THE REGULATION AND FUNCTION OF INTEGRIN αE (CD103) IN HUMAN DENDRITIC CELLS

by

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NOMENCLATURE

Aldh1a2 – retinaldehyde dehydrogenase

ALK – activing receptor-like kinase

CagA – cytotoxin-associated gene A

CD103 – αE integrin, gene name *ITGAE*

cDC – conventional DCs

DC – dendritic cell

Ecad – E-cadherin

EEA-1 – early endosome antigen 1

ERK1 – extracellular signali-regulated kinase 1

H. pylori – Helicobacter pylori

IEL – intraepithelial lymphocyte

IL - interleukin

ILT3 – Ig-like transcript 3

LAP – latency-associated protein

LPS – lipopolysaccharide

MAPK – mitogen activated protein kinase

MoDCs – monocyte-derived DCs

MOI – multiplicity of infection

NFAT – nuclear factor of activated T cells

pre-μ DCs – pre-mucosal DC progenitor

qRT-PCR – quantitative RT-PCR

NOMENCLATURE CONT.

RA – retinoic acid

RAR – retinoic acid receptor

rh – recombinant human

RT-PCR – reverse transcriptase polymerase chain reaction

RXR – retinoid X receptors

SEB – staphylococcal enterotoxin B

Smad – similar to mothers against decapentaplegic

TGF-β – transforming growth factor-β

TGF-βRII – transforming growth factor-β receptor II

TLR – toll-like receptor

TNF – tumor necrosis factor

Treg – regulatory T cell

VacA – vacuolating cytotoxin A

ABSTRACT

Retinoic acid (RA) is a master regulator of cellular signaling and function as well as an important mediator of immune development and maintenance. CD103 is a marker that is used to distinguish functional subsets of mucosal DCs. Despite the use of CD103 as a DC marker, the RA-induced pathway leading to CD103 expression is yet unknown. In addition, the function of DC CD103 has not been fully elucidated. In this dissertation research, we evaluated the regulation of CD103 expression on human DCs and investigated the function of DC CD103. We first found that CD103 expression is driven by RA and that CD103 is found in intracellular pockets in human DCs. However, the RA-induced increase of CD103 was abrogated upon stimulation of DCs with TLR ligands. To elucidate the RA-induced pathway of CD103 expression, we established the dependence on p38 MAPK signaling and NFAT through the use of specific inhibitors. Studies with RARα siRNA and the use of RAR-specific agonists show that CD103 expression is dependent on RARα signaling. To investigate further the intracellular CD103 expression, we demonstrated that CD103 was co-localized with endosomal markers and was actively internalized over time in DCs, suggesting CD103 undergoes endosomal recycling. Based upon imaging of gastric tissue showing $CD103^+$ DCs are most often within the gastric epithelial layer, we sought to understand the role of CD103 in DC adhesion. We investigated whether CD103 is involved in adhesion of DCs to the epithelium by co-culturing the DCs with HT-29 cells, which express E-cadherin on the entire cell surface. Interestingly, we found that CD103 was not a main driver of DC-epithelial adhesion, but that DC binding to the gastrointestinal epithelium was mediated by the interactions between DC E-cadherin and E-cadherin on the HT-29 cells. In summary, this research has contributed to the understanding of CD103 expression and function on human DCs. CD103 plays a minor role in the adhesion of DCs to the gastrointestinal mucosa, despite $CD103^+$ DCs close proximity to the gastric epithelium. RA drives the expression of CD103 on DCs mediated through RARα and p38 MAPK signaling and NFAT.

CHAPTER 1

INTRODUCTION

The research focus of the Bimczok laboratory is to understand the role of dendritic cells in gastrointestinal immunology, especially in the context of gastric *Helicobacter pylori* (*H. pylori*) infection. The overall goal of this dissertation work is to evaluate the function and regulation of CD103 on human dendritic cells (DCs). This includes elucidating molecular signaling pathways that induce the expression CD103. In addition, this researach investigated the functional relevance of CD103 expression by human DCs including a potential role of CD103 in DC interactions with epithelial E-cadherin. The following provides an overview on the role of DCs, CD103, and retinoic acid (RA) in the immune system, on p38 mitogen activated protein kinase (MAPK) signaling and *H. pylori* infection. Then an explanation of the overarching hypothesis for my dissertation research.

Dendritic Cells

DCs are professional antigen presenting cells that are a part of the innate immune system. Innate immune cells are generally non-specific to antigen and are the first line of immune cell defense against foreign pathogens. DCs perform immune-surveillance within the mucosa by ingesting foreign particulates. Unlike other innate immune cells, DCs do not just phagocytose for clearance, but also to initiate adaptive immunity through antigen presentation. DCs can extend dendrites into the gut lumen to phagocytose foreign antigen or migrate into the gastrointestinal epithelial layer (1, 2). Upon antigen uptake,

DCs then migrate to the lymph node to prime T cells through presentation of antigen and engagement of co-stimulatory factors. The environment and the cytokines released by the DCs drive T cell polarization towards different phenotypes (3, 4). DCs can secrete pro-inflammatory cytokines to drive inflammatory T cell subsets, Th1, Th2, Th17, Th22, and Th9 (5). It has also been well established that in the presence of transforming growth factor-β (TGF-β) and RA, DCs prime a T regulatory (Treg) response (6). Although there are other antigen presenting cell types, DCs are attributed as the main drivers of adaptive immunity through T cell priming. In this sense, DCs are the bridge between the innate and adaptive immune responses. The adaptive immune system consists of cells that retain memory of foreign antigen in order to mount a more immediate response upon repeat exposure to that specific pathogen.

Multiple subsets of conventional DCs (cDC) have been identified in the gastrointestinal mucosa. IRF8-dependent DCs are classified as cDC1 and express CD8α in mouse, CD141 in humans, and Clec9A in both species (7, 8). Human cDC1 populations stimulated with virus are able to increase the proliferation of $CD8^+$ T cells beyond other DC subsets (9). IRF4-dependent DCs are classified as cDC2 and co-express CD11b and CD172α in mouse, CD1c in human, and SIRPα in both species (7, 8). Human cDC2 populations demonstrate similar abilities as cDC1 to phagocytose antigen (10). However, cDC2 populations have increased ability to secrete IL-12 and IL-10 (11-13). Each of these cDC subsets are derived from the same pre-mucosal DC (μDC) progenitor (8). DCs destined for the cDC lineage, but not plasmacytoid DCs, can be identified by the expression of the transcription factor Zbtb46 during development (14). *In vitro*

differentiation of pre-μDCs is dependent on RA to push them to most closely reflect the tissue-specific cDC1 and cDC2 populations (15, 16).

CD103

CD103 (αE integrin) forms a heterodimer with integrin β7 (αEβ7). Both human cDC1 and cDC2 populations can express CD103 (αEβ7 integrin) within the gastrointestinal mucosa (7, 17-19). $CD103^+$ DCs are attributed to driving $Foxp3^+$ Treg cells (6) and can sample bacterial antigen (1, 2). $CD103^+$ DCs were decreased upon induction of colitis in a murine model (20). Based on this literature, CD103 is often used to distinguish different functional subsets of mucosal DCs.

The best-known ligand of CD103 is Ecad (21-23), an adhesion molecule found on multiple cell types including DCs and epithelial cells (2, 24). However, the function of CD103 on DCs has yet to be fully understood. Intraepithelial lymphocyte (IEL) CD103 binds to epithelial Ecad in order to anchor IELs to the epithelium (21-23, 25). These interactions place the IELs in an advantageous position for immune surveillance. As DCs have been shown to interact with the epithelium and extend dendrites into the lumen to sample antigen (1, 2), CD103 on DCs may also be playing this role. This role of CD103 on human DCs is evaluated within this dissertation work.

The regulation of CD103 on DCs also has not been completely elucidated. On T cells, the expression of CD103 is dependent on TGF-β (21, 25), whereas RA has been identified as a driver of CD103 expression in DCs (26). Therefore, I also analyzed how expression of CD103 on human DCs is regulated as part of this dissertation work.

RA in the Immune System

Previous research in the Bimczok laboratory supports a critical role for RA in the human gastric immune system (27). RA is the functional derivative of vitamin A. Vitamin A is a group of dietary compounds, including retinol, which is converted to RA through two enzyme mediated steps. Retinol is converted by the enzyme retinol dehydrogenase to retinaldehyde. Next, retinaldehyde is converted into RA by the enzyme retinaldehyde dehydrogenase (Aldh1a2) (28, 29). RA elicits functional responses in multiple cells types and is essential in many cellular functions. Key components of embryogenesis are dependent on RA signaling (30). In addition, adequate levels of RA are indispensable in the development and maintenance of the immune system (31). Our lab and others have shown that in the presence of RA, DCs have increased capacity to biosynthesize RA from retinols through the enzymatic activity of Aldh1a2 (27, 32, 33). This ability allows DCs to make RA available to other immune cells and induce important cellular functions (6, 27). In the gastrointestinal tract, RA and TGF-β drive the expression of $Foxp3^+$ Treg cells (6). Plasma cells exposed to RA have increased capability to secrete IgA antibodies (34, 35). In addition to RA driving these functional aspects on T cells and B cells, RA increases the expression of gut homing molecules, α4β7 and CCR9 (36-38). RA is essential in the development of the cDC2 population of mucosal DCs (15, 16, 39).

