteri* ATCC 53608.

DC-SIGN expressed on the cell surface of immature DCs appears to be a general microbial receptor, which mediates downstream T cell activation (179). DC-SIGN signaling in DCs can also induce tolerance acting as an important mechanism in the maintenance of homeostasis (77, 180, 181), which can be elicited by certain *Lactobacillus* strains including *L. rhamnosus* (182), *L. acidophilus* (183), *L. reuteri* and *L. casei* (184). Interestingly, *Lactobacillus* cell surface proteins, such as *L. rhamnosus* proteinaceous pili (185) and *L. acidophilus* S-layer proteins (183, 186) have recently been implicated in the interplay of *Lactobacillus sp.* with DC-SIGN. *L. rhamnosus* GG pili binds to mucus (187) and interacts with MΦ (188). Furthermore, 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, IL-12p40, and IL-12p35 in DCs (185). The induction of these cytokines was partially dependent on DC-SIGN (185), and in agreement with the reported impact of pili on TLR-2 signaling (189, 190), 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 (188, 190, 191). 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 (192). 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 (193, 194). 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 upregulation of co-stimulatory molecules in moDCs, which in turn results in Th1 and Th17 polarization of the 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 mucus-binding adhesins 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 (195) 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 (20), 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 (196, 197). This observation allowed us to distinguish the unique characteristics of the cell surface molecules such as CD1 and CD103 on different moDC types expressed by the $CD1d^{-}CD103^{-}$ and the $CD1a^{-}CD1d^{+}CD103^{+}$ cell populations, respectively (**Figure 28A**).

We first characterized and compared the expression levels of the contributing transcription factors including IRF4, PPARγ and RARα in moDCs. It has been previously found that DCs expressing IRF4 are less potent inducers of Tc cells than cells expressing IRF8, a DC subset localized to the gut mucosa (163, 198). Our results also revealed that $IRF4^{hi}$ moDCs act as immunogenic cells and are 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 $RAR\alpha^{hi}IRF4^{lo}$ 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 (42). Ligation of the CD40 cell surface molecule enhances the inflammatory potential of DCs (199), and resting moDCs concomitantly conditioned with ATRA up regulate the cell surface expression of CD40, which can be further increased by *E. coli Schaedler* as compared to moDCs differentiated in the absence of ATRA. This observation is also confirmed by the notion that resting DCs express high levels of CD40 on the cell surface representing a semi-activated DC population with tolerogenic features (200, 201).

