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Differential regulation of CD103 (α E integrin) expression in human dendritic cells by retinoic acid and Toll-like receptor ligands

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ABSTRACT

CD103 (α E integrin) is an important dendritic cell (DC) marker that characterizes functionally distinct DC subsets in mice and humans. However, the mechanism by which CD103 expression is regulated in human DCs and the role of CD103 for DC function are not very well understood. Here, we show that retinoic acid (RA) treatment of human monocyte-derived DCs (MoDCs) increased the ability of the DCs to synthesize RA and induced MoDC expression of CD103 and β 7 at the mRNA and protein level. In contrast, RA was unable to induce the expression of CD103 in primary human DCs isolated from the gastric mucosa. Inhibition of TGF- β signaling in MoDCs down-regulated RA-induced CD103 expression, indicating that TGF- β -dependent pathways contribute to the induction of CD103. Conversely, when RA-treated MoDCs were stimulated with live *Helicobacter pylori*, commensal bacteria, LPS, or a TLR2 agonist, the RA-induced up-regulation of CD103 and β 7 integrin expression was completely abrogated. To determine whether CD103 expression impacts DC priming of CD4⁺ T cells, we next investigated the ability of CD103⁺ and CD103⁻ DCs to induce mucosal homing and T cell proliferation. Surprisingly, RA treatment of DCs enhanced both α 4 β 7 expression and proliferation in cocultured T cells, but no difference was seen between RA-treated CD103⁺ and CD103⁻ DCs. In summary, our data demonstrate that RA, bacterial products, and the tissue environment all contribute to the regulation of CD103 on human DCs and that DC induction of mucosal homing in T cells is RA dependent but not CD103 dependent. *J. Leukoc. Biol.* 101: 000-000; 2017.

Abbreviations: 7-AAD = 7-aminoactinomycin D, ALDH1A1/2 = aldehyde dehydrogenase 1 family member A1/2, ALK = activin receptor-like kinase, DC = dendritic cell, ILT3 = Ig-like transcript 3, IRB = Institutional Review Board, ITGAE = integrin α E, ITGB7 = integrin β 7, LAP = latency-associated protein, MoDC = monocyte-derived dendritic cell, MOI = multiplicity of infection, qRT-PCR = quantitative RT-PCR, RA = retinoic acid, rh = recombinant human, SEB = staphylococcal enterotoxin B, Smad = similar to mothers against decapentaplegic, T_{reg} = regulatory T cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

CD103 is a functionally important marker for DC subsets, especially at mucosal sites [1, 2]. A number of studies have established that CD103⁺ DCs, which represent a major DC subset in murine small intestinal lamina propria and mesenteric lymph nodes [2, 3], drive both the induction of T_{reg} responses and T cell expression of mucosal homing molecules α 4 β 7 and CCR9 through an RA-dependent mechanism [1, 3–7]. Likewise, human CD103⁺ DCs from mesenteric lymph nodes selectively induce RA receptor-dependent CCR9 expression on allogenic T cells [1]. Thus, CD103⁺ DCs are considered crucial for the induction of mucosal T cell homing and the maintenance of mucosal tolerance.

However, DC CD103 expression and functional properties of CD103-expressing DC subsets seem to differ between humans and mice and among different tissue compartments. Moreover, although transcriptional profiles of intestinal CD103⁺ DC subsets are largely conserved between mice and humans, some crucial differences have been determined [8–10]. Thus, RA biosynthesis is not restricted to the CD103⁺ DC subset in the human intestine, whereas in mice, there is a tight correlation between CD103 and RA-biosynthesis gene expression [9]. Compared with the murine intestine, CD103 is also less-widely expressed on human gastrointestinal DCs, with almost 40% of DCs lacking CD103 expression in the ileum and colon [8]. Importantly, in a previous study, we discovered that the absence of CD103 expression in gastric DCs was one major difference between human small intestinal and gastric DC populations, with <5% of CD103⁺ DCs in the human stomach compared with 15–20% in the human small intestine, in spite of similar levels of retinoids in these 2 compartments [10].

The mechanism by which CD103 expression on DCs is regulated is currently not well understood, particularly in the human system. One established pathway for the induction of CD103 is through TGF- β , which drives CD103 expression in T cells [11–14]. In a recent study in human CD8⁺ T cells, Mokrani

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et al. [15] identified specific bindings sites for Smad2/3, important transcription factors activated by TGF- β , in promoter and enhancer regions of the *ITGAE* gene, which encodes CD103. However, several independent studies using human DCs derived from blood monocytes or CD34⁺ progenitor cells did not observe increased CD103 expression with rTGF- β [16–19] but found increased surface expression of CD103 in response to RA [16–18, 20].

With a long-term goal of elucidating the discrepancy in CD103 expression between human gastric and intestinal DCs, here, we sought to define factors that regulate CD103 expression in human DCs, with a focus on RA, TGF- β , and the gastric pathogen *H. pylori*. We demonstrate that RA drives expression of both CD103 and the β 7 integrin in human MoDCs, but not in primary human gastric DCs, through a mechanism that involves TGF- β signaling. Moreover, we show that MoDC stimulation with *H. pylori*, commensal bacteria, or TLR2/4 agonists significantly inhibits CD103 and β 7 integrin expression. We also show that RA treatment of DCs enhances both α 4 β 7 expression and proliferation in cocultured T cells but that induction of T cell α 4 β 7 expression and proliferation was independent of CD103 expression by the DCs.

METHODS

MoDC

Whole blood was obtained with local IRB approval (Protocol #DB092614) from healthy, adult donors in Bozeman, Montana, USA, and PBMCs were isolated by centrifugation in leukocyte separation media (Lonza, Basel, Switzerland) at 800 *g* for 25 min at room temperature. CD14⁺ monocytes were isolated by MACS sorting (Miltenyi Biotec, Cologne, Germany), as previously described [21], which resulted in an average purity of 93.1 $\pm$ 3.2% (Supplemental Fig. 1A). All monocyte preparations were analyzed for activation based on cluster formation and spontaneous TNF- α release, and preactivated cells were excluded from our analyses. To generate MoDCs, monocytes were cultured in serum-free X-VIVO (Lonza) media, supplemented with 100 U/l penicillin, 100 μ g/l streptomycin, 50 μ g/ml gentamycin, 5 mM Hepes, and 2 mM L-glutamine (all Hyclone, Logan, UT, USA) and 25 ng/ml rhGM-CSF and 7 ng/ml rhIL-4 (R&D Systems, Minneapolis, MN, USA) for 3–5 d. Duration of the DC culture did not significantly affect DC viability or phenotype (Supplemental Fig. 1B and C). Serum-free medium was used in all experiments to avoid confounding effects of retinoids or TGF- β that are present in sera. In designated cultures, RA (Sigma, St. Louis, MO, USA) was added at 100 nM from d 0. Media, cytokines, and RA were replenished every 3 d. All RA-treated cells were handled under red light to prevent RA degradation.

Human gastric DCs

Gastric tissue specimens from sleeve gastrectomy surgeries were obtained with IRB approval by the National Disease Research Interchange (Philadelphia, PA, USA) or by Dr. Kent Sasse (Sasse Surgical Associates, Reno, NV, USA). To obtain gastric DCs, mucosal tissue was subjected to 3 rounds of EDTA treatment and then digested with collagenase solution, as described previously [22]. Gastric DCs were pre-enriched for HLA-DR⁺ cells by MACS (Miltenyi Biotec), and viable (7-AAD^{neg}) CD45^{pos}/lineage^{neg}/HLA-DR^{high} DCs were purified by FACS sorting on a FACSAria II sorter (BD Biosciences, San Jose, CA, USA). The lineage cocktail contained antibodies to CD3, CD19, CD20, CD56, and CD14.