RA elicits gene regulation through genomic and non-genomic effects. Genomic regulation by RA involves RA receptors (RARs) binding to DNA and acting as transcription factors (40-43). Binding of RARs to DNA can lead to either activation or

repression of RA-response genes (44-46). Additionally, there are three isoforms of RARs; RARα, RARβ, and RARγ. RARs form heterodimers with retinoid X receptors (RXRs) to elicit these genomic effects of RA signaling. Non-genomic effects of RA signaling include the phosphorylation of kinases within the cytoplasm, including extracellular signal-regulated kinase 1 (ERK1) and p38 mitogen activated protein kinase (MAPK) (40, 41, 43, 47, 48). The phosphorylation of p38 MAPK by RA is mediated through RARα (41). These findings demonstrate the ability of RARs to have cytoplasmic and non-genomic effects, which had previously only been associated with nuclear activity.

p38 MAPK Signaling

p38 MAPK is a key intracellular signaling molecule involved in cellular responses to various external stimuli. Notably, a large number of signaling mechanisms can lead to the activation of p38 MAPK, including toll-like receptor (TLR) activation, tumor necrosis factor (TNF) signaling, TGF-β signaling, and interleukin (IL)-1 (49, 50). Many pro-inflammatory cytokines are known downstream targets of p38 MAPK signaling. It has been demonstrated that p38 MAPK is essential in the secretion of IL-12 p70 upon DC stimulation by TLR2, TLR4, or TLR5 agonists (49). Signaling through p38 MAPK also has been attributed to the increased expression of maturation markers CD40 and CD80 in DCs stimulated with lipopolysaccharide (LPS) or TNF-α (51). The blockade of p38 MAPK signaling reduced the secretion of TNF-α by DCs stimulated with LPS (51). It is also appreciated that p38 MAPK can be phosphorylated through RA signaling pathways (40, 41, 43, 48). An additional downstream target of p38 MAPK is nuclear

factor of activated T cells (NFAT). P38 MAPK activates nuclear factor of activated T cells (NFAT) (52, 53). NFAT is a transcription factor that has been associated with the enhancer region of CD103 (*ITGAE*) in T cells (54). Conversely, p38 MAPK signaling is essential in DC and T regulatory cell IL-10 secretion of DCs (55, 56). These studies suggest that p38 MAPK signaling is important in both inflammatory and homeostatic inducing pathways. Chapter 3 of this dissertation analyzes a role of p38 MAPK in the expression of CD103.

Helicobacter pylori

Mucosal DCs are crucially involved in the immune response to gastrointestinal pathogens such as *H. pylori. H. pylori* is a gram-negative pathogenic bacterium that colonizes the stomach in half of the world's population (57, 58). Infection with *H. pylori* can lead to chronic inflammation that has different disease outcomes, such as ulcers, gastric adenocarcinoma, and mucosal-associated lymphoid tissue lymphoma (59). Gastric cancer associated with *H. pylori* infection accounts for the third most common cause of cancer-related mortality in the world (57). The determining factors that lead to different *H. pylori* associated disease outcomes are not well understood. However, *H. pylori* virulence factors, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), increase the prevalence of gastric cancer and peptic ulcer development (60-63). Additionally, in order to colonize the acidic environment of the stomach *H. pylori* secretes urease. The secretion of urease by *H. pylori* enables the bacterium to colonize the gastric mucosa and is associated with negative outcomes of infection (64, 65). *H. pylori* demonstrates immune evasion tactics that can lead to a Treg response. *H. pylori* VacA

can modulate maturation and pro-inflammatory cytokine secretion in DCs (66). Kao *et al*. demonstrated that upon infection with *H. pylori* there was an increase in $CD103^+$ DCs (67), which induce Treg cells (6). *H. pylori* colonization persists when Treg responses are activated (68). Host cell recognition of *H. pylori* includes TLR2 (69) and TLR4 (70) signaling pathways. However, *H. pylori* LPS, which signals through TLR4, is shown to be less immunogenic than other forms of LPS (71), and aids in *H. pylori* immune evasion. As part of the research described in Chapter 2, we analyzed the impact of *H. pylori* and other TLR agonists on DC CD103 expression.

Hypothesis

The overall hypothesis of this dissertation research is that RA modulates the regulation and function of CD103 on human DCs. To evaluate this hypothesis, we asked the following questions: 1) What are the molecular mechanisms driving the expression of CD103 on human DCs? 2) What is the function of CD103 on human DCs?

What are the Molecular Mechanisms Driving the Expression of CD103 on Human DCs?

As discussed in Chapter 2 (72), we found that RA drives the expression of CD103 on human DCs. In addition, the inhibition of TGF-β receptor II (TGFβ-RII) decreased the expression of CD103 even in the presence of RA. RA signaling can be mediated through three different RARs. To assess the molecular pathways that drive RA-induced expression of CD103, we used agonists and siRNA to identify the RAR involved in the RA signaling pathway that leads to the expression of CD103 (Chap. 4). Non-canonical TGF-β signaling can lead to the phosphorylation of p38 MAPK in a SMAD-dependent

manner (73). We analyzed the activation of SMAD and p38 MAPK in the DCs treated with RA (Chap. 4). Based upon literature demonstrating that p38 MAPK can be phosphorylated through RA signaling (40, 41, 43, 47, 48), we used an inhibitor of p38 MAPK to evaluate the role of p38 MAPK signaling in the RA-induced expression of CD103 (Chap. 4). In addition, p38 MAPK has been shown to activate the transcription factor, NFAT (52, 53). NFAT interacts with the enhancer region of CD103 (*ITGAE*) in T cells (54). We used an inhibitor of NFAT to assess the role of NFAT in the RA-induced expression of CD103 (Chap. 4). Conversely, we determined how stimulation of DCs with bacterial ligands and *H. pylori* affects the RA-induced expression of CD103 (Chap. 2).

What is the Function of CD103 on Human DCs?

$CD103^+$ DCs induce α4β7 expression on T cells and induce a Treg response (6). We assessed these attributes in human DCs by analysis of T cell proliferation and α4β7 expression following co-culture with DCs treated with or without RA and sorted based upon CD103 expression (Chap. 2). Data from our laboratory previously showed that membrane CD103 expression is low for stomach DCs, in spite of the presence of RA (27, 72). While evaluating the expression of CD103 on human DCs, we found that both monocyte-derived and primary gastric DCs have intracellular pools of CD103 (72). It has been demonstrated, most extensively with β1 integrin, that integrins can undergo endosomal recycling (74-78). We determined whether recycling through the cell membrane might make intracellular pools of CD103 available for dynamic interactions between DCs and their environment. We analyzed intracellular CD103 through co-localization and internalization assays (Chap. 3). It has been established that IEL CD103

is important in binding IELs to the epithelium through interactions with E-cad (21). To determine the role of DC CD103 in this context, we first analyzed the proximity of $CD103^+$ DCs with the gastric epithelium in human gastric tissue samples. In addition, we used a co-culture model to elucidate the interactions between DC CD103 and epithelial-expressed E-cad, the ligand of CD103 (Chap. 3).

References Cited

1. Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, Furtado GC, Lira SA, Shakhar G. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity. 2013;38(3):581-95. doi: 10.1016/j.immuni.2013.01.009. PubMed PMID: 23395676; PMCID: PMC4115273.

2. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2001;2(4):361-7. doi: 10.1038/86373. PubMed PMID: 11276208.

3. de Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA, Yazdanbakhsh M, Kapsenberg ML. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. J Immunol. 2002;168(4):1704-9. doi: 10.4049/jimmunol.168.4.1704. PubMed PMID: 11823500.

4. Edwards AD, Manickasingham SP, Sporri R, Diebold SS, Schulz O, Sher A, Kaisho T, Akira S, Reis e Sousa C. Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. J Immunol. 2002;169(7):3652-60. doi: 10.4049/jimmunol.169.7.3652. PubMed PMID: 12244157.

5. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine. 2015;74(1):5-17. doi: 10.1016/j.cyto.2014.09.011. PubMed PMID: 25458968; PMCID: PMC4416069.

6. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007;204(8):1757-64. doi: 10.1084/jem.20070590. PubMed PMID: 17620361; PMCID: PMC2118683.

7. Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology. 2018;154(1):3-20. doi: 10.1111/imm.12888. PubMed PMID: 29313948; PMCID: PMC5904714.

8. Murphy TL, Grajales-Reyes GE, Wu X, Tussiwand R, Briseno CG, Iwata A, Kretzer NM, Durai V, Murphy KM. Transcriptional Control of Dendritic Cell Development. Annu Rev Immunol. 2016;34:93-119. doi: 10.1146/annurev-immunol-032713-120204. PubMed PMID: 26735697; PMCID: PMC5135011.

9. Cohn L, Chatterjee B, Esselborn F, Smed-Sorensen A, Nakamura N, Chalouni C, Lee BC, Vandlen R, Keler T, Lauer P, Brockstedt D, Mellman I, Delamarre L. Antigen

delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation. J Exp Med. 2013;210(5):1049-63. doi: 10.1084/jem.20121251. PubMed PMID: 23569326; PMCID: PMC3646496.

10. Segura E, Durand M, Amigorena S. Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells. J Exp Med. 2013;210(5):1035-47. doi: 10.1084/jem.20121103. PubMed PMID: 23569327; PMCID: PMC3646495.

11. Nizzoli G, Krietsch J, Weick A, Steinfelder S, Facciotti F, Gruarin P, Bianco A, Steckel B, Moro M, Crosti M, Romagnani C, Stolzel K, Torretta S, Pignataro L, Scheibenbogen C, Neddermann P, De Francesco R, Abrignani S, Geginat J. Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood. 2013;122(6):932-42. doi: 10.1182/blood-2013-04-495424. PubMed PMID: 23794066.

12. Nizzoli G, Larghi P, Paroni M, Crosti MC, Moro M, Neddermann P, Caprioli F, Pagani M, De Francesco R, Abrignani S, Geginat J. IL-10 promotes homeostatic proliferation of human CD8(+) memory T cells and, when produced by CD1c(+) DCs, shapes naive CD8(+) T-cell priming. Eur J Immunol. 2016;46(7):1622-32. doi: 10.1002/eji.201546136. PubMed PMID: 27129615.

13. Sittig SP, Bakdash G, Weiden J, Skold AE, Tel J, Figdor CG, de Vries IJ, Schreibelt G. A Comparative Study of the T Cell Stimulatory and Polarizing Capacity of Human Primary Blood Dendritic Cell Subsets. Mediators Inflamm. 2016;2016:3605643. doi: 10.1155/2016/3605643. PubMed PMID: 27057096; PMCID: PMC4761397.

14. Satpathy AT, Kc W, Albring JC, Edelson BT, Kretzer NM, Bhattacharya D, Murphy TL, Murphy KM. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. J Exp Med. 2012;209(6):1135-52. doi: 10.1084/jem.20120030. PubMed PMID: 22615127; PMCID: PMC3371733.

