

The Mucosal Factors Retinoic Acid and TGF- β 1 Induce Phenotypically and Functionally Distinct Dendritic Cell Types

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Key Words

Dendritic cell · Activation · CD103 · CCR7 · β 7 · Mucosal factors · Diet

Abstract

Non-inflammatory dendritic cell (DC) subsets play an essential role in preventing massive inflammation in mucosal tissues. We investigated whether mucosa-related factors, namely retinoic acid (RA) and transforming growth factor- β (TGF- β 1), can induce such DC types. DCs were differentiated from monocytes in the absence or presence TGF- β 1 and RA. The phenotype as well as responsiveness to bacterial ligands was studied in detail. Compared to monocyte-derived DCs (moDCs), the expression of co-stimulatory molecule CD86 and DC maturation marker CD83 were strongly reduced by RA and TGF- β 1. In addition, both RA- and TGF- β 1-induced DCs showed strongly decreased responsiveness to stimulation with the bacterial ligands lipopolysaccharide and peptidoglycan, and produced significantly lower levels of the pro-inflammatory cytokines IL-12 and TNF- α compared to moDCs, whilst IL-10 production was not significantly reduced. DCs differentiated under the influence of RA uniquely expressed markers related to intestinal homing (CD103

and integrin β 7). In addition, CCR7, which mediates homing to lymph nodes, was expressed by DCs differentiated in the presence of RA, and also to a lesser extent by the other DC types. Furthermore, whereas moDCs and TGF- β 1-derived moDCs expressed high levels of CD32, RA-derived DCs lacked CD32 expression but expressed high levels of CD64, suggesting that RA-DCs may primarily respond to soluble proteins and moDCs, and TGF- β DCs to immune complexes. The data presented here support the hypothesis that the mucosal factors TGF- β 1 and RA, which can also be provided through dietary intake of dairy products, result in functionally and phenotypically distinct DC types with non-inflammatory properties.

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Introduction

A complex network of specialized dendritic cell (DC) subsets is involved in inducing acute inflammatory responses upon invasion by pathogens on the one hand, and on the other hand in inducing tolerance to harmless dietary and inhaled antigens as well as commensal microbiota [1, 2]. Knowledge of the role of local mucosal and

dietary factors that induce these different DC types is still limited.

Intestinal epithelial cells have been shown to be able to induce tolerogenic DCs expressing integrin alpha E (CD103) [3]. Transforming growth factor- β (TGF- β) and retinoic acid (RA) are important factors for conferring a tolerogenic environment in the mucosa by inducing regulatory T cells (Treg) and IgA-producing plasma cells [4, 5]. Under specific conditions, RA is also able to induce more inflammatory CD4 T cells [6, 7]. Intestinal epithelial cells produce TGF- β 1 in steady state and can metabolize dietary vitamin A to retinoic acid [reviewed by 8]. TGF- β present in breast milk has been associated with development of gut homeostasis and prevention of allergy development in infants [9]. Several studies have indicated a role for the hematopoietic Fms-related tyrosine kinase 3 ligand (Flt3l) for induction of CD103 DCs in mice, but this has not been confirmed for human CD103+ DCs [10, 11]. Other reports have demonstrated that RA is able to induce CD103 expression on human monocyte-derived DCs [3] or mouse bone marrow cells [12], but data on responsiveness to bacterial ligands is lacking. RA has also been implicated in imprinting T cells to migrate to the intestines [13]. Both DCs and CD4+ T cells can display tolerogenic properties, which is reflected by expression of CD103 by DCs and forkhead box P3 (FoxP3) by Tregs [1, 3, 5].

DC subsets exert different functional roles in the intestines, as evidenced by an altered composition of the DC network in disease. Increased numbers of CD11b+ DCs and decreased numbers of CD103+ DCs were observed after sensitization with peanut allergens in a mouse peanut allergy model [14]. Tolerogenic CD103+ DCs are known for their different migratory capacity (homing to mesenteric lymph node; MLN) and capacity to induce peripheral tolerance by inducing FoxP3+ CD4+ Tregs [1, 5, 15]. Next to decreased numbers of CD103+ DCs, Treg-inducing capacity by CD103 expressing DCs was lost in a mouse model of colitis [16]. Those studies in mice provide insight into how DC subsets may be involved in the prevention or progression of disease, but the role of DC subsets still needs to be established in the human system [17]. Intestinal epithelial cells are also important in the maintenance of intestinal homeostasis as intestinal epithelial cells of patients suffering from colitis produce lower levels of thymic stromal lymphopoietin (TSLP), TGF- β 1 and aldehyde dehydrogenase (ALDH). ALDH is involved in the synthesis of RA, and reduced expression of ALDH may explain the altered RA-dependent induction of Tregs in those patients [3, 18].

Complete activation and differentiation of T cells in contact with antigen-presenting DCs is determined by expression levels of co-stimulatory molecules like CD80 and CD86, and the production of cytokines (TNF- α , IL-10, IL-12) and other soluble factors (e.g. RA). Next to induction of Tregs, TGF- β and the metabolite of vitamin A, RA, have been shown to play a role in the induction of IgA in vitro and mouse models, which is a major contributor to the first line of mucosal defense [4, 19, 20]. Vitamin A deficiency is associated with pathological signs in epithelia, which could be restored after supplementation with vitamin A [21], but it is not known whether the restoration of pathology was mediated by CD103+ DCs.

The currently established model for in vitro DC studies relies on differentiation of monocytes under the influence of GM-CSF and IL-4. In addition to the limitations in physiological functionality of differentiating DCs from monocytes in vitro, the conventional model generates only one subtype of DC whereas multiple DC subsets are found in vivo. As data on the direct effects of RA and TGF- β 1 on the DC phenotype and responses to bacterial ligands is limited, the current study aimed to identify how these mucosal factors affect the DC phenotype and activation upon stimulation with bacterial ligands. Conventional monocyte-derived DCs (moDCs) were compared to moDCs that were differentiated under the additional influence of TGF- β 1 or RA. The expression of DC subtype-specific cell surface markers, homing receptors and IgG receptors was evaluated, as were their functional responses to stimulation by Toll-like receptor (TLR) ligands.