TGF- β R inhibition and rhTGF- β culture

MoDCs were cultured for 3 d with or without RA, the TGF- β inhibitor SB431542 (50 μ M; Tocris Bioscience, Bristol, United Kingdom), rhTGF- β 1 or rhTGF- β 2 (0.5–5 ng/ml; R&D Systems), or a combination of these reagents added to the culture wells on d 0. Control wells were cultured with the

appropriate carrier, DMSO, or 4 mM HCl + 1 mg/ml BSA, respectively. None of the treatments significantly altered DC viability.

H. pylori, commensal bacteria, and TLR agonists

H. pylori strain 60190 (CagA⁺, VacA⁺) was plated from frozen stocks on *Brucella* agar plates, 5% horse blood (BD Biosciences), and was incubated under microaerophilic conditions. *H. pylori* were harvested into prewarmed *Brucella* broth and quantified as previously described [23]. Differentiated MoDCs generated in the presence or absence of RA were stimulated with the following: 1) *H. pylori* (MOI 10), 2) a commercially available preparation of probiotic bacteria (VSL#3: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus helveticus*; Sigma- τ Pharmaceuticals, Gaithersburg, MD, USA; MOI 10), or 3) individual TLR agonists, with continuous presence of RA and cytokines. Human TLR agonists (InvivoGen, San Diego, CA, USA) were used at the following concentrations: TLR2 agonist (heat-killed *Listeria monocytogenes*) 1 $\times$ 10⁸ cells/ml, TLR3 agonist (polyinosinic:polycytidylic acid high molecular weight) 10 μ g/ml, TLR4 agonist (*Escherichia coli* K12 LPS) 1 μ g/ml, TLR9 agonist (ODN2006 type B) 5 μ M. None of the treatments significantly altered DC viability. MoDCs were harvested after 48 h of stimulation and were then analyzed by FACS or qRT-PCR.

ELISA

Supernatants from MoDC cultures were analyzed for total TGF- β 1 or active TGF- β 1 by ELISA, following the manufacturer's protocol (BioLegend, San Diego, CA, USA). A TGF- β LAP ELISA (R&D Systems) was used to test both culture supernatants and cell lysates for LAP. Supernatants from DC–T cell cocultures were analyzed for IL-10 and IFN- γ using BioLegend kits. ELISA plates were read on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 and 570 nm, and data were analyzed with GraphPad Prism 6.05 (GraphPad Software, San Diego, CA, USA).

Antibodies and flow cytometry

MoDCs were stained using anti-human antibodies directed against CD103 (α E integrin), β 7, HLA-DR (all eBioscience, San Diego, CA, USA); TGF- β RII (R&D Systems); CD85k (ILT3; BioLegend); CD83, CD86, and CD11c (all BD Biosciences); or appropriate isotype controls. Dead cells were labeled with a LIVE/DEAD Yellow stain (Thermo Fisher Scientific, Waltham, MA, USA). A subset of cells was permeabilized with Cytofix/Cytoperm (BD Biosciences) for intracellular staining of CD103 and β 7. Following staining, cells were resuspended in FACS buffer for analysis on an LSR II or an LSR Fortessa Flow Cytometer (BD Biosciences). Sorting for CD103⁺ and CD103[−] populations was performed on a FACSAria II instrument (BD Biosciences). FACS data were analyzed using FlowJo X software (Tree Star, Ashland, OR, USA) with DCs gated based on size, single cells, and live cells. As baseline fluorescence levels differed between the 2 cytometers, data were normalized by dividing geometric fluorescence of the sample by geometric fluorescence of the appropriate isotype control (normalized geometric mean).

ImageStream analysis

For multispectral imaging flow cytometry, we used an ImageStream X Mark II (EMD Millipore, Seattle, WA, USA). Cells were labeled with predetermined optimum concentrations of antibodies, as described for FACS analysis above, and nuclei were labeled with DAPI. Data were analyzed with IDEAS software v6.1 (EMD Millipore). Images of 10,000 cells/sample were recorded in the following channels: Ch 1, brightfield; Ch 2, CD103 FITC; Ch 3, CD11c PE; and Ch 7, DAPI. DCs were gated as focused cells based on gradient root mean square Ch 1, single cells based on aspect ratio and area Ch1, nucleated cells based on DAPI staining in Ch 7, and finally, CD11c/CD103 double-positive cells in Ch 2 and Ch 3. A mask for membrane staining was created based on CD11c staining. An internalization score, reflecting CD103 staining not colocalized with CD11c membrane staining, was determined using the internalization wizard in the IDEAS software. Externalization was calculated as 100% – internalization.

qRT-PCR

Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) was used to isolate RNA from MoDC cultures. RT-PCR reactions were performed with iScript (Bio-Rad, Hercules, CA, USA). Gene-expression analysis for *ITGAE*, *ITGB7*, *TGFB1*, *TGFB2*, *TGFB3*, *ALDH1A1*, and *ALDH1A2* was performed with TaqMan Universal PCR MasterMix (Thermo Fisher Scientific) and primer/probes (Thermo Fisher Scientific) on a LightCycler 96 (Roche, Penzberg, Germany). The housekeeping genes 18s rRNA and GAPDH were used for normalization. qRT-PCR analysis was performed by the Pfaffl method [24].

Analysis of RA biosynthesis

RA production by MoDCs was analyzed using an RA bioassay, as described previously [10]. MoDCs were generated in serum-free medium and were transferred to wells containing Sil-15 monolayers and DMEM, supplemented with G418 and 20% FBS, which contains retinol, for the assay. The Sil-15 cell line used for the RA bioassay was kindly provided by Dr. Michael Wagner (The State University of New York Downstate Medical Center, Brooklyn, NY, USA) [25].

DC-T cell cocultures

DC-T cell cocultures were established using FACS-purified CD103⁺ and CD103⁻ DCs that were pulsed with SEB (1 μ g/ml; Toxin Technology, Sarasota, FL, USA) and autologous, naïve CD4⁺ T cells, as described previously [21]. T cell proliferation was determined using the CellTrace Violet (Thermo Fisher Scientific) dilution assay.

Statistical analysis

Data were analyzed using GraphPad Prism 6.05. Results are presented as means $\pm$ SEM. Differences between values were analyzed for statistical significance by the 2-tailed Student's *t* test, Mann-Whitney *U* test, or ANOVA with Tukey's post hoc test, as appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

RA drives RA biosynthesis by human MoDCs

RA is known to enhance RA biosynthesis in murine DCs through a positive-feedback loop [26–28]. We previously showed that exposure of human blood monocytes to RA-producing gastric epithelial cells or RA drives RA biosynthesis in the monocytes. To determine whether human MoDCs generated in the presence of RA also have an increased capacity for RA biosynthesis, we analyzed gene expression of 2 important RA biosynthesis enzymes—*ALDH1A1* and *ALDH1A2*—by qRT-PCR and DC release of RA using an RA bioassay [25]. MoDCs were generated from purified human blood monocytes in the presence of physiologic levels of all-trans RA (100 nM) [29] for 3–5 d. MoDCs generated in the presence of RA had a slightly, but not significantly, elevated expression of *ALDH1A2* compared with untreated cells (Fig. 1A). Importantly, we observed significant release of RA by live, RA-treated MoDCs but not by DCs generated in medium only or by fixed MoDCs, confirming that exposure to RA during DC development enhances their ability to release RA (Fig. 1B).