15. Zeng R, Bscheider M, Lahl K, Lee M, Butcher EC. Generation and transcriptional programming of intestinal dendritic cells: essential role of retinoic acid. Mucosal Immunol. 2016;9(1):183-93. doi: 10.1038/mi.2015.50. PubMed PMID: 26129652; PMCID: PMC4698111.

16. Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, Butcher EC. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. Mucosal Immunol. 2013;6(4):847-56. doi: 10.1038/mi.2012.123. PubMed PMID: 23235743; PMCID: PMC3612556.

17. Bain CC, Montgomery J, Scott CL, Kel JM, Girard-Madoux MJH, Martens L, Zangerle-Murray TFP, Ober-Blobaum J, Lindenbergh-Kortleve D, Samsom JN, Henri S, Lawrence T, Saeys Y, Malissen B, Dalod M, Clausen BE, Mowat AM. TGFbetaR signalling controls CD103(+)CD11b(+) dendritic cell development in the intestine. Nat

Commun. 2017;8(1):620. doi: 10.1038/s41467-017-00658-6. PubMed PMID: 28931816; PMCID: PMC5607002.

18. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, Segura E, Tussiwand R, Yona S. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol. 2014;14(8):571-8. doi: 10.1038/nri3712. PubMed PMID: 25033907; PMCID: PMC4638219.

19. Watchmaker PB, Lahl K, Lee M, Baumjohann D, Morton J, Kim SJ, Zeng R, Dent A, Ansel KM, Diamond B, Hadeiba H, Butcher EC. Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. Nat Immunol. 2014;15(1):98-108. doi: 10.1038/ni.2768. PubMed PMID: 24292363; PMCID: PMC3942165.

20. Strauch UG, Grunwald N, Obermeier F, Gurster S, Rath HC. Loss of CD103+ intestinal dendritic cells during colonic inflammation. World J Gastroenterol. 2010;16(1):21-9. doi: 10.3748/wjg.v16.i1.21. PubMed PMID: 20039445; PMCID: PMC2799913.

21. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature. 1994;372(6502):190-3. doi: 10.1038/372190a0. PubMed PMID: 7969453.

22. Corps E, Carter C, Karecla P, Ahrens T, Evans P, Kilshaw P. Recognition of E-cadherin by integrin alpha(E)beta(7): requirement for cadherin dimerization and implications for cadherin and integrin function. J Biol Chem. 2001;276(33):30862-70. doi: 10.1074/jbc.M101712200. PubMed PMID: 11413131.

23. Higgins JM, Cernadas M, Tan K, Irie A, Wang J, Takada Y, Brenner MB. The role of alpha and beta chains in ligand recognition by beta 7 integrins. J Biol Chem. 2000;275(33):25652-64. doi: 10.1074/jbc.M001228200. PubMed PMID: 10837471.

24. Swain S, Roe MM, Sebrell TA, Sidar B, Dankoff J, VanAusdol R, Smythies LE, Smith PD, Bimczok D. CD103 (alphaE Integrin) Undergoes Endosomal Trafficking in Human Dendritic Cells, but Does Not Mediate Epithelial Adhesion. Front Immunol. 2018;9:2989. doi: 10.3389/fimmu.2018.02989. PubMed PMID: 30622531; PMCID: PMC6308147.

25. Russell GJ, Parker CM, Cepek KL, Mandelbrot DA, Sood A, Mizoguchi E, Ebert EC, Brenner MB, Bhan AK. Distinct structural and functional epitopes of the alpha E beta 7 integrin. Eur J Immunol. 1994;24(11):2832-41. doi: 10.1002/eji.1830241138. PubMed PMID: 7525307.

26. Iliev ID, Spadoni I, Mileti E, Matteoli G, Sonzogni A, Sampietro GM, Foschi D, Caprioli F, Viale G, Rescigno M. Human intestinal epithelial cells promote the

differentiation of tolerogenic dendritic cells. Gut. 2009;58(11):1481-9. doi: 10.1136/gut.2008.175166. PubMed PMID: 19570762.

27. Bimczok D, Kao JY, Zhang M, Cochrun S, Mannon P, Peter S, Wilcox CM, Monkemuller KE, Harris PR, Grams JM, Stahl RD, Smith PD, Smythies LE. Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells. Mucosal Immunol. 2015;8(3):533-44. doi: 10.1038/mi.2014.86. PubMed PMID: 25249167; PMCID: PMC4372513.

28. Gyongyosi A, Szatmari I, Pap A, Dezso B, Pos Z, Szeles L, Varga T, Nagy L. RDH10, RALDH2, and CRABP2 are required components of PPARgamma-directed ATRA synthesis and signaling in human dendritic cells. J Lipid Res. 2013;54(9):2458-74. doi: 10.1194/jlr.M038984. PubMed PMID: 23833249; PMCID: PMC3735943.

29. Kam RK, Deng Y, Chen Y, Zhao H. Retinoic acid synthesis and functions in early embryonic development. Cell Biosci. 2012;2(1):11. doi: 10.1186/2045-3701-2-11. PubMed PMID: 22439772; PMCID: PMC3325842.

30. Vermot J, Gallego Llamas J, Fraulob V, Niederreither K, Chambon P, Dolle P. Retinoic acid controls the bilateral symmetry of somite formation in the mouse embryo. Science. 2005;308(5721):563-6. doi: 10.1126/science.1108363. PubMed PMID: 15731404.

31. Erkelens MN, Mebius RE. Retinoic Acid and Immune Homeostasis: A Balancing Act. Trends Immunol. 2017;38(3):168-80. doi: 10.1016/j.it.2016.12.006. PubMed PMID: 28094101.

32. Agace WW, Persson EK. How vitamin A metabolizing dendritic cells are generated in the gut mucosa. Trends Immunol. 2012;33(1):42-8. doi: 10.1016/j.it.2011.10.001. PubMed PMID: 22079120.

33. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, Mebius RE. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. J Immunol. 2011;186(4):1934-42. doi: 10.4049/jimmunol.1001672. PubMed PMID: 21220692.

34. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science. 2006;314(5802):1157-60. doi: 10.1126/science.1132742. PubMed PMID: 17110582.

35. Watanabe K, Sugai M, Nambu Y, Osato M, Hayashi T, Kawaguchi M, Komori T, Ito Y, Shimizu A. Requirement for Runx proteins in IgA class switching acting downstream of TGF-beta 1 and retinoic acid signaling. J Immunol. 2010;184(6):2785-92. doi: 10.4049/jimmunol.0901823. PubMed PMID: 20142360.

36. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med. 2008;205(9):2139-49. doi: 10.1084/jem.20080414. PubMed PMID: 18710932; PMCID: PMC2526207.

37. Jaensson-Gyllenback E, Kotarsky K, Zapata F, Persson EK, Gundersen TE, Blomhoff R, Agace WW. Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. Mucosal Immunol. 2011;4(4):438-47. doi: 10.1038/mi.2010.91. PubMed PMID: 21289617; PMCID: PMC3130189.

38. Bakdash G, Vogelpoel LT, van Capel TM, Kapsenberg ML, de Jong EC. Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. Mucosal Immunol. 2015;8(2):265-78. doi: 10.1038/mi.2014.64. PubMed PMID: 25027601.

39. Klebanoff CA, Spencer SP, Torabi-Parizi P, Grainger JR, Roychoudhuri R, Ji Y, Sukumar M, Muranski P, Scott CD, Hall JA, Ferreyra GA, Leonardi AJ, Borman ZA, Wang J, Palmer DC, Wilhelm C, Cai R, Sun J, Napoli JL, Danner RL, Gattinoni L, Belkaid Y, Restifo NP. Retinoic acid controls the homeostasis of pre-cDC-derived splenic and intestinal dendritic cells. J Exp Med. 2013;210(10):1961-76. doi: 10.1084/jem.20122508. PubMed PMID: 23999499; PMCID: PMC3782040.

40. Canon E, Cosgaya JM, Scsucova S, Aranda A. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell. 2004;15(12):5583-92. doi: 10.1091/mbc.e04-05-0439. PubMed PMID: 15371543; PMCID: PMC532036.

41. Piskunov A, Rochette-Egly C. A retinoic acid receptor RARalpha pool present in membrane lipid rafts forms complexes with G protein alphaQ to activate p38MAPK. Oncogene. 2012;31(28):3333-45. doi: 10.1038/onc.2011.499. PubMed PMID: 22056876.

42. Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res. 2002;43(11):1773-808. doi: 10.1194/jlr.r100015-jlr200. PubMed PMID: 12401878.

43. Bruck N, Vitoux D, Ferry C, Duong V, Bauer A, de The H, Rochette-Egly C. A coordinated phosphorylation cascade initiated by p38MAPK/MSK1 directs RARalpha to target promoters. EMBO J. 2009;28(1):34-47. doi: 10.1038/emboj.2008.256. PubMed PMID: 19078967; PMCID: PMC2633082.

44. Brelivet Y, Rochel N, Moras D. Structural analysis of nuclear receptors: from isolated domains to integral proteins. Mol Cell Endocrinol. 2012;348(2):466-73. doi: 10.1016/j.mce.2011.08.015. PubMed PMID: 21888944.

45. Dilworth FJ, Chambon P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene. 2001;20(24):3047-54. doi: 10.1038/sj.onc.1204329. PubMed PMID: 11420720.

46. Osz J, Brelivet Y, Peluso-Iltis C, Cura V, Eiler S, Ruff M, Bourguet W, Rochel N, Moras D. Structural basis for a molecular allosteric control mechanism of cofactor binding to nuclear receptors. Proc Natl Acad Sci U S A. 2012;109(10):E588-94. doi: 10.1073/pnas.1118192109. PubMed PMID: 22355136; PMCID: PMC3309777.

47. Tokuda H, Hatakeyama D, Akamatsu S, Tanabe K, Yoshida M, Shibata T, Kozawa O. Involvement of MAP kinases in TGF-beta-stimulated vascular endothelial growth factor synthesis in osteoblasts. Arch Biochem Biophys. 2003;415(1):117-25. doi: 10.1016/s0003-9861(03)00225-x. PubMed PMID: 12801520.