Materials and Methods

Peripheral Blood Mononuclear Cells and Monocyte Isolation

Peripheral blood mononuclear cells (PBMCs) obtained from buffy coats of healthy blood donors (Sanquin Blood Bank, Nijmegen, The Netherlands) were diluted 1:1 in IMDM (Gibco-BRL, Paisley, UK) and isolated by gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) for 5 min at 200 g and subsequently for 15 min at 500 g (without brake at 20°C). The PBMCs were harvested from the Ficoll layer, gently resuspended in IMDM and washed twice in IMDM.

Freshly isolated PBMCs were labeled with MicroBeads conjugated to mouse IgG2a monoclonal anti-human CD14 antibodies (130-050-201; Myltenyi Biotec, Bergisch Gladbach, Germany) and incubated for 15 min at 4°C, washed with MACS buffer (0.5% BSA, 2 mM EDTA in PBS, pH 7.2), centrifuged (10 min, 200 g) and resuspended in MACS buffer. The MACS columns were placed in the QuadroMACS (Myltenyi Biotec) and rinsed with MACS buffer. Subsequently, the cell suspension was added, the columns were rinsed three times with MACS buffer and removed from the quad-

roMACS; labeled cells were collected in a new tube by rinsing with MACS buffer and the supplied plunger.

The purity of the CD14⁺ cell population was between 90 and 95%, as determined by flow cytometric analysis (FACS Canto II; BD Biosciences, San Jose, Calif., USA) by labeling the cells with mouse IgG2a anti-human CD14 APC or an isotype control (clone M5E2, 555399 or 555576; BD Biosciences).

DC Generation and Stimulation

Isolated monocytes were cultured in RPMI (Lonza, Basel, Switzerland) containing 10% standardized FCS (A15–551; PAA laboratories) and 1% pen/strep (Gibco, 15140) at a concentration of $0.5\text{--}1 \times 10^6$ cells/ml. 50 ng/ml recombinant human GM-CSF (300-03; Peprotech) and IL-4 (200-04; Peprotech) were added and refreshed every second or third day. On day 7, expression of cell surface markers was analyzed using flow cytometry (FACS Canto II; BD Biosciences). Alternatively, DC subsets were generated by adding 10^{-5} M all-trans retinoic acid (Sigma-Aldrich, St. Louis, Mo., USA) or 10 ng/ml recombinant human TGF- β 1 (Peprotech) in addition to IL-4 and GM-CSF. Like IL-4 and GM-CSF, RA and TGF- β 1 were refreshed every second or third day. The concentrations used for RA and TGF- β 1 were based on figure 6 of Iliev et al. [3]. TSLP (50 ng/ml, Peprotech) was also tested as a factor for CD103 induction.

After differentiation DCs were stimulated with medium or 2 μ g/ml of lipopolysaccharide (LPS; *Salmonella typhimurium*, L7261; Sigma-Aldrich) or peptidoglycan (PGN; *S. aureus*, tlr1-pgn-sa; InvivoGen, Toulouse, France). Cell-free supernatants were collected and stored at -80°C until analysis. DCs were collected and cell surface marker expression was analyzed.

DC-T Cell Co-Culture

In order to evaluate the functional consequences of RA-induced expression on CD4⁺ cells, DCs and autologous or allogeneic T cells (CD14-depleted PBMCs) were co-cultured in a 1:10 ratio (DC:T cell) [22]. At day 5 of DC differentiation, T cells were added and incubated for another 5 days. Some T cells were pre-incubated with 10^{-5} M RA receptor antagonist LE136 (Tocris Bioscience, Bristol, UK) and/or during culture 150 ng/ml or 100 ng/ml of monoclonal antibodies to CD3 and CD28 (BD Biosciences) were added. Cells were harvested and stained for CD4 and CD127, followed by fixation and permeabilization and staining with FoxP3 according to the manufacturer's descriptions (Treg staining kit 560131; BD Biosciences). Cells were gated on CD4-positivity, and the percentage of FoxP3+CD127[−] cells were extracted by batch processing using FlowJo.

Cell Surface Marker Analysis

DCs were stained with antibodies against CD14 (APC-H7, 560270; BD Biosciences), CD103 (PE, 550260; BD Biosciences), CX-3CR1 (PerCP-eFluor710, 46-6099; eBioscience), HLA-DR (APC-eFluor780, 47-9956-41; eBioscience), CD11b (PE-Cy7, 25-0118; eBioscience), CD11c (V450, 560370; BD Biosciences), CD83 (FITC, 11-0839-42; eBioscience), CD86 (V450, 560357; BD Biosciences), β 7 chain (APC, 551082; BD Biosciences), CCR7 (FITC, 561271; BD Biosciences), CD209 (PerCP-Cy5.5, 558263; BD Biosciences), CD16 (PE, 332779; BD Biosciences), CD32 (GTX74628; GeneTex, Irvine, Calif., USA), CD64 (305015; BioLegend), FcRn (sc-271745, Santa Cruz Biotechnology; Santa Cruz, Calif., USA). For FcRn, CD32 and CD64, a secondary antibody labeled with FITC was used (goat anti-

mouse IgG/IgM, 555988; BD Biosciences). A control sample of each sample was analysed, stained with control antibodies (using the same concentrations as for positive staining) of the same host species and isotype, and conjugated with the same fluorochrome.

Cells were incubated at room temperature for 20 min in the dark. Subsequently, cells were centrifuged, supernatant was discarded, 200 μ l FACS Buffer (BD Biosciences) was added and analysed for surface marker expression by flow cytometric analysis (10,000 cells were analyzed with BD Canto II using DIVA software; BD Biosciences). Data was batch processed and percentages and delta mean fluorescence intensity (MFI) values (surface marker staining – isotype control staining) were obtained.

Human Cytokine Measurements

Human cytokine concentrations (IL-10, IL-12 and TNF- α , 558274, 558283 and 558273, respectively; BD Biosciences) in cell culture supernatants were determined using the cytometric bead array flex sets (BD Biosciences) and incubations were performed in the recommended Protein Master Buffer Kit (BD Biosciences). Briefly, 50 μ l of supernatant was incubated with capture beads diluted in 50 μ l of capture bead diluent for 1 h at room temperature in the dark. Next, PE detection reagent was diluted in 50 μ l of detection reagent diluent, added and incubated for 2 h at room temperature in the dark. For the standard curve, the provided lyophilized recombinant cytokines were used and assayed together with the samples. After incubation the samples were centrifuged, supernatant was discarded and 200 μ l of wash buffer was added. The samples were analyzed by flow cytometric analysis (FACS Canto II; BD Biosciences).