RA induces intracellular and extracellular expression of CD103 and β 7 integrin in human MoDCs

We next analyzed the effect of RA on DC CD103 expression. Generation of MoDCs in the presence of RA resulted in a

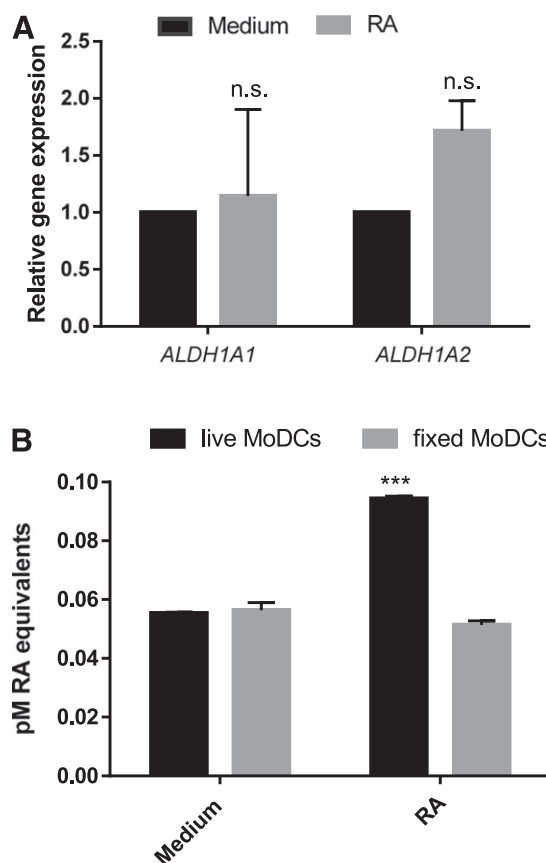


Figure 1. MoDCs generated in the presence of RA show increased RA biosynthesis. (A) MoDCs were cultured for 3–5 d in the presence of RA, harvested, washed, and then analyzed for *ALDH1A1* and *ALDH1A2* gene expression using qRT-PCR; cumulative data from 3 independent experiments (means $\pm$ SEM). (B) RA release by RA-treated and untreated MoDCs was analyzed using an RA reporter assay based on the Sil-15 cell line, as previously reported [10]. Means $\pm$ SD of triplicate wells; *** $P \leq 0.001$, Student's *t* test. Experiment shown is representative of $n = 3$.

sharp increase in surface expression of CD103 ($P \leq 0.05$; Fig. 2A and B and Supplemental Fig. 2). Expression of β 7 integrin was also significantly increased upon RA treatment (Fig. 2B).

Our data confirmed previous reports of only low levels ($6.8 \pm 2.8\%$) of CD103 surface expression on human MoDCs generated in the presence of medium alone [16, 30]. However, when we stained for CD103 expression after membrane permeabilization, which allows detection of both extracellular and intracellular proteins, an average of $50.8 \pm 17.5\%$ of DCs was CD103 positive. A significantly increased CD103 signal following permeabilization is consistent with additional intracellular CD103 expression and was seen in untreated and RA-treated MoDCs ($P \leq 0.05$; Fig. 2A and B). Surprisingly, we found no significant intracellular expression of β 7 in either RA-treated or untreated MoDCs (data not shown).

RA-treated and control MoDCs were also analyzed for gene expression of *ITGAE* and *ITGB7* (Fig. 2C). Consistent with the observed increase in CD103 and β 7 protein expression,

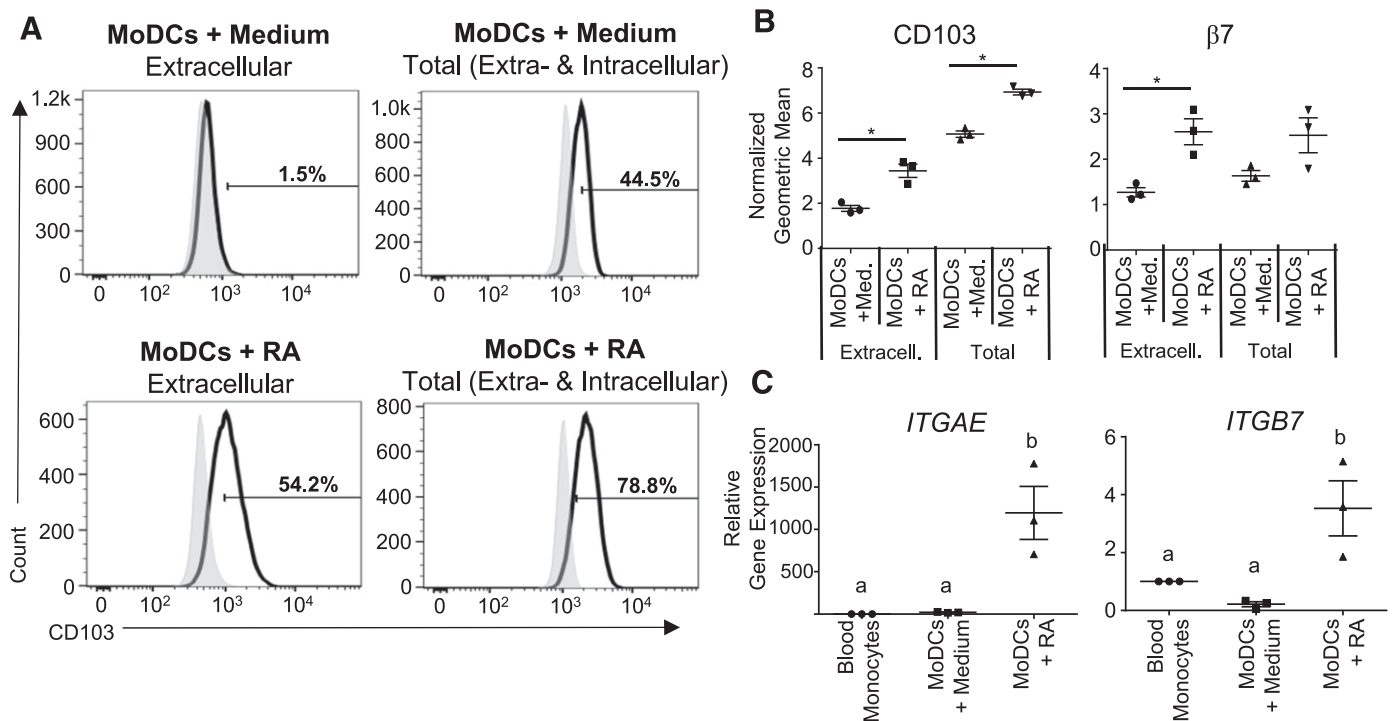


Figure 2. RA increases protein and gene expression of CD103 and $\beta 7$ in human MoDCs. MoDCs were cultured with RA or medium only for 5 d and harvested for FACS or gene-expression analysis. (A) Representative histograms of MoDCs cultured in the presence of RA or medium alone. CD103 expression was analyzed without cell permeabilization (extracellular) or after permeabilization (total). Gray histograms, Isotype controls; black lines, samples. (B) Cumulative FACS data for CD103 and $\beta 7$ expression from 3 independent experiments; individual data points (means $\pm$ SEM) are shown. Geometric mean fluorescence was normalized by dividing geometric mean fluorescence intensity of the sample by geometric mean fluorescence intensity of the isotype control. Different letters denote statistically significant differences (* $P < 0.05$, ANOVA). (C) qRT-PCR analysis of fresh monocytes and RA-treated and untreated DCs for CD103 (*ITGAE*) and $\beta 7$ (*ITGB7*) expression. Cumulative data from 3 independent experiments; different letters denote statistically significant differences ($P < 0.05$).

RA-treated MoDCs expressed significantly higher levels of *ITGAE* and *ITGB7* mRNA than control MoDCs. These experiments confirm and extend previous studies, which indicate that RA enhances CD103 expression in human DCs [16, 17].