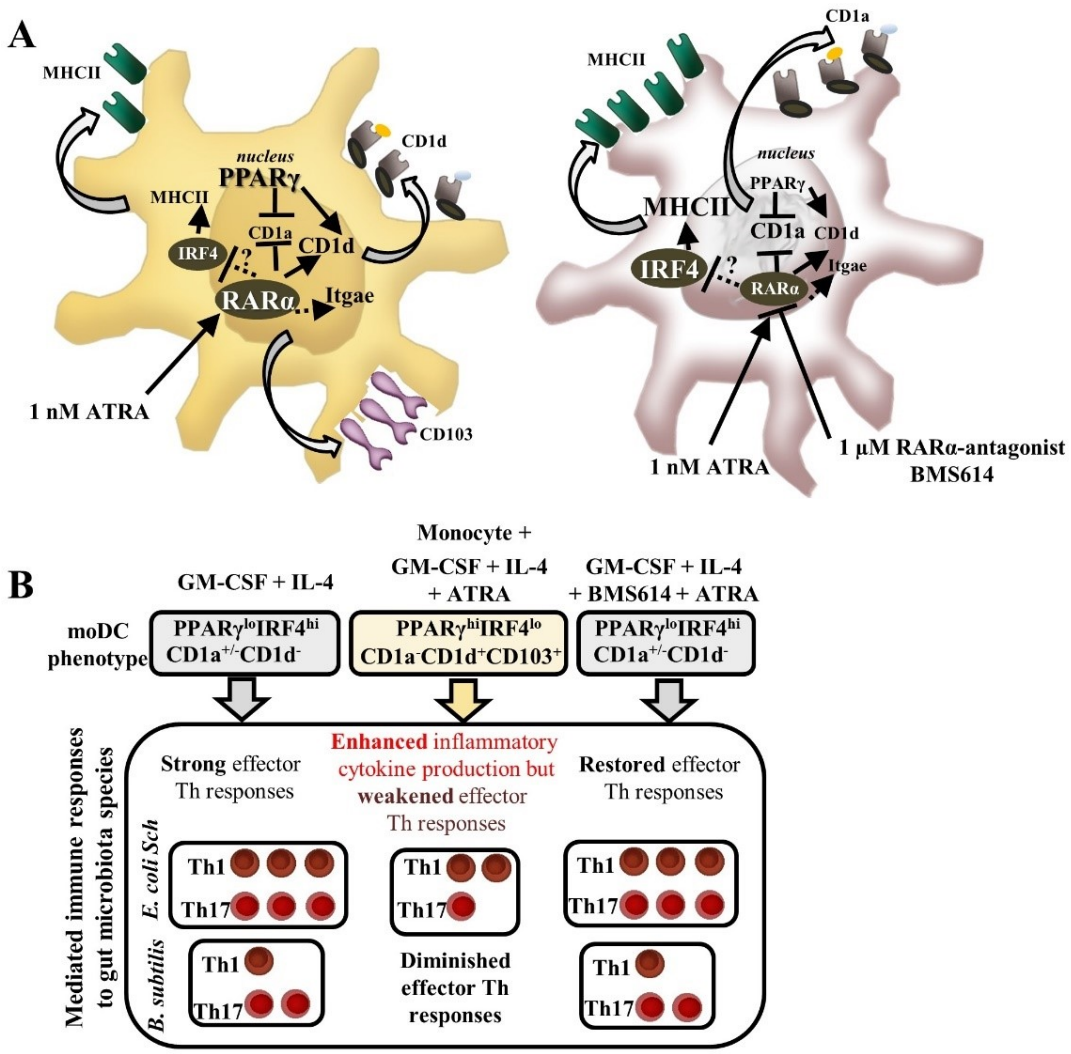


Figure 28. The role of RARα in guiding moDC development and microbiota-specific immune responses ATRA modifies the differentiation program of moDCs that could be prevented by the selective inhibition of RARα. *(**A**) In the presence of GM-CSF and IL-4 monocytes differentiate to CD1d$^-$CD1a$^+$ DCs (20). In human moDCs PPARγ and RARα regulate the gene expression levels of CD1d and ALDH1A2 both directly and indirectly (42). IRF4 mediates the differentiation and antigen presenting capacity of human moDCs, which can be down regulated by the ligation of RARα resulting in decreased mRNA and protein levels of IRF4 together with CD1a. Selected microbiota species provoke different types of immune responses mediated by moDCs. (**B**) E. coli Schaedler induces full maturation in moDCs leading to strong inflammatory and microbiota-specific effector responses, while B. subtilis induces inflammation in the absence of IL-12 and IL-23, while provokes decreased effector Th1/Th17 immune responses. ATRA down modulates the immunogenicity of moDCs resulting in diminished Th1 and undetectable Th17 responses, but in moDCs this effect can be restored by prior inhibition of RARα. Solid lines represent known mechanisms; dotted lines indicate unknown molecular interactions.*

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 PAMP (LPS) stimuli while the expression level of TNF-α and IL12 genes were up regulated (202).

It has also been demonstrated that in the presence of heat-killed *E. coli Schaedler* and *B. subtilis* bacteria, the phagocytic capacity of moDCs could be facilitated by ATRA, similar to a previous work showing increased PPARγ activity in moDCs upon internalizing corpuscular antigens more efficiently than moDCs with low PPARγ activity (23). 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. Moreover, ATRA-primed moDCs in the presence of selected bacterial strains induced pro-inflammatory cytokine secretion leading to high level secretion of TNF-α, IL-1ß, IL-6 and IL-23. Considering that these inflammatory cytokines play central role in the maintenance and/or disruption of mucosal integrity, exemplified by secreted IL-23 of both DC and MΦ origin. These regulatory circuits may serve as double-edged swords during the maintenance of balance in health and disease. The increased level of secreted IL-23 could directly be associated with chronic inflammatory diseases including IBD (203). However, in the *lamina propria* the presence of microbiota provide 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 (129, 204). 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 (123).