48. Alsayed Y, Uddin S, Mahmud N, Lekmine F, Kalvakolanu DV, Minucci S, Bokoch G, Platanias LC. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to all-trans-retinoic acid. J Biol Chem. 2001;276(6):4012-9. doi: 10.1074/jbc.M007431200. PubMed PMID: 11060298.

49. Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, Pulendran B. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J Immunol. 2003;171(10):4984-9. doi: 10.4049/jimmunol.171.10.4984. PubMed PMID: 14607893.

50. Nakahara T, Uchi H, Urabe K, Chen Q, Furue M, Moroi Y. Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. Int Immunol. 2004;16(12):1701-9. doi: 10.1093/intimm/dxh171. PubMed PMID: 15477228.

51. Arrighi JF, Rebsamen M, Rousset F, Kindler V, Hauser C. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. J Immunol. 2001;166(6):3837-45. doi: 10.4049/jimmunol.166.6.3837. PubMed PMID: 11238627.

52. Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S, Hisatake K, Nogi Y. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. J Biol Chem. 2004;279(44):45969-79. doi: 10.1074/jbc.M408795200. PubMed PMID: 15304486.

53. Wu CC, Hsu SC, Shih HM, Lai MZ. Nuclear factor of activated T cells c is a target of p38 mitogen-activated protein kinase in T cells. Mol Cell Biol. 2003;23(18):6442-54. doi: 10.1128/mcb.23.18.6442-6454.2003. PubMed PMID: 12944472; PMCID: PMC193716.

54. Mokrani M, Klibi J, Bluteau D, Bismuth G, Mami-Chouaib F. Smad and NFAT pathways cooperate to induce CD103 expression in human CD8 T lymphocytes. J Immunol. 2014;192(5):2471-9. doi: 10.4049/jimmunol.1302192. PubMed PMID: 24477908.

55. Jarnicki AG, Conroy H, Brereton C, Donnelly G, Toomey D, Walsh K, Sweeney C, Leavy O, Fletcher J, Lavelle EC, Dunne P, Mills KH. Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. J Immunol. 2008;180(6):3797-806. doi: 10.4049/jimmunol.180.6.3797. PubMed PMID: 18322186.

56. Adler HS, Kubsch S, Graulich E, Ludwig S, Knop J, Steinbrink K. Activation of MAP kinase p38 is critical for the cell-cycle-controlled suppressor function of regulatory T cells. Blood. 2007;109(10):4351-9. doi: 10.1182/blood-2006-09-047563. PubMed PMID: 17244678.

57. Venerito M, Vasapolli R, Rokkas T, Malfertheiner P. Gastric cancer: epidemiology, prevention, and therapy. Helicobacter. 2018;23 Suppl 1:e12518. doi: 10.1111/hel.12518. PubMed PMID: 30203589.

58. Zamani M, Ebrahimtabar F, Zamani V, Miller WH, Alizadeh-Navaei R, Shokri-Shirvani J, Derakhshan MH. Systematic review with meta-analysis: the worldwide prevalence of Helicobacter pylori infection. Aliment Pharmacol Ther. 2018;47(7):868-76. doi: 10.1111/apt.14561. PubMed PMID: 29430669.

59. Roesler BM, Rabelo-Goncalves EM, Zeitune JM. Virulence Factors of Helicobacter pylori: A Review. Clin Med Insights Gastroenterol. 2014;7:9-17. doi: 10.4137/CGast.S13760. PubMed PMID: 24833944; PMCID: PMC4019226.

60. Figueiredo C, Van Doorn LJ, Nogueira C, Soares JM, Pinho C, Figueira P, Quint WG, Carneiro F. Helicobacter pylori genotypes are associated with clinical outcome in Portuguese patients and show a high prevalence of infections with multiple strains. Scand J Gastroenterol. 2001;36(2):128-35. doi: 10.1080/003655201750065861. PubMed PMID: 11252403.

61. Parsonnet J, Friedman GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. Gut. 1997;40(3):297-301. doi: 10.1136/gut.40.3.297. PubMed PMID: 9135515; PMCID: PMC1027076.

62. Atherton JC, Peek RM, Jr., Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. Gastroenterology. 1997;112(1):92-9. doi: 10.1016/s0016-5085(97)70223-3. PubMed PMID: 8978347.

63. Miehlke S, Yu J, Schuppler M, Frings C, Kirsch C, Negraszus N, Morgner A, Stolte M, Ehninger G, Bayerdorffer E. Helicobacter pylori vacA, iceA, and cagA status and pattern of gastritis in patients with malignant and benign gastroduodenal disease. Am J Gastroenterol. 2001;96(4):1008-13. doi: 10.1111/j.1572-0241.2001.03685.x. PubMed PMID: 11316139.

64. Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology. 1996;111(2):419-25. doi: 10.1053/gast.1996.v111.pm8690207. PubMed PMID: 8690207.

65. Megraud F, Neman-Simha V, Brugmann D. Further evidence of the toxic effect of ammonia produced by Helicobacter pylori urease on human epithelial cells. Infect Immun. 1992;60(5):1858-63. PubMed PMID: 1563774; PMCID: PMC257085.

66. Kim JM, Kim JS, Yoo DY, Ko SH, Kim N, Kim H, Kim YJ. Stimulation of dendritic cells with Helicobacter pylori vacuolating cytotoxin negatively regulates their maturation via the restoration of E2F1. Clin Exp Immunol. 2011;166(1):34-45. doi: 10.1111/j.1365-2249.2011.04447.x. PubMed PMID: 21910723; PMCID: PMC3193917.

67. Kao JY, Zhang M, Miller MJ, Mills JC, Wang B, Liu M, Eaton KA, Zou W, Berndt BE, Cole TS, Takeuchi T, Owyang SY, Luther J. Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. Gastroenterology. 2010;138(3):1046-54. doi: 10.1053/j.gastro.2009.11.043. PubMed PMID: 19931266; PMCID: PMC2831148.

68. Oertli M, Sundquist M, Hitzler I, Engler DB, Arnold IC, Reuter S, Maxeiner J, Hansson M, Taube C, Quiding-Jarbrink M, Muller A. DC-derived IL-18 drives Treg differentiation, murine Helicobacter pylori-specific immune tolerance, and asthma protection. J Clin Invest. 2012;122(3):1082-96. doi: 10.1172/JCI61029. PubMed PMID: 22307326; PMCID: PMC3287234.

69. Ding SZ, Torok AM, Smith MF, Jr., Goldberg JB. Toll-like receptor 2-mediated gene expression in epithelial cells during Helicobacter pylori infection. Helicobacter. 2005;10(3):193-204. doi: 10.1111/j.1523-5378.2005.00311.x. PubMed PMID: 15904477.

70. Kabisch R, Mejias-Luque R, Gerhard M, Prinz C. Involvement of Toll-like receptors on Helicobacter pylori-induced immunity. PLoS One. 2014;9(8):e104804. doi: 10.1371/journal.pone.0104804. PubMed PMID: 25153703; PMCID: PMC4143222.

71. Wang G, Ge Z, Rasko DA, Taylor DE. Lewis antigens in Helicobacter pylori: biosynthesis and phase variation. Mol Microbiol. 2000;36(6):1187-96. doi: 10.1046/j.1365-2958.2000.01934.x. PubMed PMID: 10931272.

72. Roe MM, Swain S, Sebrell TA, Sewell MA, Collins MM, Perrino BA, Smith PD, Smythies LE, Bimczok D. Differential regulation of CD103 (alphaE integrin) expression in human dendritic cells by retinoic acid and Toll-like receptor ligands. J Leukoc Biol. 2017;101(5):1169-80. doi: 10.1189/jlb.1MA0316-131R. PubMed PMID: 28087652; PMCID: PMC5380378.

73. Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J. 2002;21(14):3749-59. doi: 10.1093/emboj/cdf366. PubMed PMID: 12110587; PMCID: PMC126112.

74. Bretscher MS. Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. EMBO J. 1992;11(2):405-10. PubMed PMID: 1531629; PMCID: PMC556468.

75. Chao WT, Kunz J. Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. FEBS Lett. 2009;583(8):1337-43. doi: 10.1016/j.febslet.2009.03.037. PubMed PMID: 19306879; PMCID: PMC2801759.

76. Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. J Cell Biol. 2009;187(5):733-47. doi: 10.1083/jcb.200904054. PubMed PMID: 19951918; PMCID: PMC2806590.

77. Roberts MS, Woods AJ, Dale TC, Van Der Sluijs P, Norman JC. Protein kinase B/Akt acts via glycogen synthase kinase 3 to regulate recycling of alpha v beta 3 and alpha 5 beta 1 integrins. Mol Cell Biol. 2004;24(4):1505-15. doi: 10.1128/mcb.24.4.1505-1515.2004. PubMed PMID: 14749368; PMCID: PMC344170.

78. Sonnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol. 2000;149(4):901-14. doi: 10.1083/jcb.149.4.901. PubMed PMID: 10811830; PMCID: PMC2174575.

CHAPTER 2

DIFFERENTIAL REGULATION OF CD103 (αE INTEGRIN) EXPRESSION IN HUMAN DENDRITIC CELLS BY RETINOIC ACID AND TOLL-LIKE RECEPTOR LIGANDS

Contribution of Authors and Co-Authors

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Abstract

CD103 (αE 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. We here 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 β7 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 downregulated 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 *H. pylori*, commensal bacteria, LPS or a TLR2 agonist, the RA-induced upregulation of CD103 and β7 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 α4β7 expression and proliferation in co-cultured T cells, but no difference was seen between RA-treated $CD103^+$ and $CD103^-$ DCs. In summary, our data demonstrate that RA, bacterial products and 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.