RA does not alter cellular distribution of CD103

To confirm our observations of intracellular CD103 expression, we used imaging flow cytometry (ImageStream) to visualize the distribution of CD103 on the cell membrane and at intracellular sites. MoDCs were labeled with an anti-CD11c antibody and then were permeabilized for CD103 labeling. Gating strategy and masks used for analyzing ImageStream data are shown in Supplemental Fig. 3. ImageStream analysis confirmed our observations of intracellular CD103 expression in untreated and RA-treated MoDCs (Fig. 3A) and of increased overall CD103 expression upon RA treatment (Supplemental Fig. 3C).

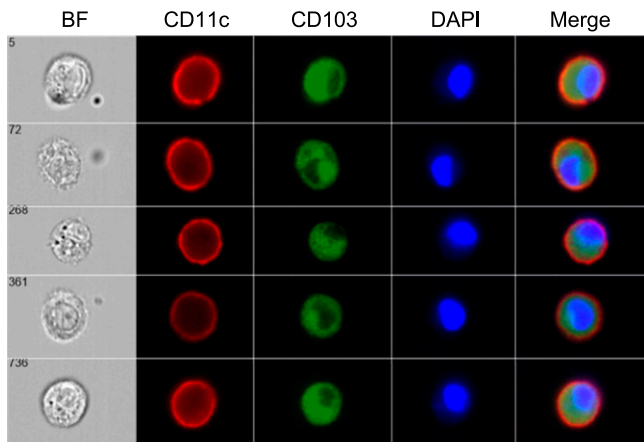
We next asked whether RA modulates the distribution of CD103 between intracellular compartments and the cell surface. However, we found no significant differences for externalization of CD103 between RA-treated and untreated MoDCs (Fig. 3B and C). With the use of data from the flow cytometry experiments shown in Fig. 2, we also detected no difference in the ratio of extracellular compared with total CD103 expression between RA-treated and untreated MoDCs (Fig. 3D). Thus, our

data indicate that RA increases both gene expression and protein expression of CD103 in MoDCs but does not alter relative distribution of CD103 within the cell.

RA does not induce expression of CD103 in primary human gastric DCs

We previously showed that primary human DCs isolated from the gastric mucosa express surprisingly low levels of CD103 (0–10%), in spite of high levels of retinoids present in the gastric mucosa [10]. Therefore, we asked whether exposure of gastric DCs to exogenous RA would increase their CD103 expression. Primary DCs were FACS purified from resected human gastric tissue (Fig. 4A) and were cultured in the presence of RA for 24 h. A longer treatment was not possible as a result of the limited lifespan of the primary DCs in vitro; however, a 16 h treatment was sufficient to induce significant up-regulation of CD103 in monocytes (data not shown). In contrast to the MoDCs, primary human gastric DCs increased their surface CD103 expression by a small amount only (5.1 ± 2.0 to $7.1 \pm 2.4\%$; $n = 3$; $P = 0.25$, Mann-Whitney *U* test; Fig. 4B). qRT-PCR analysis similarly revealed only a minor, insignificant increase in *ITGAE* expression following RA treatment of gastric DCs (data not shown). Thus, differentiation of DCs within the gastric mucosa seems to down-regulate their susceptibility to RA-induced regulation of CD103 expression.

A MoDCs + Medium



MoDCs + RA

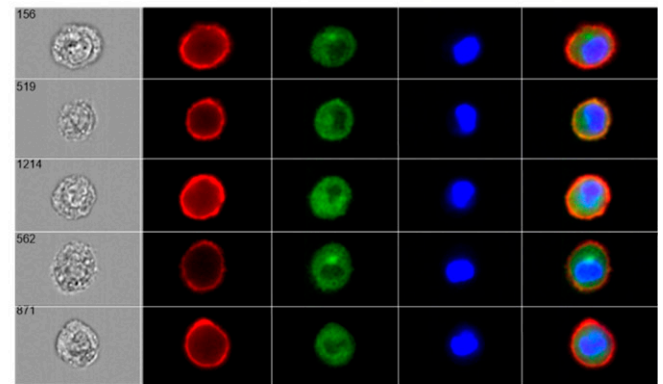
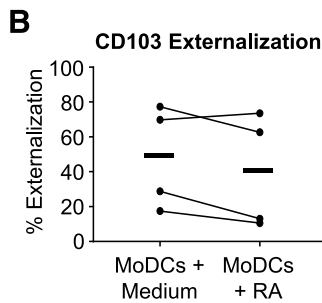
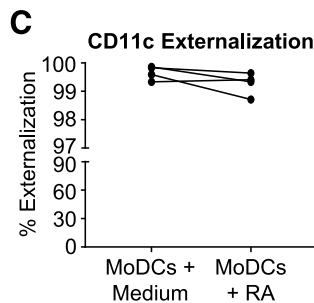
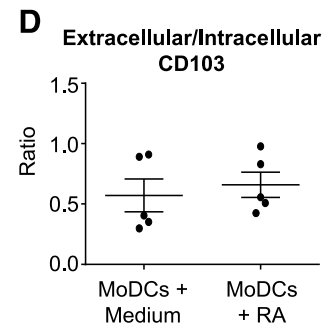


Figure 3. Imaging flow cytometry analysis of CD103 expression in RA-treated and untreated MoDCs. Imaging flow cytometry (ImageStream) analysis of MoDCs generated in the presence of RA or medium alone. MoDCs were permeabilized before addition of the anti-CD103 antibody to reveal intracellular CD103. (A) Representative images of control MoDCs (left panel) and RA-treated MoDCs (right panel) labeled for CD11c (PE, red), CD103 (FITC, green), and nuclei (DAPI, blue). BF, Brightfield. (B) Percentage of surface compared with intracellular CD103 (externalization score). A mask for cell surface staining was created based on CD11c surface labeling. Data from 4 independent experiments are shown. Lines connect control and RA-treated DCs from the same experiment. Thick, black lines, mean externalization scores. (C) Calculated externalization scores for CD11c obtained using the CD11c mask. (D) Ratio of extracellular versus total CD103 expression calculated based on the FACS data (geometric mean fluorescence intensity) shown in Fig. 2. Cumulative data from 5 independent experiments; individual data points (means ± SEM) are shown.

TGF-βRI signaling contributes to RA-induced up-regulation of CD103

To elucidate the mechanism by which RA induces CD103 expression, we focused on a potential role of TGF-β, as RA regulates both TGF-β and TGF-βR expression [31, 32], and TGF-β is a strong driver of CD103 expression in T cells [11–13]. However, the presence of RA in the culture medium had no significant impact on DC secretion of active or total TGF-β1 or on DC expression of cell-associated LAP, which is noncovalently bound to inactive TGF-β1, -2, and -3 [33] (Supplemental Fig. 4A). Moreover, gene expression of *TGFB1*, -2, and -3 was not significantly modified by RA treatment of DCs (Supplemental Fig. 4B), and we also found no significant changes in surface protein expression of TGF-βRII or in gene expression of *TGFBRI* and *TGFBRII* in response to MoDC RA treatment (Supplemental Fig. 4C).

To determine whether exogenous TGF-β increases the expression of CD103 or β7 integrin in human MoDCs, we derived MoDCs in the presence of 0.5 or 5 ng/ml rhTGF-β1 or rhTGF-β2. None of the TGF-β treatments caused a significant

change in CD103 or β7 expression (Supplemental Fig. 5), confirming previous studies in which TGF-β did not induce CD103 expression in human MoDCs when present during DC development [16, 17].