Statistical Analysis

Statistical tests were performed in IBM SPSS Statistics v.19. After log transforming the data to improve its distribution, the independent samples t test was used to test mean differences between DC types or treatments. Depending on the equality of variances between test conditions, the p value of equal or unequal variances assumed was reported.

Results

Effects of RA and TGF- β on DC Differentiation

IL-4 and GM-CSF are generally used to generate DCs from monocytes. moDCs are often used as model DCs in studies on intestinal immunomodulation. As the intestines contain several DC subsets, of which tolerogenic subsets are crucial for maintaining homeostasis, we developed two additional DC subsets using the relevant mucosal factors RA and TGF- β 1. These factors were added to the differentiating DCs of at least 4 different donors in addition to IL-4 and GM-CSF.

All three DC subsets expressed HLA-DR and lacked CX3CR1 and CD14 expression (table 1). Conventionally differentiated moDCs and TGF- β 1-derived DCs (TGFmoDCs) expressed CD1a, but RA-derived DCs (RAMoDCs) did not express CD1a (table 1).

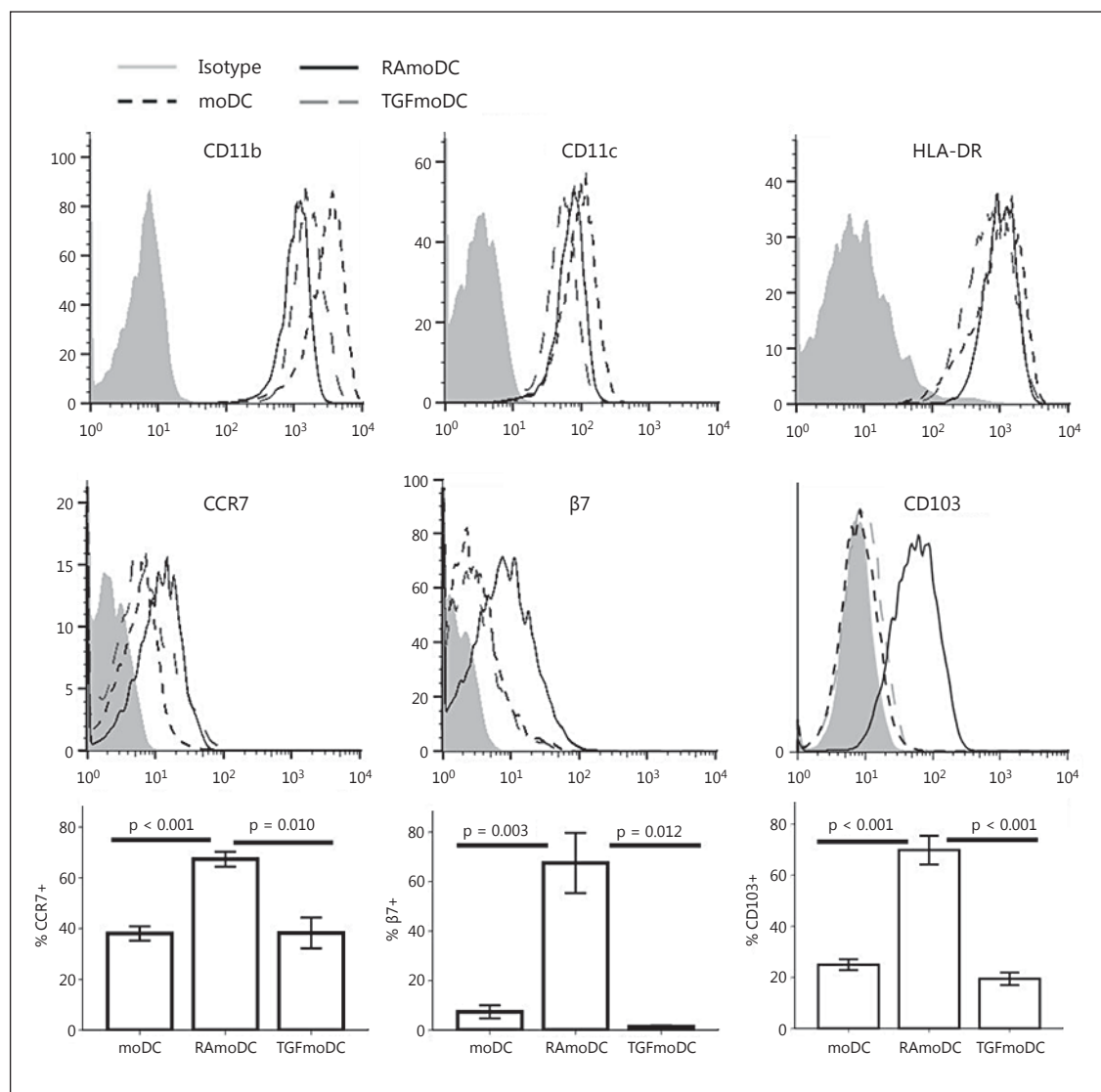


Fig. 1. Surface marker expression by the moDCs differentiated under three different regimes. FACS histograms (above) show a representative example of at least 4 donors tested. The grey shaded area is isotype control, black dashed line is moDCs (IL-4 and GM-CSF only), black line is RAmoDCs and grey long-dashed line is

TGFmoDCs. Bar graphs (below) are shown indicating the average percentage of cells expressing those markers (isotype control values are subtracted) and standard error of 4 different donors. p values shown are based on double-sided t tests, performed after log transformation of the data.

All three DC types expressed CD11b and CD11c (fig. 1). Analysis of the MFI of CD11b revealed that TGF- $\beta 1$ -derived DCs, and even more so the RA-derived DCs expressed reduced levels of CD11b compared to moDCs ($p = 0.006$ and $p = 0.001$, respectively). CD11c expression was also reduced ($p = 0.001$, $p = 0.006$, respectively; fig. 1). This was more prominent for TGF-derived moDCs than for RA-derived moDCs, resulting in a significantly reduced CD11b/CD11c ratio for RA-derived moDCs compared to moDCs ($p = 0.020$; data not shown).