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 (168). 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 (13). 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.

Based on the known regulatory functions of DCs, this study also demonstrates that the selected commensal bacteria also secrete IL-10, an inhibitory cytokine acting

independently on the bacterial species. At our experimental conditions the cell surface expression of PD-L1 protein became up regulated in a bacterial strain dependent manner, which could also be demonstrated in ATRA-primed moDCs even though its expression level was significantly lower as compared to the respective ATRA free moDC counterpart. In addition to these results, the secretion of IL-12 cytokine with known inflammatory properties was down modulated by ATRA as shown before by others (58). In contrast to these findings, we could demonstrate that ATRA had no effect on IL-10 secretion in moDCs. Collectively, these data indicate that decreased levels of IL-12, reduced co-stimulatory and antigen-presenting capacity by RARα^{hi}IRF4^{lo} moDCs together with the production of inhibitory IL-10 create a local cellular milieu, which is inefficient to induce potent effector T helper cell responses upon targeting the selected gut microbiota species.

Our results clearly demonstrated that in resting moDCs ATRA is able to up regulate the relative mRNA levels of RARα previously confirmed also by others (58). 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 (data not shown). 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 critical role in regulating moDC differentiation and guiding mucosal immune responses. In addition, the gut microbiota has an impact on retinoid signaling-mediated immune homeostasis transmitted by microbial metabolites such as SCFAs (205) and retinoid supplementation through diet also acts on the composition of the gut microbiota and on energy metabolism of the host (206). 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 (207). 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 (61). 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 including 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 (208).

Human moDCs not only provoke antigen-specific immune responses, but also induce the activation and expansion of T cells with semi-invariant TCR, among them lipid reactive iNKT cells (42, 209), and also present lipid antigens via cell surface glycolipid receptors. Remarkably, the levels of CD1a and CD1d expression can be modified by commensal bacteria to different extents supporting the notion that this effect is not even related to the local lipid/retinoid environment, the activity of PPARγ (51) or the presence of pathogenic microbes (210), however their activities may resemble some microbiota species such as *E. coli Schaedler* and *B. subtilis*. MoDCs with increased PPARγ-activity also induce the expansion of IFNγ-secreting iNKT cells at high levels as compared to moDCs with low PPARγ activity (23). Surprisingly, we were unable to detect changes in the number of iNKT cells in moDCs stimulated by the selected commensal bacteria in the activated moDC – T cell cultures. Instead, moDCs generated processed lipid antigens derived from commensal bacteria indicating that these lipids are unable to provide ligands for CD1a or CD1d proteins.

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 (**Figure 28B)**.

7. 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.

L. 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 $CD1a^{-}CD1d^{+}CD103^{+}/RAR\alpha^{hi}IRF4^{lo}$ moDCs on a RARα-dependent manner, while in the absence of ATRA monocytes differentiate to $CD1a^{med}CD1d^{-}CD103^{-}/RAR\alpha^{lo}IRF4^{hi}$ 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 $RAR\alpha^{lo}IRF4^{hi}$ moDCs polarize Th1 and Th17 cells effectively on a strain dependent manner, while $RAR\alpha^{hi}IRF4^{lo}$ 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.

ÖSSZEFOGLALÁS

A táplálkozás során bevitt tápanyagok és a kommenzális bélbaktériumok meghatározó szereppel bírnak a nyálkahártyában zajló és a szisztémás immunológiai folyamatokra, úgymint a specializált dendritikus sejt (DC) és segítő T-limfocita populációk kialakulására, a kostimulátor molekulák megjelenítésére és a citokinek illetve a kemokinek termelésére. A DC funkcionális elemzése során ezeket a sejtélettani mechanizmusokat nyomon követhetjük, amelyek kimenete szorosan összefügg a sejtfejlődés aktuális állapotától és a mikrobák által létrehozott gyulladásos jelátviteli pályák aktiváltsági szintjétől. Célunk olyan érzékeny *in vitro* rendszer kialakítása volt, amely alkalmas a mikrobióta és a humán immunrendszer alkotói közötti kölcsönhatások megfigyelésére.