To investigate whether TGF-β-dependent signaling pathways are involved in RA-mediated up-regulation of CD103 in human MoDCs, we next cultured MoDCs with or without RA in the presence of the small molecule TGF-βR inhibitor SB431542. TGF-βR signal transduction involves phosphorylation of Smad2/3, which then recruit Smad4 to form the active transcription factor complex [34]. SB431542 inhibits TGF-βR signaling and phosphorylation of Smad2 by blocking the TGF-βRI ALK5 [35]. The Smad2/3 transcription factors were recently shown to contribute to the induction of CD103 expression in CD8 T cells through interactions with Smad-binding sites in the *ITGAE* promoter/enhancer region [15]. Here, MoDCs were cultured for 3 d in medium alone, medium and SB431542, RA only, or RA and SB431542. Interestingly, the observed significant increase in surface CD103 and β7 integrin expression induced by RA (Fig. 2) disappeared in the presence of the TGF-βR inhibitor (Fig. 5A

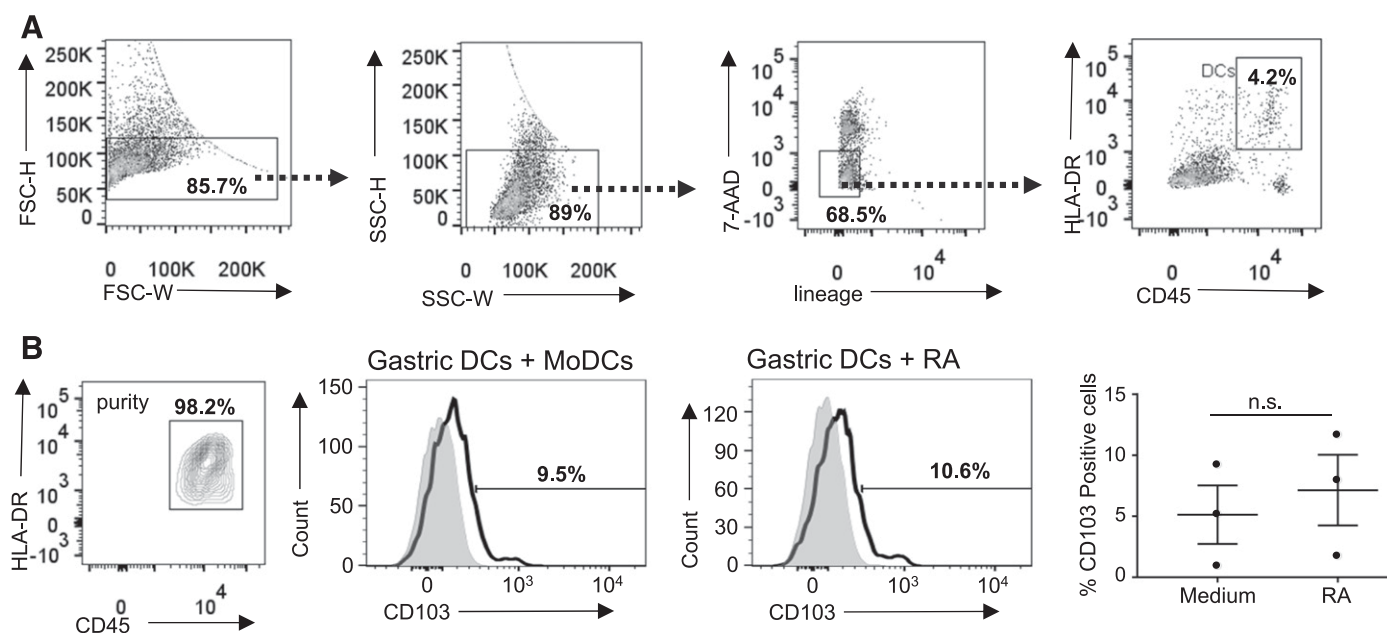


Figure 4. RA does not increase expression of CD103 on primary human gastric DCs. Gastric DCs were sorted as live HLA-DR⁺/CD45⁺/lin⁻ single cells and were incubated in the presence of RA (100 nM) for 24 h. (A) Sorting strategy, representative FACS plots. (B) Purity of sorted population and representative histograms of CD103 expression (left) and cumulative FACS data from 3 experiments (right; means $\pm$ SEM). FSC-H, Forward-scatter-height; FSC-W, forward-scatter-width; SSC-H, side-scatter-height; SSC-W, side-scatter-width.

and B). Importantly, MoDC gene expression of *ITGAE*, but not *ITGB7*, was also significantly reduced in the presence of both RA and the TGF- β R inhibitor compared with RA alone (Fig. 5C), suggesting that TGF- β signaling contributes to RA regulation of CD103 expression in human MoDCs.

TLR engagement abrogates RA-induced CD103 and β 7 expression

RA may interfere with the response of DCs to pathogenic stimuli by decreasing DC costimulatory molecule expression, proinflammatory cytokine release, and T cell stimulatory capacity [16, 36]. Here, we asked whether bacterial stimulation modulates DC CD103 expression. MoDCs were cultured with or without RA for 3 d and then stimulated with *H. pylori*, an important mucosal pathogen that induces DC activation [21, 22, 37], in the continued presence or absence of RA. As previously reported [16, 36, 38], RA caused a decreased expression of the DC activation markers CD83 and CD86, both in the presence and absence of *H. pylori* stimulation (Fig. 6A). RA also significantly reduced HLA-DR expression in *H. pylori*-treated MoDCs (Fig. 6A). In contrast, expression of ILT3 (CD85k), a regulatory DC marker, was significantly increased following RA treatment, independent of *H. pylori* stimulation (Fig. 6A). These observations are consistent with an increased tolerogenic DC phenotype following RA treatment.

Surprisingly, *H. pylori* stimulation completely abrogated the RA-induced increase in surface expression (Fig. 6B and C) and intracellular expression (data not shown) of CD103 and β 7. Likewise, gene expression of *ITGAE* and *ITGB7* was significantly reduced in RA-treated MoDCs stimulated with *H. pylori* compared with nonstimulated, RA-treated MoDCs

(Fig. 6D). As a 3-d exposure of developing MoDCs to RA was sufficient to induce a significant increase in CD103 and β 7 expression, as shown in Supplemental Fig. 1C, our observations suggest that *H. pylori* stimulation actively down-regulates CD103 and β 7 expression. However, the mechanism for this down-regulation remains unclear, as *H. pylori*, like RA, had no significant effect on DC TGF- β secretion (Supplemental Fig. 4A).

One major pathway by which *H. pylori* interacts with and stimulates DCs is through activation of TLR2, -4, and -9 [39–41]. To determine whether downmodulation of CD103 and β 7 in RA-treated DCs was specific to *H. pylori* or mediated by global pattern recognition receptor activation, we compared *H. pylori* bacteria with a panel of TLR agonists, as well as a commercially available preparation of commensal bacteria (VSL#3) for their ability to block RA-induced CD103 expression. Stimulation of DCs generated in the presence of RA with agonists for TLR2 and -4 or with commensal bacteria (MOI 10) resulted in a significant reduction of both CD103 and β 7 expression, as also seen after *H. pylori* stimulation (Fig. 6E). These observations indicate that bacterial ligands block RA-induced expression of CD103, likely through a pathway that involves TLR2 and TLR4 activation.