As *in vivo* functionality of CD103+ DCs depends on co-expression of ($\alpha 4$) $\beta 7$ to adhere to E-cadherin and migrate to the intestine and on CCR7 for homing to the MLN, we assessed expression of those cell surface markers on the different DC types. The percentage of CCR7-expressing cells increased under the influence of RA but not under the influence of TGF- $\beta 1$ (fig. 1). The MFI level of CCR7 was significantly higher on RA-derived moDCs compared to moDCs, but not compared to TGF-derived moDCs. In addition to expression of CD103, the percent-

Table 1. Basal expression (%) of cell surface molecules on the three moDC types

	moDC	RAmoDC	TGFmoDC
CD14	–	–	–
HLA-DR	++	++	++
CD1a	+	–	+
CD103	–	+	–
CX3CR1	–	–	–
CCR7	+/ $\square$	+	+/ $\square$
β 7	+/ $\square$	+	+/ $\square$
CD11b	++	++	++
CD11c	++	++	++
CD86	+	$\square$	$\square$
CD83	+/ $\square$	$\square$	$\square$
FcRn	–	–	–
CD16	–	+/-	–
CD32	+	–	+
CD64	$\square$	+	$\square$

Data was calculated by subtracting isotype percentages and is based on at least 4 different donors, except for Fc receptor staining, which is based on 3 donors. – = <5%; $\square$ = 5–25%; +/ $\square$ = 25–50%; + = 50–90%; ++ = >90%.

age of DCs expressing the integrin β 7 was significantly higher for RA compared to moDCs and TGF- β 1-differentiated DCs (fig. 1). TSLP and Flt3 ligand (implicated in CD103 induction in mice) did not induce expression of CD103 (data not shown). The basal expression data of the three DC subsets are summarized in table 1. We conclude that especially RA gave rise to moDCs resembling tolerogenic DCs observed in vivo in humans.

Differential Expression of Fc γ Receptors

Fc γ receptors (Fc γ R) have different affinities for IgG. Low affinity Fc γ R (CD16) and high affinity Fc γ R (CD64) can bind to monomeric IgG, whereas low affinity Fc γ R (CD32) binds to immune complexes only [23]. CD32 expression was observed for moDCs and TGF-derived moDCs, but not for RA-derived moDCs (fig. 2). CD64 was expressed by all DC types, but its expression was highest on RA-derived moDCs. The Fc γ R expression pattern is similar for moDCs and TGF-derived moDCs, although expression of CD32 and CD64 seems to be higher on TGF-derived moDCs. RA-derived moDCs have a different Fc γ R expression profile by expressing low levels of CD16 and high levels of CD64, but no CD32. Expression of the neonatal receptor (FcRn) could not be shown on any of the DC types.

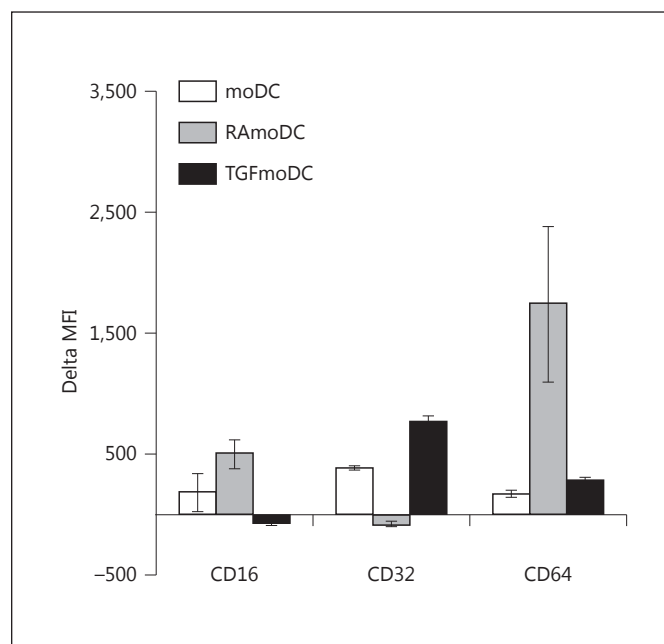


Fig. 2. Fc γ R expression by the three DC types. Mean MFI and standard errors are shown of 3 different donors.

Bacterial Ligands Reduce Expression Levels of CD103 on RA-Derived DCs

CD103+ DCs are believed to migrate to the MLN upon activation, for which reduction of CD103 expression levels may be essential, next to upregulation of CCR7, which is responsible for homing to the MLN. To test if this also occurs in these in vitro-generated CD103+ DCs, RA-derived DCs were stimulated with several bacterial ligands and changes in CD103 expression level were measured after 48 h. Addition of bacterial PGN (TLR 2/6 ligand) and LPS (TLR4 ligand) significantly reduced expression of CD103 (data not shown).

DC Subsets Vary in Expression of CD86 and CD83

The expression of co-stimulatory molecules is essential for complete activation of T cells. Unstimulated conventional moDCs were slightly pre-activated as evidenced by increased CD83 and CD86 expression, but this was not observed for RAmoDCs or TGFmoDCs. Even so, CD83 and CD86 expression increased significantly after stimulation with bacterial ligands. The percentage of CD86-expressing cells was reduced in RA- and TGF-derived moDCs relative to moDCs ($p = 0.013$ and $p = 0.007$, respectively), whereas the MFI of CD86 was only reduced in RA-derived moDCs (not significant; fig. 3). All CD83-expressing cells also expressed CD86. RA- and TGF- β 1-derived moDCs