Introduction

CD103 is a functionally important marker for dendritic cell (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 regulatory T cell responses and T cell expression of mucosal homing molecules α4β7 and CCR9 through a retinoic acid (RA)-dependent mechanism [1, 3-7]. Similarly, 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 between 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 to the murine intestine, CD103 is also less widely expressed on human gastrointestinal DCs, with almost 40% of DCs lacking CD103 expression in ileum and colon [8]. Importantly, in a previous study, we discovered that absence of CD103 expression in gastric DCs was one major difference between human small intestinal and gastric DC populations, with less than 5% of $CD103^+$

DCs in human stomach compared to 15 – 20% in human small intestine, in spite of similar levels of retinoids in these two 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 et al. 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 [15]. However, several independent studies using human DCs derived from blood monocytes or CD34+ progenitor cells did not observe increased CD103 expression with recombinant TGF-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, we here sought to define factors that regulate CD103 expression in human DCs, with a focus on RA, TGF-β and the gastric pathogen *Helicobacter pylori*. We demonstrate that RA drives expression of both CD103 and β7 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 β7 integrin expression. We also show that RA-treatment of DCs enhances both α4β7 expression and proliferation in co-cultured T cells, but that induction of T cell α4β7 expression and proliferation was independent of CD103 expression by the DCs.

Methods

Monocyte-Derived Dendritic Cell (MoDC) Culture

Whole blood was obtained with local IRB approval (protocol #DB092614) from healthy adult donors in Bozeman, MT, and peripheral blood mononuclear cells were isolated by centrifugation in leukocyte separation media (Lonza, Basel, Switzerland) at 800 *g* for 25 minutes 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 ± 3.2% (Suppl. Fig. 1A). All monocyte preparations were analyzed for activation based on cluster formation and spontaneous TNF-α release, and pre-activated 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 μg/L streptomycin, 50 μg/mL gentamycin, 5 mM Hepes, and 2 mM L-glutamine (all Hyclone, Logan, UT), 25 ng/mL rh GM-CSF and 7 ng/mL rh IL-4 (R&D systems, Minneapolis, MN) for 3 - 5 days. Duration of DC culture did not significantly affect DC viability or phenotype (Suppl. 1B, 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) was added at 100 nM from day 0. Media, cytokines, and RA were replenished every three days. All RA-treated cells were handled under red light to prevent RA degradation.

Human Gastric DC

Gastric tissue specimens from sleeve gastrectomy surgeries were obtained with Institutional Review Board (IRB) approval by the National Disease Research Interchange

(NDRI; Philadelphia, PA) or by Dr. Kent Sasse (Sasse Surgical Associates, Reno, NV). To obtain gastric DCs, mucosal tissue was subjected to three 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, Auburn, CA), and viable (7-AAD) CD45^{pos}/lineage^{neg}/HLA-DR^{high} DCs were purified by FACS sorting on a FACSAria II sorter (Becton Dickinson). The lineage cocktail contained antibodies to CD3, CD19, CD20, CD56 and CD14.

TGF-βR Inhibition and rhTGF-β Culture

MoDCs were cultured for 3 days with or without RA, the TGF-β inhibitor SB431542 (50 μM; Tocris Bioscience, Bristol, UK), recombinant human (rh)TGFβ-1 or rhTGFβ-2 (0.5 - 5 ng/mL; R&D systems) or a combination of these reagents added to the culture wells on day 0. Control wells were cultured with the appropriate carrier, DMSO or 4mM HCl + 1mg/mL BSA, respectively. None of the treatments significantly altered DC viability.

Helicobacter 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, San Jose, CA) and was incubated under microaerophilic conditions. *H. pylori* were harvested into pre-warmed Brucella broth and quantified as previously described [23]. Differentiated MoDCs generated in the presence or absence of RA were stimulated with (1) *H. pylori (*MOI 10*),* (2) a commercially available preparation of probiotic bacteria (VSL#3: *S. thermophilus, B. breve, B. lactis, L. acidophilus, L. plantarum, L. paracasei, L. helveticus;* Sigma-Tau Pharmaceuticals Inc.,

Gaithersburg, MD; MOI 10) or (3) individual TLR agonists, with continuous presence of RA and cytokines. Human TLR agonists (InvivoGen, San Diego, CA) were used at the following concentrations: TLR2 agonist (heat killed *Listeria monocytogenes*) $1x10^8$ cells/mL, TLR3 agonist (Poly (I:C) HMW) 10 μg/mL, TLR4 agonist (*E. coli* K12 LPS) 1 μg/mL, TLR9 agonist (ODN2006 type B) 5 μM. 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 TGFβ-1 or active TGFβ-1 by ELISA, following the manufacturer's protocol (Biolegend, San Diego, CA). A TGFβ latency-associated protein (LAP) ELISA (R&D Systems, Minneapolis, MN) was used to test both culture supernatants and cell lysates for LAP. Supernatants from DC – T cell co-cultures were analyzed for IL-10 and IFN-γ using Biolegend kits. ELISA plates were read on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA) at 450nm and 570nm, and data were analyzed with GraphPad Prism 6.05 (Graphpad Software, Inc., San Diego, CA).

Antibodies and Flow Cytometry

MoDCs were stained using anti-human antibodies directed against CD103 (αE integrin), β7, HLA-DR (all eBioscience, San Diego, CA), TGF-βRII (R&D systems), CD85k (ILT3, Biolegend), CD83, CD86, CD11c (all BD Biosciences) or appropriate isotype controls. Dead cells were labeled with a LIVE/DEAD yellow stain (Invitrogen, Carlsbad, CA). A subset of cells were permeabilized with Cytofix/Cytoperm (BD

Bioscience) for intracellular staining of CD103 and β7. 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 FACS Aria II instrument (BD Biosciences). FACS data were analyzed using FlowJo X software (Tree Star, Inc., Ashland, OR), with DCs gated based upon size, single cells, live cells. Since baseline fluorescence levels differed between the two cytometers, data were normalized by dividing geomean fluorescence of the sample by geomean fluorescence of the appropriate isotype control (normalized geometric mean).

ImageStream® Analysis

For multispectral imaging flow cytometry, we used an ImageStreamX Mark II (EMD Millipore, Seattle, WA). Cells were labelled with pre-determined optimum concentrations of antibodies as described for FACS analysis above, and nuclei were labelled with DAPI. Data were analyzed with IDEAS software v6.1 (EMD Millipore). Images of 10,000 cells per 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 RMS 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 co-localized with CD11c membrane staining, was determined using the internalization wizard in the IDEAS® software. Externalization was calculated as 100% – % internalization.

Quantitative RT-PCR

Direct-zol Mini-RNA prep kit (Zymo Research, Irvine, CA) was used to isolate RNA from MoDC cultures. Reverse transcriptase PCR reactions were performed with iScript (Bio-Rad, Hercules, CA). Gene expression analysis for *ITGAE*, *ITGB7*, *TGFBR1*, *TGFBR2*, *TGFB1*, *TGFB2*, *TGFB3*, *ALDH1A1* and *ALDH1A2* were performed with Taqman Universal PCR mastermix (Applied Biosystems, Waltham, MA) and primer/probes (Applied Biosystems) on a Lightcycler 96 (Roche, Penzberg, Germany). The house keeping genes 18s rRNA and GAPDH were used for normalization. qRT-PCR analysis was performed by the Pfaffl method [24].

Analysis of Retinoic Acid Biosynthesis

RA production by MoDCs was analyzed using an RA bioassay, as previously described [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, SUNY Downstate Medical Center, Brooklyn, NY [25].

DC–T Cell Co-Cultures

DC-T cell co-cultures were established using FACS-purified $CD103^+$ and $CD103^-$ DCs that were pulsed with SEB (1 µg/mL, Toxin Technology, Sarasota, FL) and autologous naïve $CD4^+$ T cells, as described previously [21]. T cell proliferation was determined using the CellTrace Violet (Invitrogen, Carlsbad, CA) dilution assay.

Statistical Analysis

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

Results

Retinoic acid (RA) Drives RA Biosynthesis by Human Monocyte-Derived DCs (MoDCs)

Retinoic acid 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 monocyte-derived DCs generated in the presence of RA also have an increased capacity for RA biosynthesis, we analyzed gene expression of two important RA biosynthesis enzymes, *ALDH1A1* and *ALDH1A2,* by quantitative RT-PCR and DC release of RA using an RA-bioassay [25]. MoDCs were generated from purified human blood monocytes in the presence of physiological levels of all-trans RA (100 nM) [29] for 3 – 5 days. MoDCs generated in the presence of RA had a slightly, but not significantly, elevated expression of *ALDH1A2* compared to 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).

Figure 1:

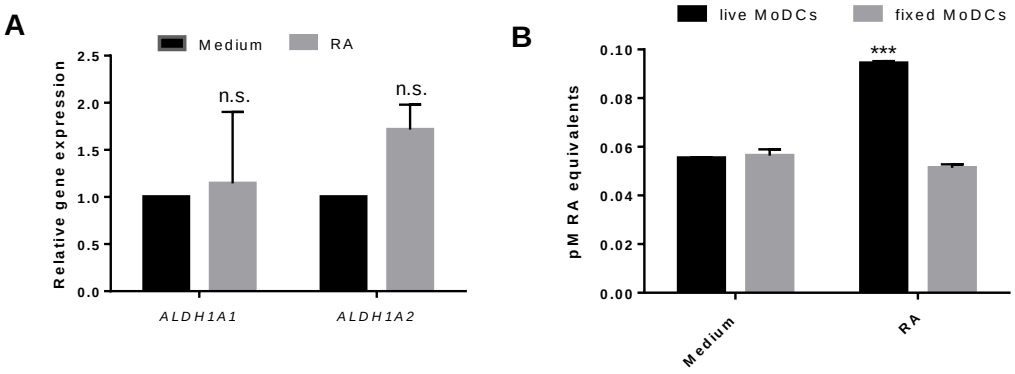

Fig. 1: MoDCs generated in the presence of RA show increased RA biosynthesis. (A) MoDCs were cultured for 3 to 5 days in the presence of RA, harvested, washed and were then analyzed for *ALDH1A1* and *ALDH1A2* gene expression using qRT-PCR; cumulative data from 3 independent experiments mean±SEM. (B) 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]. Mean±SD of triplicate wells, *** $P \leq 0.001$, Student's *t* test. Experiment shown is representative of n=3.

RA Induces Intracellular and Extracellular Expression of CD103 and β7 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 sharp increase in surface expression of CD103 ($P \leq 0.05$; Fig. 2A,B and Suppl. Fig. 2). Expression of β7 integrin was also significantly increased upon RA treatment (Fig. 2B).