Az *Lactobacillus reuteri* tejsavbaktériumok a bél mikrobióta közösség előnyös tagjai közé tartoznak, amelyek evolúciósan alkalmazkodtak a gerincesek bélrendszeréhez a nyálkahártyát kötő adhezinjeik segítségével. Eredményeink szerint, nem várt módon a *L. reuteri* baktériumok monocita-eredetű (mo) DC-vezérelt gyulladásos T sejtes választ indítottak be, amelyet a bakteriális mucin-kötő fehérje CmbA és a MUB tovább fokozott. Ezenfelül bizonyítást nyert, hogy a C-típusú lektin receptorok és a bakteriális MUB adhezin kölcsönhatásának gátlása csökkenti az moDC gyulladásos citokintermelését és az moDC által irányított immunválasz Th1 irányú polarizációját. A MUB, mint természetes adjuváns hozzájárul és erősíti a két szervezet közötti szimbiotikus kapcsolat fenntartását és fokozza a szervezet moDC-vezérelt, előnyös mikrobák által kiváltott effektor immunválaszát és immunológiai memóriát kialakító képességét.

További megfigyelésünk szerint a fiziológiásan releváns koncentrációjú A-vitamin származék, az ATRA RARα-függő módon irányítja a tolerogén $CD1a^{-}CD1d^{+}CD103^{+}$/ $RAR\alpha^{hi}IRF4^{lo}$ moDC differenciációját, míg ATRA hiányában a monociták $CD1a^{med}CD1d^{-}$ $CD103^{-}/RAR\alpha^{lo}IRF4^{hi}$ DC-vé fejlődnek. Az *E. coli Schaedler* és a *M. morganii* kommenzális bélbaktériumok az immunogén $RAR\alpha^{lo}IRF4^{hi}$ moDC által Th1 és Th17 típusú immunválaszt polarizálnak, míg a probiotikus hatású *B. subtilis* jelenlétében a Th1 válasz elmarad. Továbbá megállapíthatjuk, hogy a $RAR\alpha^{hi}IRF4^{lo}$ sejtek egy nem-vándorló, de ezzel együtt egy tolerogén és gyulladásos DC alpopulációt alkotnak.

Összefoglalva, a bél mikrobióta tagok által kiváltott, moDC-vezérelt adaptív immunválasz erősíthető mucint kötő adhezinekkel, mint természetes adjuvánsokkal, valamint a nyálkahártyákra jellemző retinoid környezetben a RARα specifikus gátlásával.

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8.2 Publication list prepared by the Kenézy Life Sciences Library

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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.
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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.
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DEBRECENI EGYETEM KENÉZY ÉLETTUDOMÁNYI EGYETEMI ÉS NEMZETI KÖNYVTÁRA

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.
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Total IF of journals (all publications): 16,911
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26 April, 2017

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

9. KEYWORDS

9.1 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.*

9.2 Kulcsszavak

Dendritikus sejt, gyulladás, T-limfocita, mikrobióta, RARα, all-transz retinsav, C-típusú lektin receptorok, mucint kötő adhezinek, *Lactobacillus reuteri*, *Escherichia coli Schaedler*, *Morganella morganii*, *Bacillus subtilis.*

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11. APPENDIX

Bene, KP., Varga, Z., Petrov, V., Boyko, N., Rajnavölgyi, É.: *Gut microbiota species can provoke both inflammatory and tolerogenic immune responses in human dendritic cells mediated by retinoic acid receptor alpha ligation*. Frontiers in Immunology, Volume 8, article 427, April 2017, doi: 10.3389/fimmu.2017.00427.

Bene, KP, Kavanaugh, DW, Leclaire, C., Gunning, A.P., MacKenzie, DA, 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*. Frontiers in Microbiology, Volume 8, article 321, March 2017, doi: 10.3389/fmicb.2017.003217.