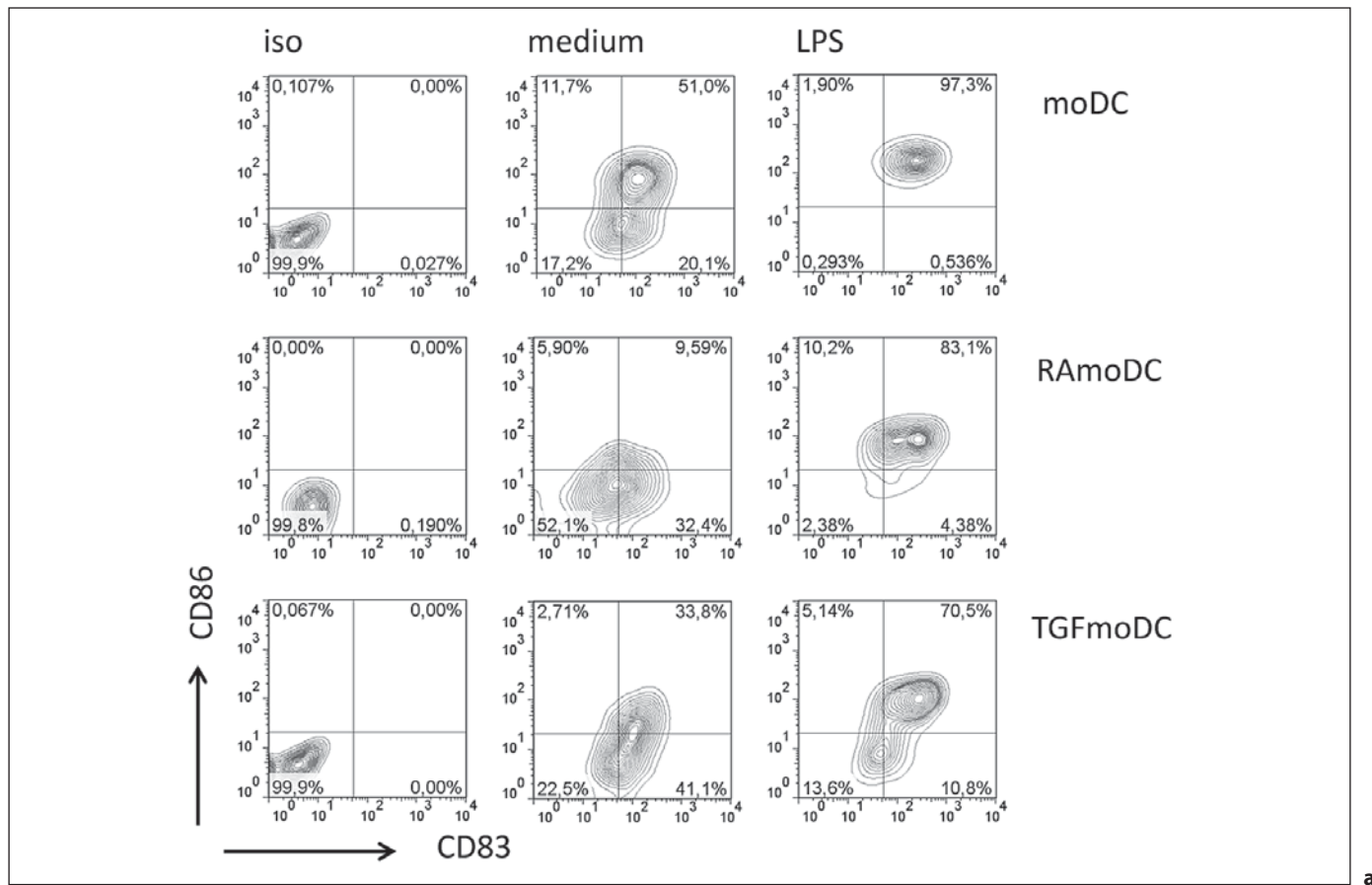
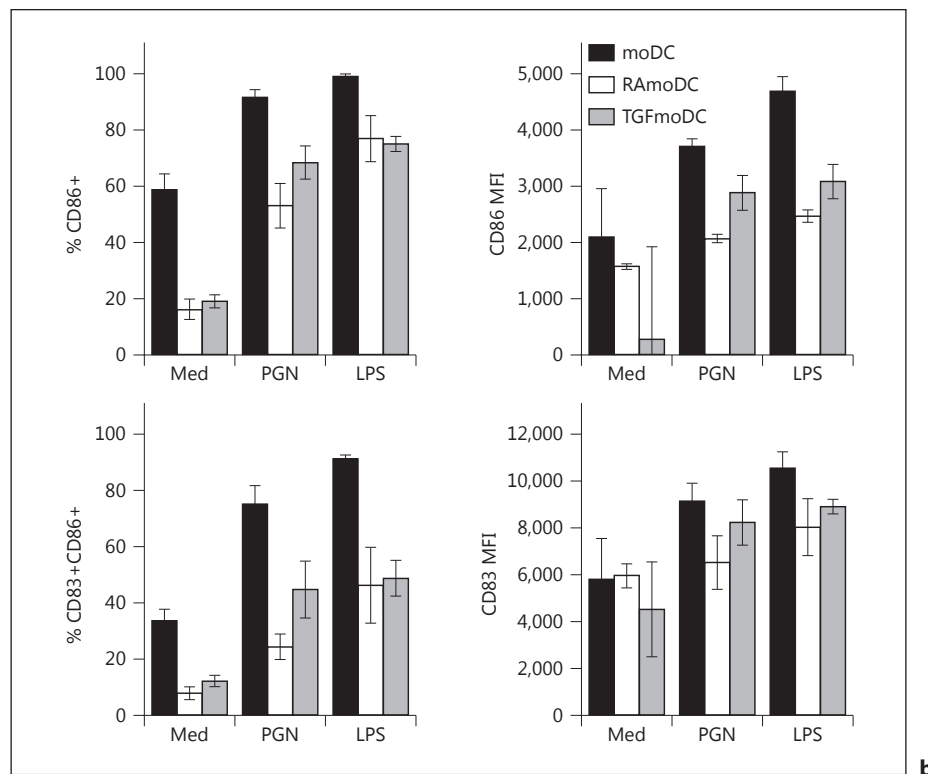


Fig. 3. Expression of maturation and activation marker CD83 and CD86 by the three DC types. **a** Isotype control staining (left column), followed by surface marker staining of non-stimulated (medium, shown in the middle) or LPS-stimulated (right column) moDCs (top), RAmoDCs (middle) and TGFmoDCs (bottom) are displayed. **b** Mean percentage, MFI and standard error are shown of 4 different donors after 48 h of incubation with medium or bacterial ligands.



	moDC versus RAmoDC				moDC versus TGFmoDC				RAmoDC versus TGFmoDC			
	CD86		CD83		CD86		CD83		CD86		CD83	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
PGN	0.004	0.002	0.001	0.113	0.012	0.049	0.049	0.503	0.172	0.158	0.117	0.301
LPS	0.072	0.053	0.017	0.13	0.000	0.087	0.005	0.112	0.826	0.194	0.876	0.511

Fig. 3. Expression of maturation and activation marker CD83 and CD86 by the three DC types. **c** p values of t test between DC subsets after activation with bacterial ligands. Statistical differences between DC subtypes are shown (t test). Similar results (not shown) were obtained with DCs stimulated for 24 h from 3 independent donors.

showed a reduced percentage of CD83+ cells compared to moDCs ($p = 0.002$ and 0.004 , respectively), whereas the level of expression (MFI) was the same.