CD103 expression on RA-treated MoDCs does not affect the ability of the DCs to induce T cell proliferation and α 4 β 7 expression

The induction of the mucosal homing molecules α 4 β 7 and CCR9 in responder T cells through a RA-dependent mechanism is considered a major functional property of CD103⁺ DCs [1, 3, 27, 42]. Having confirmed that MoDCs generated in the presence of

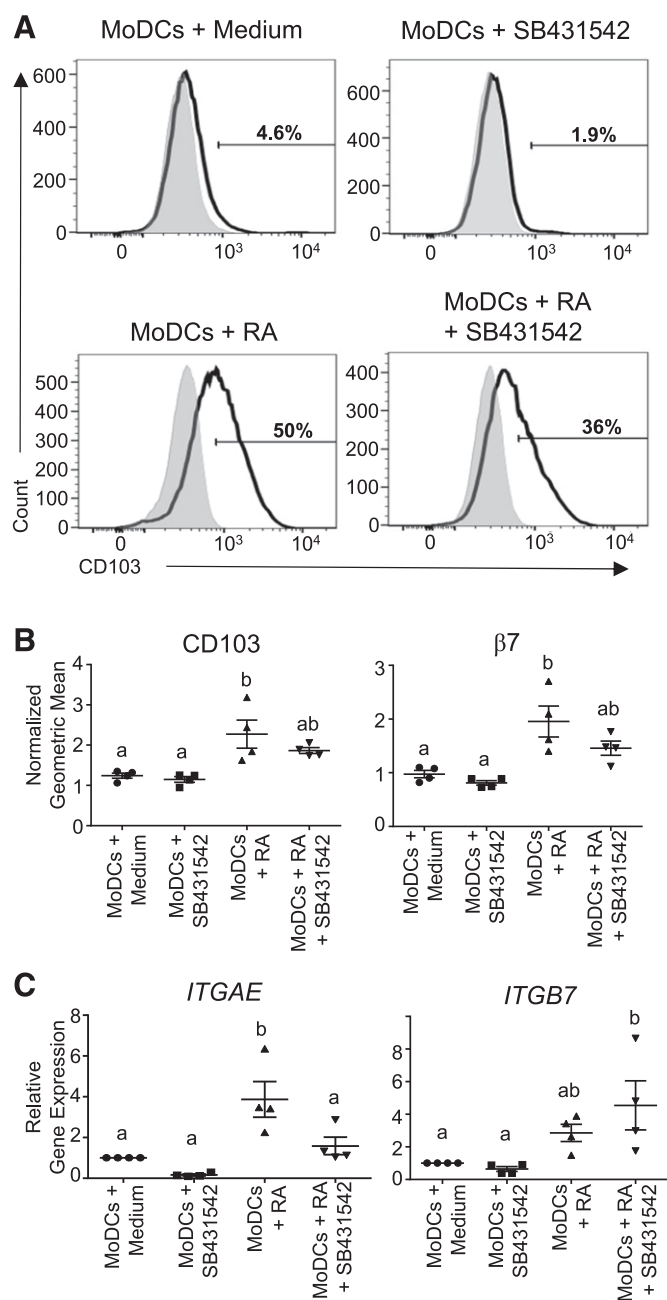


Figure 5. Inhibition of TGF- β signaling blocks RA-induced up-regulation of CD103. MoDCs were cultured with or without RA and/or the TGF- β R inhibitor SB431542 for 3 d and harvested for FACS and gene-expression analysis. (A) Representative histograms of MoDCs cultured in the presence of medium, RA, SB431542, or RA + SB431542. Gray histograms, Isotype controls; black lines, samples. (B) Cumulative FACS data from 4 independent experiments; individual data points (means $\pm$ SEM) are shown. Different letters denote statistical significance ($P < 0.05$, ANOVA). (C) qRT-PCR analysis of RA-treated and untreated DCs for CD103 (*ITGAE*) and $\beta 7$ (*ITGB7*) expression. Cumulative data from 4 independent experiments; different letters denote statistically significant differences ($P < 0.05$).

RA synthesize and release RA (Fig. 1B), we asked whether these RA-treated DCs induce T cell $\alpha 4\beta 7$ expression and whether induction of T cell $\alpha 4\beta 7$ depends on DC expression of CD103. MoDCs were generated in the presence or absence of RA,

sorted into CD103⁺ and CD103⁻ cells (Supplemental Fig. 6A), loaded with SEB, and cocultured with autologous, naïve CD4⁺ T cells. The T cells were analyzed for $\alpha 4\beta 7$ integrin expression and proliferation after 4 d (Fig. 7A). As anticipated, SEB-loaded MoDCs, but not MoDCs without antigen, consistently induced $\alpha 4\beta 7$ expression and T cell proliferation (Fig. 7B and C). Importantly, MoDCs generated in the presence of RA induced higher expression of T cell $\alpha 4\beta 7$ expression (Fig. 7B). However, both CD103⁺ and CD103⁻ DCs from RA-treated cultures drove similar levels of $\alpha 4\beta 7$ expression. Interestingly, RA-treated DCs also induced slightly higher levels of T cell proliferation than control DCs, again with no significant difference between CD103⁺ and CD103⁻ DCs (Fig. 7C). Bakdash et al. [20] recently showed that RA conditioning of human DCs did not substantially enhance T cell forkhead box p3 expression but induced $\alpha 4\beta 7$ ⁺ T cells expressing high levels of IL-10. Analysis of supernatants from our experiments similarly revealed a trend for increased levels of IL-10, but not IFN- γ , in cocultures from T cells and RA-treated MoDCs compared with control MoDCs, with CD103⁺ and CD103⁻ DCs again inducing similar levels of cytokine secretion (Supplemental Fig. 6B and C). Overall, our data suggest that RA exposure, rather than direct interactions between DC CD103 and T cells, contributes to the induction of mucosal homing molecules, proliferation, and cytokines on responder T cells.

DISCUSSION

CD103 (αE integrin) is considered a functionally important marker of DCs, particularly in the intestinal tract [1, 2, 43]. However, the mechanism by which CD103 expression is regulated in human DCs is not well understood [11]. Here, we investigated the regulation of CD103 and $\beta 7$ integrin expression by RA and by bacterial pathogen-associated molecular patterns. We show that CD103 was up-regulated by RA in MoDCs but not in primary human gastric DCs and that DC CD103 and $\beta 7$ integrin expression was down-regulated following TLR2/4 stimulation.

Our data confirm and extend previous reports of CD103 up-regulation by RA in human MoDCs [16–18]. Importantly, we used an improved culture system with physiologic concentrations of RA (100 nM), as well as a serum-free medium, thereby eliminating confounding effects of retinoids and TGF- β found in human and animal sera. Moreover, we show that $\beta 7$ integrin, which forms a constitutive heterodimer with the αE integrin subunit [11], was similarly regulated by RA, confirming the physiologic relevance of our data. Interestingly, our experiments revealed that human MoDCs contained significant pools of intracellular CD103 but not $\beta 7$ integrin that were also up-regulated upon RA treatment. Several other integrins are known to recirculate through the cell membrane by endosomal trafficking and thus, exist temporarily at intracellular sites, although this had not been shown previously for CD103 (αE) [44, 45]. Notably, RA did not enhance the relative distribution of surface-expressed versus intracellular CD103, as our imaging flow cytometry analysis showed. As we were