Our data confirmed previous reports of only low levels (6.8±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±17.5% of DCs were CD103 positive. Significantly increased CD103 signal

following permeabilization is consistent with additional intracellular CD103 expression and was seen in both untreated and RA-treated MoDCs ($P \leq 0.05$) (Fig. 2A,B). Surprisingly, we found no significant intracellular expression of β7 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 β7 protein expression, RA-treated MoDCs expressed significantly higher levels of both *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].

Retinoic Acid 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 labelled 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 both 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,C). Using data from the flow cytometry experiments shown in Fig. 2, we also detected

no difference in the ratio of extracellular compared to 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.

Figure 2:

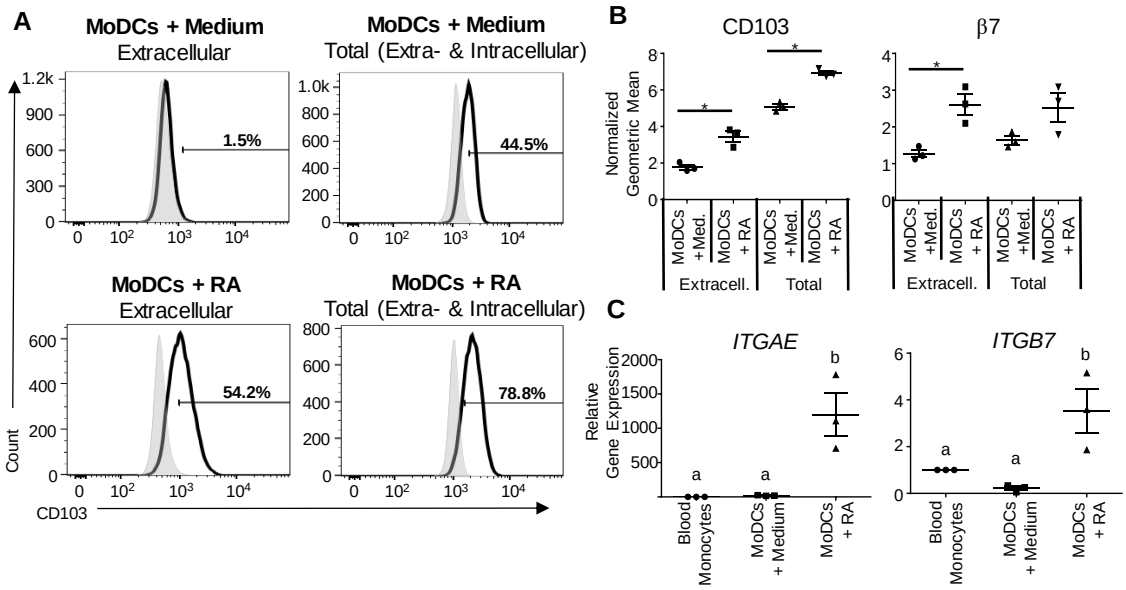

Fig 2: RA increases protein and gene expression of CD103 and β7 in human MoDCs. MoDCs were cultured with RA or medium only for 5 days and harvested for FACS or gene expression analysis. (A) Representative histograms of MoDCs cultured in the presence of RA or medium alone. CD103 expression was analyzed without cell permeabilization (extracellular) or after permeabilization (total). Grey histograms: isotype controls; black lines: samples. (B) Cumulative FACS data for CD103 and β7 expression from 3 independent experiments, individual data points, mean ± 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 (ANOVA, $P<0.05$). (C) Quantitative RT-PCR analysis of fresh monocytes and RA-treated and untreated DCs for CD103 (*ITGAE*) and β7 (*ITGB7)* expression. Cumulative data from 3 independent experiments; different letters denote statistically significant differences ($P<0.05$).

<u>RA does not Induce Expression of CD103 in Primary Human Gastric DCs</u>

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]. We therefore 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 hours. A longer treatment was not possible due to the limited lifespan of the primary DCs *in vitro;* however, a 16 h treatment was sufficient to induce significant upregulation 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±2.4%; n=3; *P*=0.25, Mann-Whitney U test, Fig. 4B). Quantitative 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 downregulate their susceptibility to RA-induced regulation of CD103 expression.

TGF-β Receptor I Signaling Contributes to RA-Induced Upregulation of CD103

To elucidate the mechanism by which RA induces CD103 expression, we focused on a potential role of TGF-β, since RA regulates both TGF-β and TGF-β receptor (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 latency associated protein (LAP), which is non-covalently bound to inactive TGF-β1, 2 and 3 [33] (Suppl. Fig. 4A). Moreover, gene expression of *TGFB1, 2,* and *3* was not significantly modified by RA treatment of the DCs (Suppl. Fig. 4B), and we also found

no significant changes in surface protein expression of TGF-βRII or in gene expression of *TGFBR1* and *TGFBR2* in response to MoDC RA treatment (Suppl. Fig. 4C).

Figure 3:

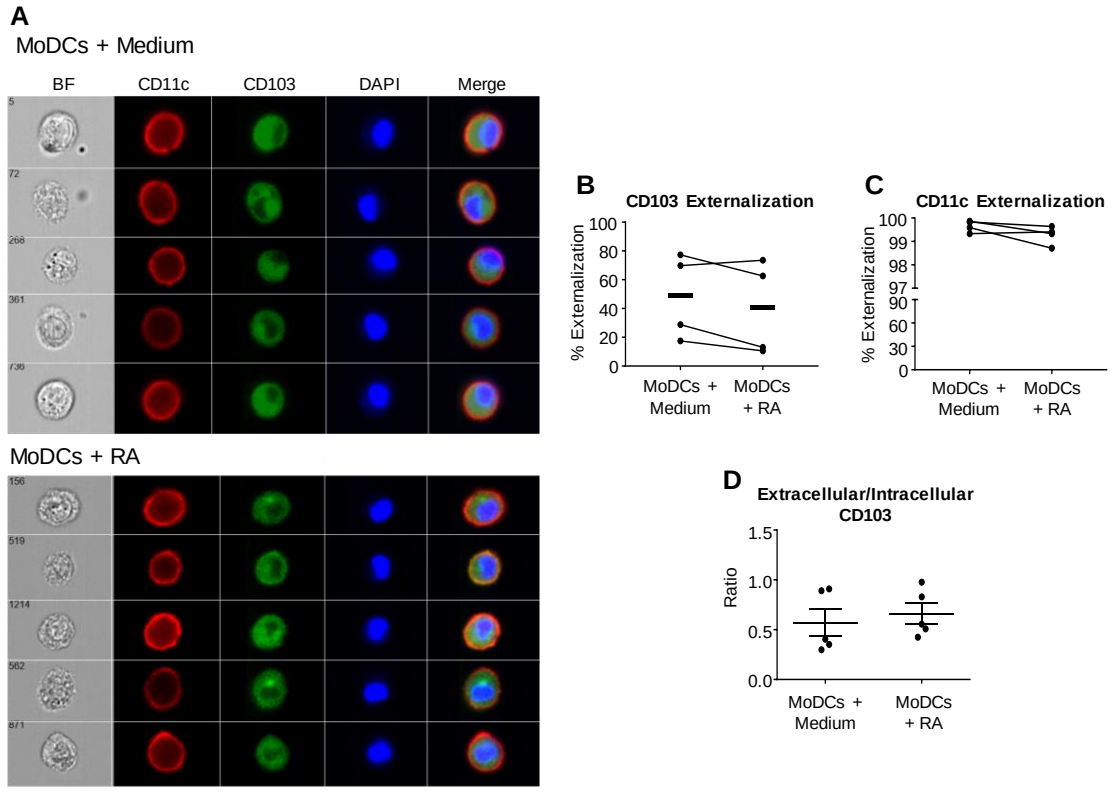

Fig. 3. Imaging flow cytometry analysis of CD103 expression in RA-treated and untreated MoDCs. Imaging flow cytometry (ImageStream®) analysis of MoDCs generated in the presence of RA or medium alone. MoDCs were permeabilized before addition of the anti-CD103 antibody to reveal intracellular CD103. (A) Representative images of control MoDCs (top panels) and RA-treated MoDCs (bottom panels) labeled for CD11c (PE, red), CD103 (FITC, green) and nuclei (DAPI, blue). (B) Percentage of surface compared to intracellular CD103 (externalization score). A mask for cell surface staining was created based on CD11c surface labeling. Data from four independent experiments are shown. Lines connect control and RA-treated DCs from the same experiment, thick black lines indicate 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, mean ± SEM are shown.

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 of rhTGF-β1 or rhTGF-β2. None of the TGF-β treatments caused a significant change in CD103 or β7 expression (Suppl. 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 upregulation of CD103 in human MoDCs, we next cultured MoDCs with or without RA in the presence of the small-molecule TGFβR 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 TGFβR signaling and phosphorylation of Smad2 by blocking the TGF-β type I receptor activin receptor-like kinase (ALK)5 [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* promotor/enhancer region [15]. Here, MoDCs were cultured for 3 days 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 TGFβR inhibitor (Fig. 5A,B). Importantly, MoDC gene expression of *ITGAE*, but not *ITGB7*, was also significantly reduced in the presence of both RA and TGF-βR inhibitor compared to RA alone (Fig. 5C), suggesting that TGF-β signaling contributes to RA regulation of CD103 expression in human MoDCs.