Although the percentage of basal expression of CD86 on TGF- β 1 and RA-derived moDCs was reduced, the addition of bacterial ligands (3 donors, 24 h, and 4 donors, 48 h) resulted in a pronounced upregulation of CD86 expression on all DC types with the most significant changes induced by exposure to LPS (fig. 3; table 2). However, after stimulation with the TLR ligands PGN and LPS, the MFI of CD86 of RA- and TGF-derived moDCs was still significantly lower compared to conventional moDCs, especially for RA-derived moDCs (fig. 3), indicating a state of non-responsiveness. Also, the percentage CD83+CD86+ cells upon activation with bacterial ligands was significantly reduced when DCs were differentiated in the presence of TGF- β 1 and RA. However, no differences in MFI of CD83 were observed.

Production of the Pro-Inflammatory Cytokines TNF- α and IL-12 Is Reduced in RA and TGF- β 1-Derived DCs

After stimulation with the bacterial ligands LPS and PGN for 24 or 48 h, cell culture supernatants were collected and cytokine production levels (TNF- α , IL-10, IL-12) were determined. Production levels of all cytokines were decreased in cultures with DCs differentiated in the presence of RA and TGF- β 1 (fig. 4). RA and TGF- β 1-derived moDCs showed significantly reduced production of the pro-inflammatory cytokines TNF- α and IL-12 compared to moDCs (RA: $p = 0.017$ and $p = 0.021$, respectively; TGF- β 1: $p = 0.013$ and $p = 0.017$, respectively). TGF-derived moDCs did not produce detectable IL-12, and strongly reduced amounts of IL-12 were produced by RA-derived moDCs when PGN or LPS was added. IL-10 production by RA- and TGF-derived DCs had a tendency to reduction upon stimulation with PGN, but this did not reach significance.

Table 2. Modulation of cell surface marker expression and cytokine production after 24 or 48 h of stimulation with PGN or LPS

	moDC	RAmoDC	TGFmoDC
CD103	–	↓	–
CD83	↑↑	↑	↑
CD86	↑↑	↑	↑
IL-10	↑↑	↑	↑↑
IL-12	↑	–	–
TNF- α	↑↑	↑	↑

Data is based on 4 donors for 48 h and 3 donors for 24 h. – = Absent (not measurable); ↓ = decrease; ↑ = increase; ↑↑ = strong increase.

Induction of FoxP3 Expression

As CD103+ DCs have been implicated in inducing FoxP3+ Tregs, moDCs and RAmoDCs were co-cultured with autologous or allogeneic PBMCs that were depleted of monocytes and B cells for 5 days at a ratio of 1:10. Cells were stimulated with suboptimal concentrations of monoclonal antibodies to CD3 and CD28 (150 and 100 ng/ml) or medium. The cells were harvested and stained for CD4 and CD127 (which should be absent on Tregs) followed by fixation and permeabilization and staining for FoxP3. The percentage of CD127–FoxP3+ CD4+ cells was determined by flow cytometric analysis. PBMCs cultured in the presence of allogeneic RAmoDCs and CD3 and CD28-specific antibodies showed increased percentage of FoxP3+CD127+ CD4+ cells (fig. 5). This could be abrogated when PBMCs were pre-incubated with RA receptor antagonist LE135 (10^{-5} M). This increased percentage of FoxP3+CD127– cells was not observed when autologous RAmoDCs or autologous or allogeneic moDCs were used.

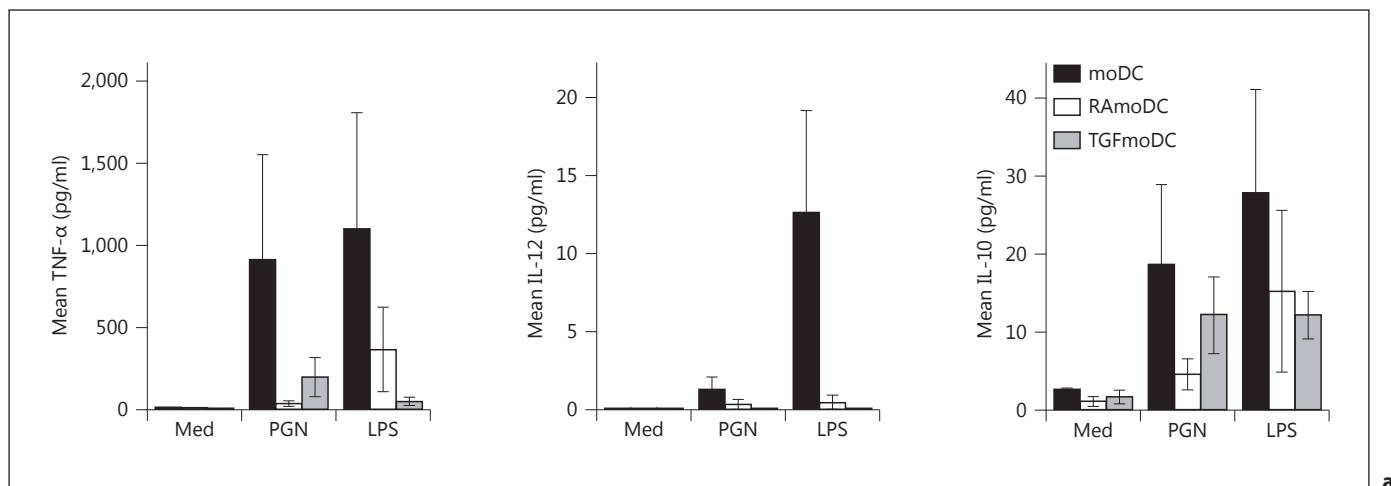


Fig. 4. Cytokine production by the three DC types upon 48 h of incubation with medium or bacterial ligands. **a** Mean cytokine concentration (pg/ml) and standard error of 4 different donors after 48 h of incubation with medium or bacterial ligands. **b** p values of t tests between DC subsets after activation with bacterial ligands (relative cytokine levels). Statistical differences between DC subtypes are shown (t test).