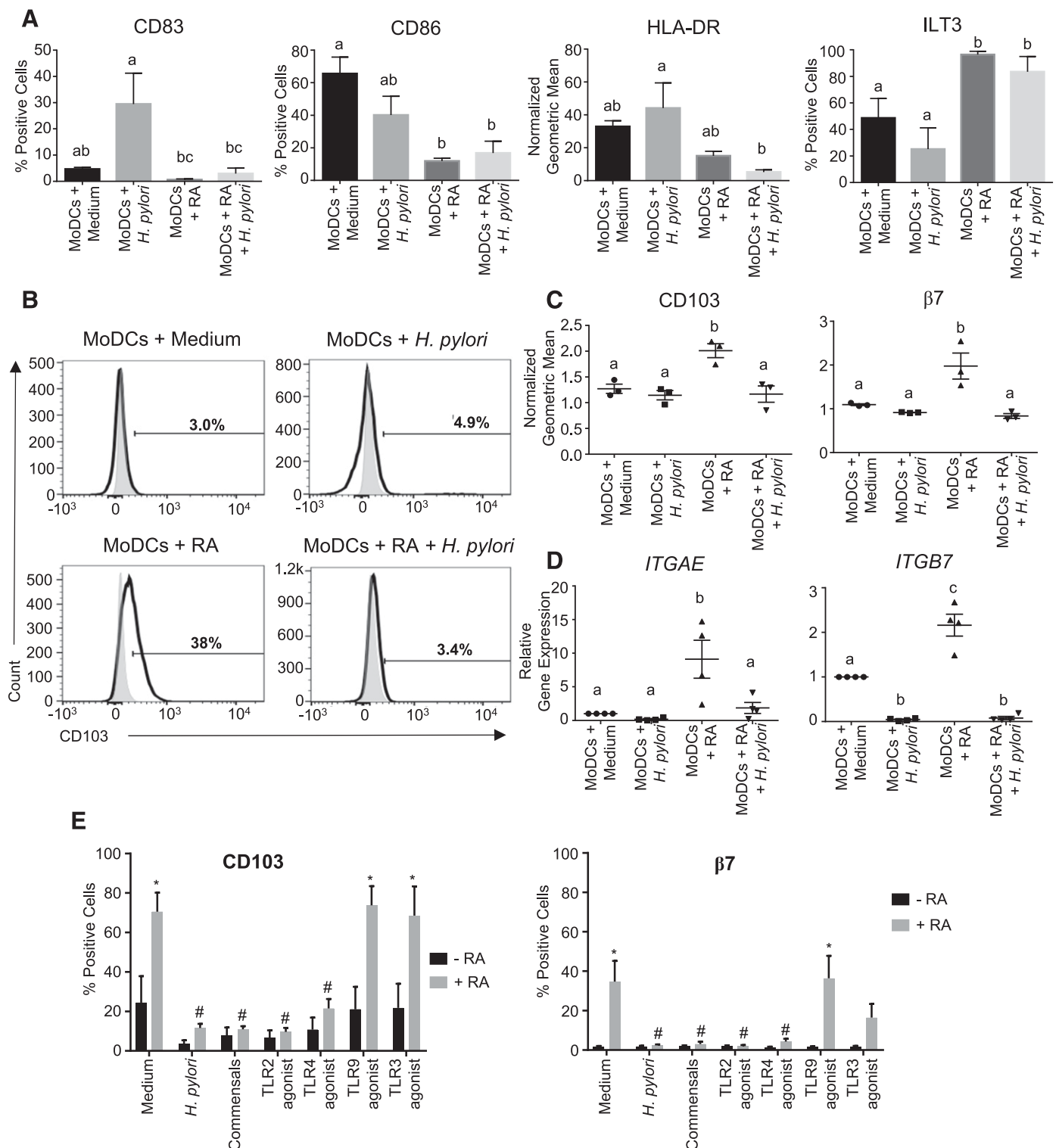


Figure 6. Stimulation of MoDCs with *H. pylori*, commensal bacteria, or TLR2/4 ligands abrogates RA-induced up-regulation of CD103 and β7. MoDCs were cultured with RA or medium for 3 d and then stimulated for 48 h with *H. pylori* 60190 (MOI 10), with continued exposure to RA or medium. (A) Cells were analyzed for surface expression of DC maturation/activation markers CD83, CD86, and HLA-DR and the regulatory marker ILT3 after *H. pylori* treatment. The percentage of cells expressing CD83, CD86, and ILT3 or geometric mean fluorescence of HLA-DR is shown as means ± SEM from 3 or more independent experiments. (B) DCs were analyzed for surface expression of CD103. Representative histograms of MoDCs cultured in the presence of medium or RA and then stimulated with *H. pylori* or *Brucella* broth control. Gray histograms, Isotype controls; black lines, samples. (C) DC CD103 and β7 expression; cumulative FACS data from 3 independent experiments; individual data points (means ± SEM) are shown. (continued on next page)

unable to detect a significant amount of intracellular $\beta 7$ integrin, the intracellular CD103 may correspond to abortive expression of the CD103 monomer.

A number of reports have investigated the influence of human DC treatment with RA on the ability of DCs to prime T cells [18, 20, 36, 38]. Overall, human DCs exposed to RA display a more anti-inflammatory phenotype, with increased IL-10 and decreased IL-12 production [36], consistent with our observations of decreased CD83, CD86, and HLA-DR expression and increased ILT3 expression. Furthermore, RA-treated human DCs have been shown to promote the expansion of T_{regs} with strong T cell IL-10 production [18, 20] and to induce expression of intestinal homing molecules $\alpha 4\beta 7$ and CCR9 on the T cells [38]. In our hands, T cells cocultured with RA-treated DCs likewise expressed increased levels of $\alpha 4\beta 7$, consistent with a mucosal homing phenotype, and secreted increased levels of IL-10, consistent with a more regulatory phenotype. Surprisingly, RA-treated MoDCs also induced higher levels of naïve CD4⁺ T cell proliferation, which was contrary to expectations and other published reports [20].

Numerous murine studies have revealed differential roles for CD103⁺ and CD103⁻ DCs in T cell priming [2, 3, 7, 9, 46]. Although T cells likely do not express E-cadherin for interacting with DC CD103, additional cellular ligands for CD103/ $\beta 7$ have been proposed [47, 48]. Therefore, we asked whether CD103 is directly involved in T cell priming by DCs. Engagement of CD103 may trigger intracellular signaling through integrin-linked kinase and downstream AKT or through Src and/or Syk-ZAP70-dependent pathways that conceivably modulate DC function and resulting T cell responses [49, 50]. However, in our hands, RA-treated CD103⁺ and CD103⁻ DCs induced similar levels of T cell $\alpha 4\beta 7$ expression, cytokine release, and proliferation. Thus, our current data do not support the hypothesis that CD103 is actively involved in the priming of human CD4 T cells.

Importantly, our study provides novel evidence for dynamic regulation of CD103 and $\beta 7$ integrin expression in human DCs. Whereas RA up-regulated CD103 expression on MoDCs, bacterial TLR agonists down-regulated RA-induced CD103 expression. Interestingly, both a gram-negative pathogen (*H. pylori*) and gram-positive commensal bacteria were similarly efficient at blocking CD103 and $\beta 7$ expression, as was pure LPS and heat-killed *Listeria* but not TLR3 or TLR9 agonists, implicating a common pathway downstream of TLR2/4 in CD103 down-regulation. If CD103 mediates interactions between DCs and the mucosal epithelium through binding to epithelial E-cadherin, as shown for intraepithelial lymphocytes [51, 52] and as suggested for gastrointestinal DCs [11], then down-regulation of CD103 may enable migration of DCs to other sites. Intriguingly, primary human gastric DCs that have presumably differentiated within the gastric mucosa from DC

precursors were unable to up-regulate surface CD103 expression in response to exogenous RA or in response to the retinoids naturally present in human stomach [10], suggesting that additional mucosal factors may contribute to CD103 regulation. Further experiments are needed to determine whether a shared pathway down-regulates CD103 expression following TLR engagement and blocks CD103 expression in primary gastric DCs. In this context, we have reported that baseline cytokine content of the gastric stroma differs from cytokine content in the small intestinal mucosa, where DC expression of CD103 is significantly higher [10, 21]. Furthermore, samples of gastric but not intestinal stromal-conditioned media routinely contained high concentrations of LPS (12.2 ± 2 U/ml) [unpublished results]. High baseline levels of LPS in gastric mucosa may conceivably contribute to reduced CD103 expression by the primary gastric DCs, as shown in our in vitro experiments.