Figure 4:

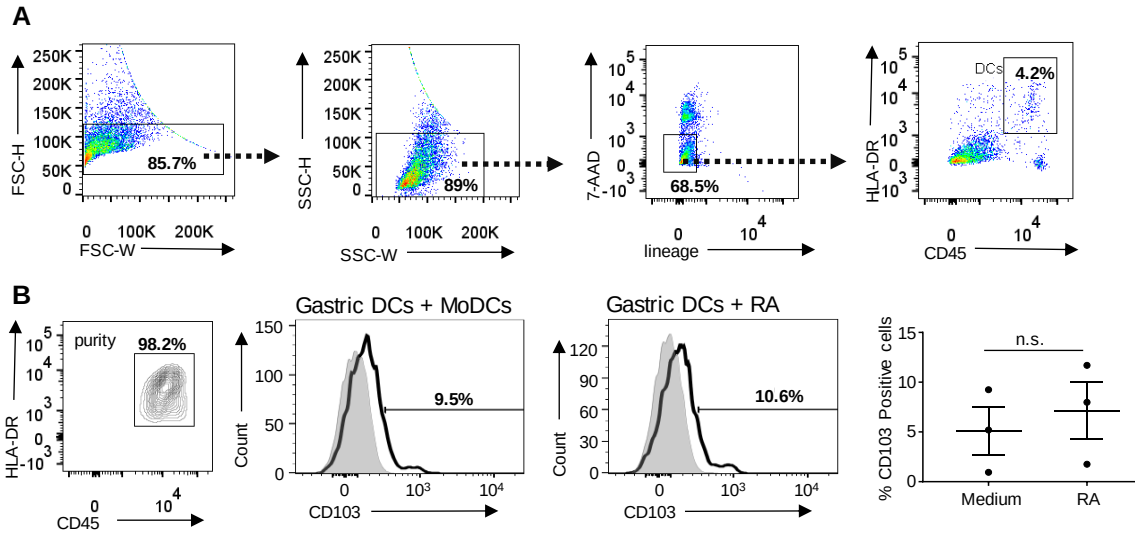

Fig 4: RA does not increase expression of CD103 on primary human gastric DCs. Gastric DCs were sorted as live HLA-DR^{+}/CD45^{+}/lin^{-} single cells and were incubated in the presence of RA (100 nM) for 24 h. (A) Sorting strategy, representative FACS plots; (B) Purity of sorted population and representative histograms of CD103 expression (left) and cumulative FACS data from three experiments (right; mean ± SEM).

Toll-Like Receptor Engagement Abrogates RA-Induced CD103 and β7 Expression

RA may interfere with the response of DCs to pathogenic stimuli by decreasing DC co-stimulatory molecule expression, pro-inflammatory 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 days 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.

Figure 5:

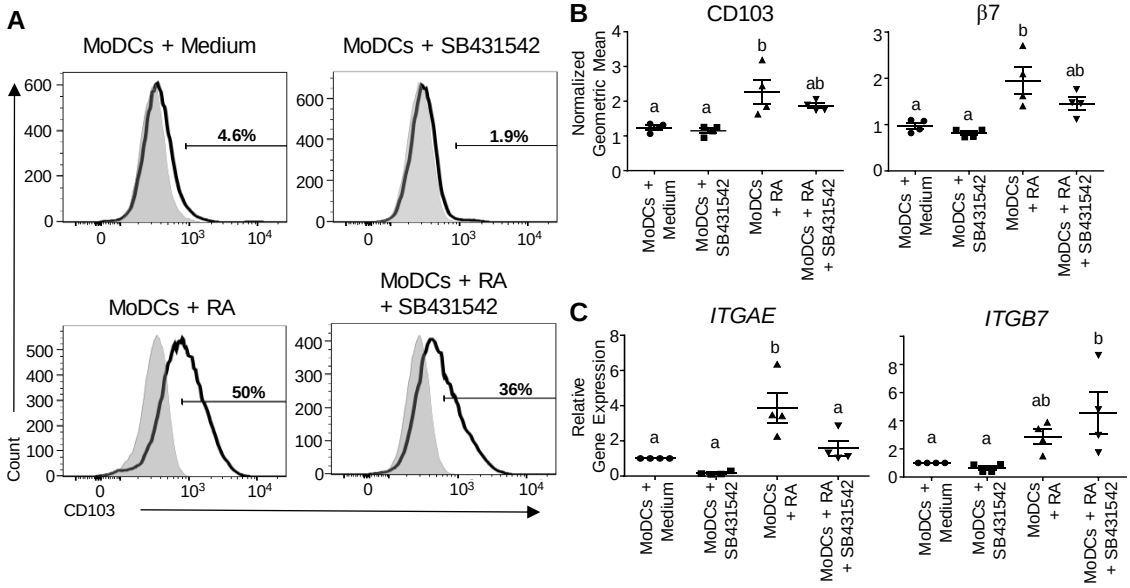

Fig. 5: Inhibition of TGF-β signaling blocks RA-induced upregulation of CD103. MoDCs were cultured with or without RA and/or the TGF-βR inhibitor SB431542 for 3 days and harvested for FACS and gene expression analysis. (**A**) Representative histograms of MoDCs cultured in the presence of medium, RA, SB431542 or RA + SB431542. Grey histograms: isotype controls; black lines: samples. (**B**) Cumulative FACS data from four independent experiments, individual data points, mean ± SEM are shown. Different letters denote statistical significance (ANOVA, $P<0.05$). (**C**) Quantitative RT-PCR analysis of RA-treated and untreated DCs for CD103 (*ITGAE*) and β7 (*ITGB7)* expression. Cumulative data from four independent experiments; different letters denote statistically significant differences ($P<0.05$).

Surprisingly, *H. pylori* stimulation completely abrogated the RA-induced increase in surface expression (Fig. 6B,C) and intracellular expression (data not shown) of CD103 and β7. Similarly, gene expression of *ITGAE* and *ITGB7* as significantly reduced in RA-treated MoDCs stimulated with *H. pylori* compared to non-stimulated RA-treated MoDCs (Fig. 6D). Since a 3-day exposure of developing MoDCs to RA was sufficient to induce a significant increase in CD103 and β7 expression, as shown in Suppl. Fig. 1C,

our observations suggest that *H. pylori* stimulation actively downregulates CD103 and β7 expression. However, the mechanism for this downregulation remains unclear, since *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 down-modulation of CD103 and β7 in RA-treated DCs was specific to *H. pylori* or mediated by global pattern recognition receptor activation, we compared *H. pylori* bacteria to 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 β7 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 α4β7 Expression

The induction of the mucosal homing molecules α4β7 and CCR9 in responder T cells through a retinoic acid (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 RA synthesize and release RA (Fig. 1B), we asked whether these RA-treated DCs induce T cell α4β7 expression and whether induction of T cell α4β7 depends on DC

Figure 6:

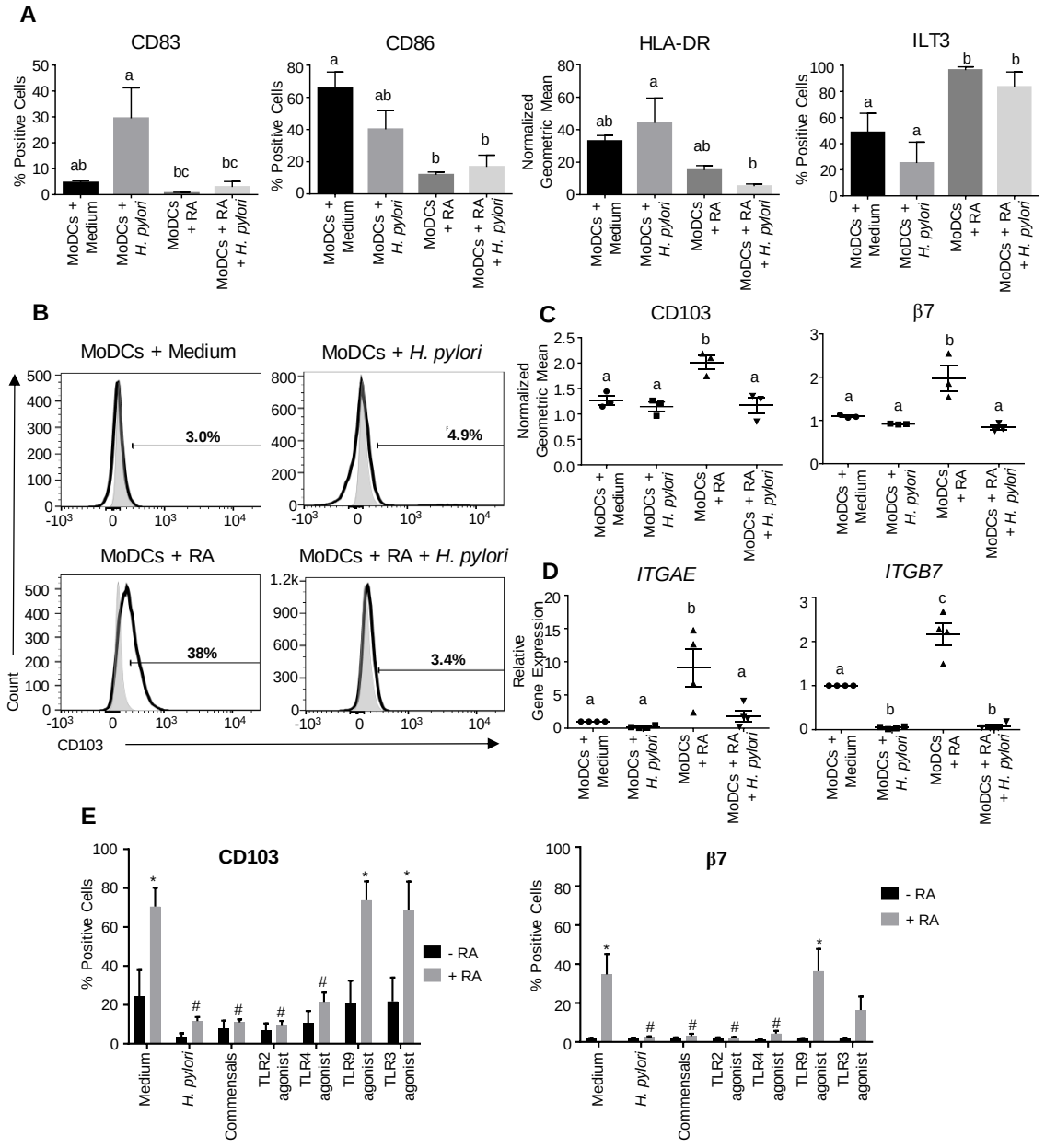

Fig. 6: Stimulation of MoDCs with *H. pylori*, commensal bacteria or TLR2/4 ligands abrogates RA-induced upregulation of CD103 and β7. Continued on next page. MoDCs were cultured with RA or medium for 3 d and then stimulated for 48 h with*H. Pylori* 60190 (MOI 10), with continued exposure to RA or medium. (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. Continued on next page.