Discussion

The generation of moDCs is well established, whilst moDCs do not fully reflect the complex network of mucosal DCs found in vivo, that is partly conditioned by local factors in the mucosa. In this report we describe two DC subsets that can be induced by the mucosal ligands RA and TGF- β 1. RA- and TGF- β 1-derived DCs were compared to the commonly used moDCs that are differentiated with IL-4 and GM-CSF only. Addition of RA gave rise to a CD103 and integrin β 7-expressing subset that also expressed CCR7 and resembles non-inflammatory DCs observed in vivo. Expression of CCR7 was also found to be increased on CD11c+CD103+ DCs isolated from human MLN, compared to CD11c+CD103- MLN DCs, indicating that our in vitro-generated RA-derived moDCs might be a relevant model for CD11c+CD103+CCR7+ DCs found in vivo [3]. DCs differentiated under the influence of IL-4 and GM-CSF in the presence or absence of TGF- β did not express CD103 and integrin β 7. Differentiation in the presence of RA or TGF- β 1 resulted in reduced responsiveness to bacterial ligands.

All three regimens used to differentiate peripheral monocytes seem to result in differentiation of immature

DC types as expression of CD14 was lost and HLA-DR, CD11b and CD11c were upregulated. CD103- and CD103+ DCs expressed CD209, but CD103+ DCs lacked CD1a. The in vitro-differentiated CD103+ DCs expressed CD11b, but at lower levels compared to CD103- moDCs. The cytokine TSLP did not induce expression of CD103. Although much progress has been made, the precise factors differentially inducing CD11c+CD103+ and CD11^{lo}CD103- DCs have not been revealed to date [24]. Even though functional consequences of different expression levels of CD11b and CD11c by DCs are not understood well, CD11b may correlate with more inflammatory DC subsets, whereas CD11c might be more expressed on tolerogenic CD103+ subsets in vivo [25]. None of the moDCs generated, neither basally, nor after addition of bacterial ligands, expressed CX3CR1. CX3CR1 expression could also not be induced by differentiation of DCs in the presence of IL-1 β . This might be explained by the finding that HLA-DR-positive colonic cells that expressed CX3CR1 also expressed CD14, which suggests that CX3CR1 expression in humans might be limited to macrophages and is lacking on DCs [26].

Compared to moDCs, differentiation using additional RA and TGF- β 1 resulted in reduced expression of

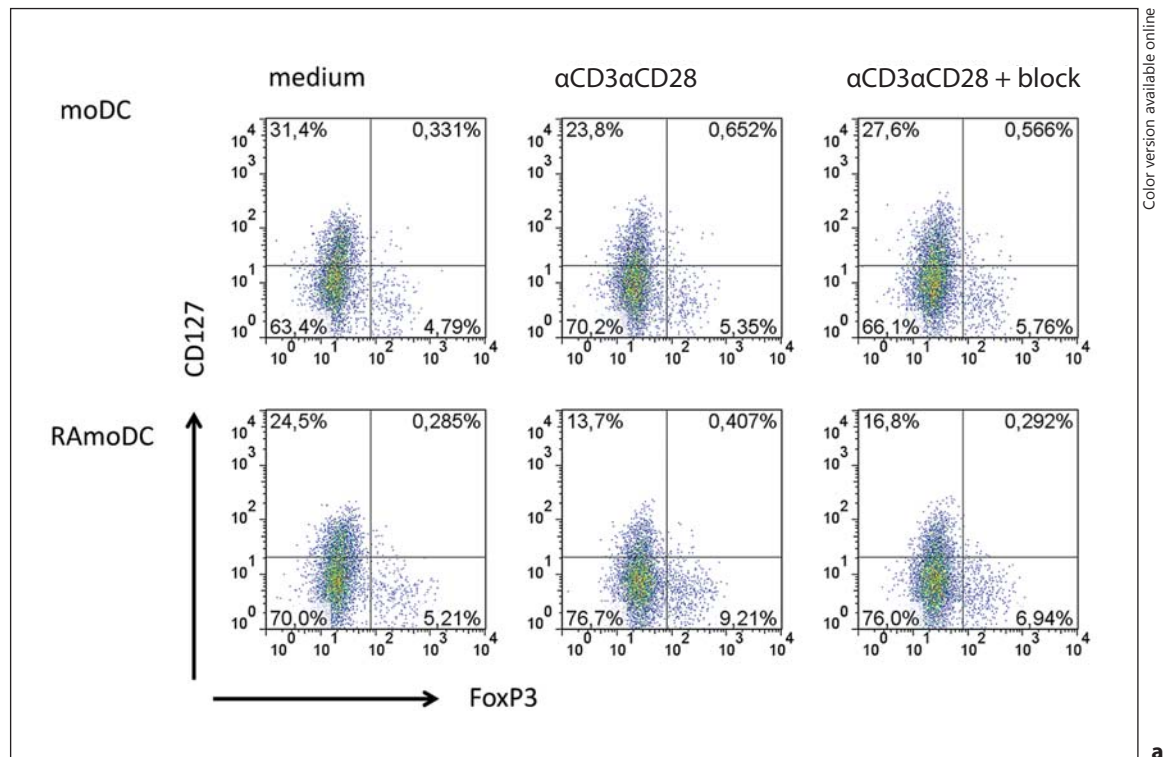
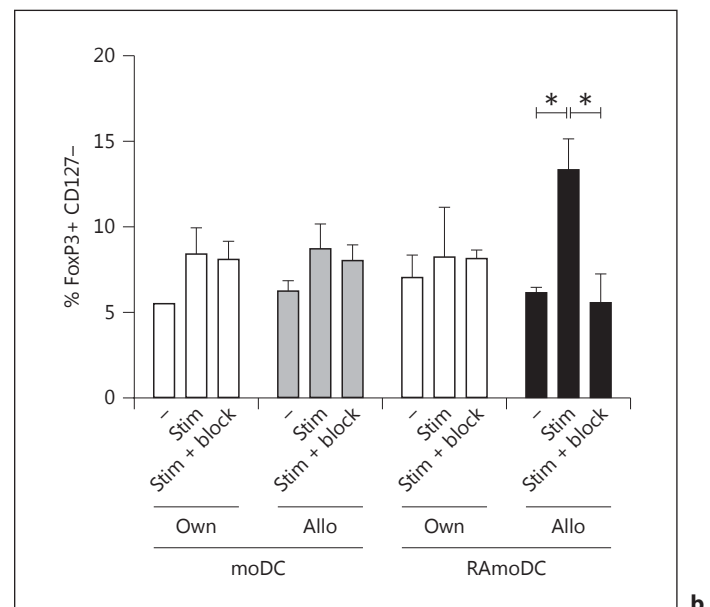