The pathways that regulate CD103 and $\beta 7$ integrin expression in DCs are largely unclear. TGF- β is commonly recognized as the major regulator of CD103 expression [11–14], and areas of interaction of Smad2/3 with the CD103 promotor site were recently identified [15]. RA has been shown to induce TGF- β and TGF- β R in other model systems [31, 32, 53, 54], and our experiments using the TGF- β R1 inhibitor SB431542 prevented RA-induced up-regulation of CD103 but not $\beta 7$ integrin expression in the DCs, indicating that RA modulation of TGF- β pathways contributes to RA induction of CD103. Notably, baseline TGF- β levels in human gastric mucosa are very low, as is CD103 expression by primary human gastric DCs [10, 21]. However, our experiments with RA-treated MoDCs did not reveal an increase in total or active TGF- β 1 release or cell-associated LAP, which tightly correlates with membrane-bound TGF- β . Moreover, we were unable to induce CD103 or $\beta 7$ integrin expression in the MoDCs using exogenous TGF- β , confirming earlier reports [16, 17]. Our failure to detect a direct role for TGF- β in our experiments may have been a result of an analysis of suboptimal time points. Alternatively, TGF- β family members, such as inhibins or nodal, also signal through ALK receptors that are inhibited by SB431542 (ALK4, ALK7), share downstream signaling pathways with TGF- β , and might thus be involved in altered CD103 expression following RA treatment [35, 55]. Thus, further work is needed to elucidate the role for TGF- β -associated signaling in the RA-dependent regulation of induction of the CD103/ αE integrin.

In summary, we report a dynamic regulation of CD103 and $\beta 7$ integrin expression in human DCs by RA, *H. pylori* bacteria, and other TLR2/4 agonists and possibly additional factors present in the gastric mucosal environment. Our data also indicate that RA, but not CD103 itself, contributes to the

Different letters denote statistically significant differences ($P < 0.05$, ANOVA). (D) qRT-PCR analysis of RA-treated and untreated DCs for CD103 (*ITGAE*) and $\beta 7$ (*ITGB7*) expression. Cumulative data from 4 independent experiments; different letters denote statistically significant differences ($P < 0.05$). (E) MoDCs generated in the presence or absence of RA were stimulated for 48 h with *H. pylori*, VSL#3 commensal bacteria, or TLR2, -4, -9, or -3 agonists. Cumulative FACS data of CD103 and $\beta 7$ expression from 3 independent experiments. *Significant difference from medium only; #significant difference between RA-treated, unstimulated cells and RA-treated cells with bacterial stimulation ($P < 0.05$).

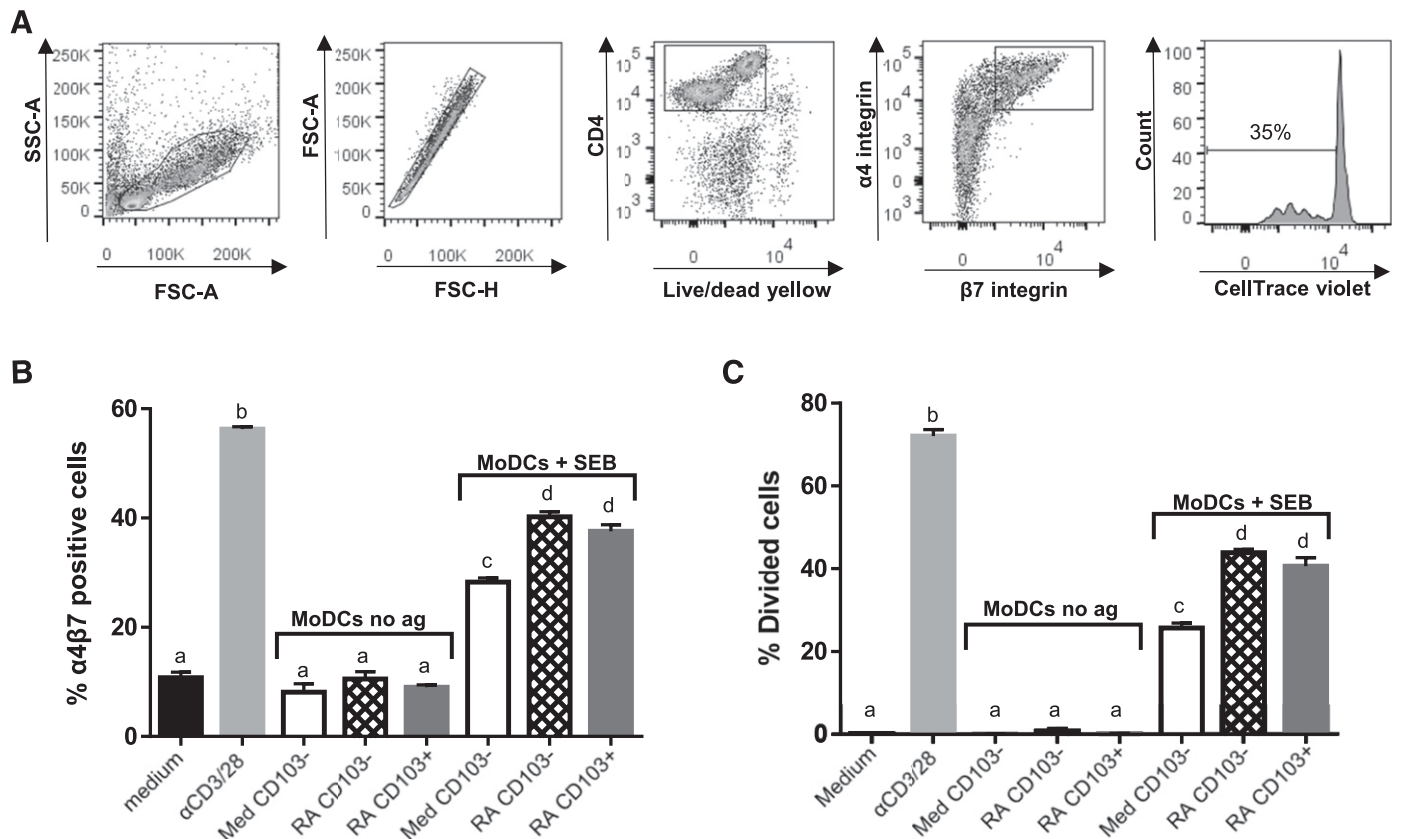


Figure 7. RA-treated DCs drive increased T cell $\alpha 4 \beta 7$ expression and proliferation, independent of CD103 expression. MoDCs were cultured with RA or medium alone for 3 d and were then sorted by FACS as CD103⁺ or CD103⁻ DCs. As a result of low numbers of CD103⁺ DCs in samples without RA treatment, this population was excluded from the analysis. Sorted DCs were loaded with SEB (1 μ g/ml) for 1 h and then cocultured with CellTrace Violet-labeled autologous, naïve CD4⁺ T cells for 4 d. Cocultured T cells were harvested and analyzed for expression of $\alpha 4 \beta 7$ and proliferation. (A) T cell gating strategy and representative proliferation and $\alpha 4 \beta 7$ expression. SSC-A, Side-scatter-area; FSC-A, forward-scatter-area. (B) Percent of CD4⁺ T cells coexpressing $\alpha 4$ and $\beta 7$ integrin. (C) Percent of divided T cells. Means $\pm$ SEM of triplicate wells; experiment shown is representative of $n = 4$. Different letters denote statistically significant differences ($P < 0.05$).

induction of a mucosal homing phenotype in responder T cells.

AUTHORSHIP

D.B., P.D.S., and L.E.S. developed the project. D.B., M.M.R., and S.S. designed the experiments and analyzed the data. M.M.R., S.S., T.A.S., M.M.C., M.A.S., and D.B. performed experiments. B.A.P. provided gastric tissue samples. M.M.R., S.S., D.B., P.D.S., and L.E.S. critically interpreted the data. M.M.R. and D.B. wrote the manuscript. S.S., P.D.S., and L.E.S. revised the manuscript. D.B., P.D.S., and L.E.S. provided funding for the project. All authors have approved the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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