Fig. 5. Induction of FoxP3 expression by CD4⁺ T cells. T cells were added at day 5 of differentiation and incubated for another 5 days before cells were harvested for FACS staining. **a** CD4⁺ cells are gated on CD127 (y-axes) and FoxP3 (x-axes); medium (left), α CD3 α CD28 (middle) and α CD3 α CD28 plus RA block (LE135) and α TGF- β (right) for moDCs (top) and RAmoDCs (bottom) are presented. **b** Mean and standard error of 3 donors are shown for percentage FoxP3⁺CD127⁻ CD4 cells. Own = Autologous; Allo = allogeneic. * $p < 0.05$.



CD86 and CD83, and reduced production of cytokines, indicating that these cells may have tolerogenic properties, which was confirmed by their reduced activation by TLR ligands. Those findings are similar to data obtained after co-culturing moDCs with CaCo-2 cells for 24 h [3, 27].

CD103⁺ DCs are believed to migrate to the lymph node upon activation in a CCR7-dependent manner [28]. CD103⁺ DCs present in the MLN express CCR7 [3, 24], which was also found in a significant proportion of RA-derived moDCs. Expression of ALDH does not depend on TLR-signaling [29], which is in line with our finding

that expression of CD103 was downregulated after stimulation with bacterial ligands. It can be envisaged that CCR7-dependent migration is more efficient when expression of CD103 is reduced upon activation. An effect of the downregulation of CD103 expression upon stimulation with bacterial ligands may be that CD103+ DCs lose their non-inflammatory properties [16]. The functional relevance of CCR7 expression by RAmoDCs is not clear yet. CCR7 expression by CD103+ DCs may allow induction of peripheral tolerance, or enhance inflammation as CCR7-expressing DCs were observed in the colon of Crohn's disease patients, which were absent in healthy individuals [30].

Another interesting difference observed between the three DC types was the level of expression of FcγR on the cell membrane. Addition of TGF-β1 during differentiation resulted in a similar pattern of FcγR expression as on moDCs (CD32^{hi}, CD16^{lo}, CD32^{lo}) but expression levels of low affinity CD32 and high affinity FcγR CD64 were increased. RA, however, resulted in a lack of CD32 expression and relatively high expression of CD64. This may imply that moDCs and TGF-derived moDCs have a higher capacity to bind to immune complexes, including bacteria, whereas RA-derived moDCs may be specialized in binding soluble antigens (e.g. dietary proteins). Immune complex binding (e.g. bacteria) by moDCs may preferentially result in the activation of T cells, whereas those complexes bound by non-inflammatory TGF-derived DCs may result in tolerance to those bacteria. It has been established that ligation of CD64 by antigen-IgG complexes could result in activation of T cells by DCs [31]. As RA-derived DCs show non-inflammatory characteristics, ligation of CD64 on RA-derived DCs (CD103+) may result in induction of oral tolerance as those cells migrate to the MLN and induce Tregs in vivo. Currently, data describing FcγR expression of DC subsets is limited. As IL-4 downregulates FcγR expression, it is worthwhile evaluating the effect of RA and TGF-β1 on FcγR expression in the absence of IL-4 [32].

Like CD103+ DCs found in vivo, the RA-differentiated DCs described here could induce increased percentages of FoxP3+ in allogeneic CD4+ cells under the influence of suboptimal doses of anti-CD3 and anti-CD28 monoclonal antibodies (fig. 5). This increased expression was not observed when T cells were co-cultured with conventional moDCs, and could be abrogated by blocking TGF-β and RA simultaneously (fig. 5). It was previously shown that the RA- and TGF-β-dependent induction of FoxP3 expression by CD4+ T cells is abrogated when IL-15 is also present, and RA even enhances IFN-γ

production by CD4+ T cells in the presence of RA [6]. The different levels of cytokines produced by the DC types may result in differential skewing of T helper cells, as was observed for the induction of Th2 cells by DCs conditioned by epithelial cells [33]. The absence of production of Th1 inducing IL-12 by TGF-derived DCs may promote the development of Th2 cells, especially since IL-10 expression was largely maintained [34, 35]. Recently, DCs have been identified as important players in induction of mucosally produced IgA. CD103+ DCs are a candidate IgA-inducing DC subset [12] as these cells are able to produce the major IgA switch factor TGF-β, and RA which induces IgA plasma cell homing to the intestines [3, 4]. For synthesis of RA, humans depend on dietary intake of RA precursors. These precursors are present in several dietary ingredients, including a number of vegetables like carrot (β-carotene) and cow's milk. Induction of peripheral antigen-specific FoxP3+ Tregs may indirectly depend on dietary intake of RA. Also, TGF-β is present in physiologically relevant amounts (several nanograms per milliliter) in cow's milk. The bioactive form of TGF-β1 found in milk is largely conserved and TGF-β2 is identical to human TGF-β, and, interestingly, TGF-β bioactivity is induced by acidic conditions in the stomach rather than inhibited [36]. Also, bovine IL-10 is cross-reactive with the human IL-10R, though IL-10 is mainly present in colostrum and lacking in the majority of commercially available dairy products [37]. Presence of IL-10 during terminal differentiation into immature DCs gives rise to DCs with increased capacity to induce type 1 regulatory T cells [38, 39]. Thus, several mucosal and dietary factors may contribute to a non-inflammatory intestinal milieu.

In conclusion, the RA- and TGF-β1-differentiated DCs described here induce distinct mucosal DC subsets, including CD103+ DCs. The phenotype of CD103+ DCs resembles the CD103+ DCs described in in vivo studies, and can advance widely used DC-type in vitro models with a different functional response to bacterial ligands. However, it still needs to be further established to what extent those CD103+ moDCs resemble the functionality of CD103+ DCs in vivo. Importantly, the expression of homing markers and FcγR differed between the DC types, suggesting different functional roles in intestinal immunity. Induction of these non-inflammatory DC types may very well be modulated by the dietary intake of vitamin A and TGF-β.

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