Fig. 6 cont. 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. Different letters denote statistically significant differences ($P < 0.05$, ANOVA). (D) qRT-PCR analysis of RA-treated and untreated DCs for CD103 (*ITGAE*) and β7 (*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 β7 expression from 3 independent experiments. *Significant difference from medium only; #significant difference between RA-treated, unstimulated cells and RA-treated cells with bacterial stimulation ($P< 0.05$).

expression of CD103. MoDCs were generated in the presence or absence of RA, sorted into $CD103^+$ and $CD103^-$ cells (Suppl. Fig. 6A), loaded with SEB and co-cultured with autologous naïve $CD4^+$ T cells. The T cells were analyzed for α4β7 integrin expression and proliferation after 4 days (Fig. 7A). As anticipated, SEB-loaded MoDCs, but not MoDCs without antigen, consistently induced α4β7 expression and T cell proliferation (Fig. 7B,C). Importantly, MoDCs generated in the presence of RA induced higher expression of T cell α4β7 expression (Fig. 7B). However, both $CD103^+$ and $CD103^-$ DCs from RA-treated cultures drove similar levels of α4β7 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. recently showed that RA-conditioning of human DCs did not substantially enhance T cell Foxp3 expression, but induced $\alpha4\beta7^+$ T cells expressing high levels of interleukin (IL)-10 [20]. Analysis of supernatants from our experiments similarly revealed a trend for increased levels of IL-10, but not IFN-γ, in co-cultures from T cells and RA-treated MoDCs compared to control MoDCs, with $CD103^+$ and $CD103^-$ DCs

again inducing similar levels of cytokine secretion (Suppl. Fig. 6B,C). Overall, our data suggest that RA exposure rather than direct interactions between DC CD103 and T cells contribute to the induction of mucosal homing molecules, proliferation and cytokines on responder T cells.

Figure 7:

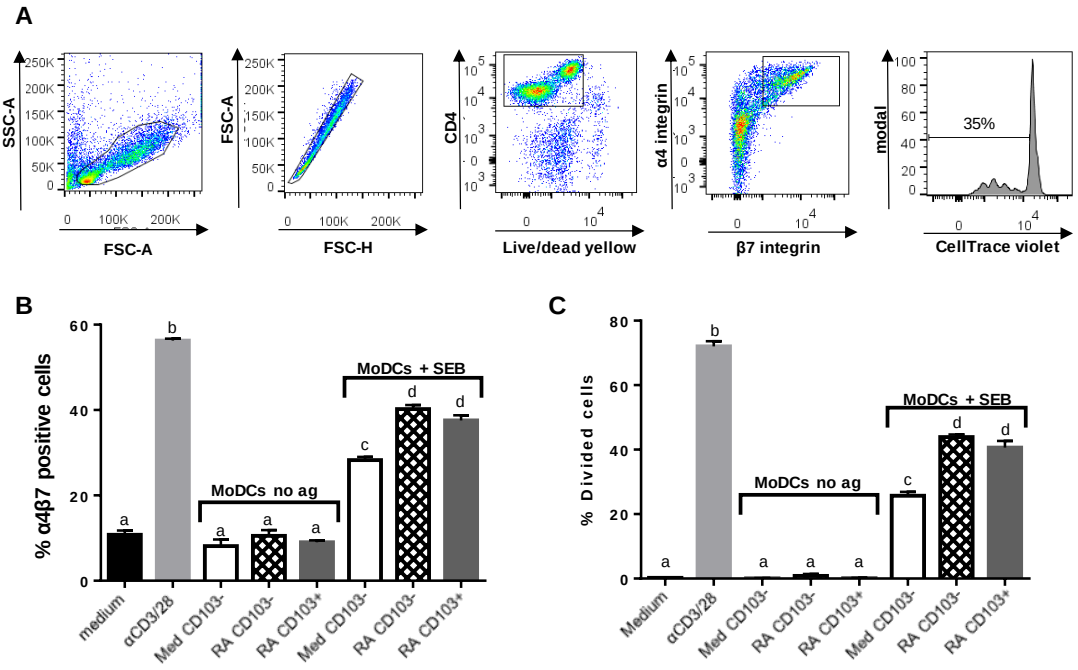

Fig. 7: RA-treated DCs drive increased T cell α4β7 expression and proliferation independent of CD103 expression. MoDCs were cultured with RA or medium alone for 3 days and were then sorted by FACS as $CD103^+$ or $CD103^-$ DCs. Due to low numbers of $CD103^+$ DCs in samples without RA-treatment, this population was excluded from the analysis. Sorted DCs were loaded with staphylococcal enterotoxin B (SEB, 1 µg/mL) for 1 h and then co-cultured with CellTrace Violet-labeled autologous naïve $CD4^+$ T cells for 4 days. Co-cultured T cells were harvested and analyzed for expression of α4β7 and proliferation. (A) T cell gating strategy and representative proliferation and α4β7 expression; (B) % of $CD4^+$ T cells co-expressing α4 and β7 integrin; (C) % of divided T cells. Mean ± SEM of triplicate wells; experiment shown is representative of n=4. Different letters denote statistically significant differences (P<0.05).

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 β7 integrin expression by RA and by bacterial pathogen-associated molecular patterns. We show that CD103 was upregulated by RA in monocyte-derived DCs (MoDCs), but not in primary human gastric DCs, and that DC CD103 and β7 integrin expression was downregulated following TLR2/4 stimulation.

Our data confirm and extend previous reports of CD103 upregulation by RA in human MoDCs [16-18]. Importantly, we used an improved culture system with physiological 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 β7 integrin, which forms a constitutive heterodimer with the αE integrin subunit [11], was similarly regulated by RA, confirming the physiological relevance of our data. Interestingly, our experiments revealed that human MoDCs contained significant pools of intracellular CD103 but not β7 integrin that were also upregulated 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 previously been shown 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. Since we were unable to detect

significant intracellular β7 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 DCs' ability 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 regulatory cells with strong T cell IL-10 production [18, 20] and to induce expression of intestinal homing molecules α4β7 and CCR9 on the T cells [38]. In our hands, T cells co-cultured with RA-treated DCs likewise expressed increased levels of α4β7, 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 $CD103^-$ DC 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/β7 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 (ILK) 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 α4β7 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 β7 integrin expression in human DCs. While RA upregulated CD103 expression on MoDCs, bacterial TLR agonists downregulated RA-induced CD103 expression. Interestingly, both a gram-negative pathogen (*H. pylori*) and gram-positive commensal bacteria were similarly efficient at blocking CD103 and β7 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 downregulation. 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], downregulation 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 upregulate 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 downregulates 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; Bimczok and Smythies, unpublished observations). 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 β7 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 both TGF-β and TGF-β receptor in other model systems [31, 32, 53, 54], and our experiments using the TGF-βRI inhibitor SB431542 prevented RA-induced upregulation of CD103 but not β7 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, as we have shown [10, 21]. However, our experiments with RA-treated MoDCs did not reveal an increase in either total or active TGF-β1 release or cell-associated LAP, which tightly correlates with membrane-bound TGF-β. Moreover, we were unable to induce CD103 or β7 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 due to analysis of sub-optimal time points. Alternatively, TGF-β family members such as inhibins or nodal also signal through ALK receptors that are inhibited by SB431542 (ALK4, ALK7) and 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 CD103/αE integrin.

In summary, we report a dynamic regulation of CD103 and β7 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 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.

References Cited

1. Jaensson, E., Uronen-Hansson, H., Pabst, O., Eksteen, B., Tian, J., Coombes, J. L., Berg, P. L., Davidsson, T., Powrie, F., Johansson-Lindbom, B., Agace, W. W. (2008) Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp.Med. 205, 2139-2149.

2. del Rio, M. L., Bernhardt, G., Rodriguez-Barbosa, J. I., Forster, R. (2010) Development and functional specialization of CD103+ dendritic cells. Immunol Rev 234, 268-81.

3. Jaensson-Gyllenback, E., Kotarsky, K., Zapata, F., Persson, E. K., Gundersen, T. E., Blomhoff, R., Agace, W. W. (2011) Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. Mucosal Immunol 4, 438-47.

4. McDole, J. R., Wheeler, L. W., McDonald, K. G., Wang, B., Konjufca, V., Knoop, K. A., Newberry, R. D., Miller, M. J. (2012) Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature 483, 345-9.

5. Schulz, O., Jaensson, E., Persson, E. K., Liu, X., Worbs, T., Agace, W. W., Pabst, O. (Dec 21) Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med 206, 3101-14.

6. Muzaki, A. R., Tetlak, P., Sheng, J., Loh, S. C., Setiagani, Y. A., Poidinger, M., Zolezzi, F., Karjalainen, K., Ruedl, C. (2015) Intestinal CD103CD11b dendritic cells restrain colitis via IFN-gamma-induced anti-inflammatory response in epithelial cells. Mucosal Immunol.

7. Coombes, J. L., Siddiqui, K. R., Arancibia-Carcamo, C. V., Hall, J., Sun, C. M., Belkaid, Y., Powrie, F. (2007) A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp.Med. 204, 1757-1764.

8. Mann, E. R., Bernardo, D., English, N. R., Landy, J., Al-Hassi, H. O., Peake, S. T., Man, R., Elliott, T. R., Spranger, H., Lee, G. H., Parian, A., Brant, S. R., Lazarev, M., Hart, A. L., Li, X., Knight, S. C. (2015) Compartment-specific immunity in the human gut: properties and functions of dendritic cells in the colon versus the ileum. Gut.

9. Watchmaker, P. B., Lahl, K., Lee, M., Baumjohann, D., Morton, J., Kim, S. J., Zeng, R., Dent, A., Ansel, K. M., Diamond, B., Hadeiba, H., Butcher, E. C. (2014) Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. Nat Immunol 15, 98-108.

10. Bimczok, D., Kao, J. Y., Zhang, M., Cochrun, S., Mannon, P., Peter, S., Wilcox, C. M., Monkemuller, K. E., Harris, P. R., Grams, J. M., Stahl, R. D., Smith, P. D., Smythies, L. E. (2014) Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells. Mucosal Immunol.

11. Hadley, G. A. and Higgins, J. M. (2014) Integrin alphaEbeta7: molecular features and functional significance in the immune system. Advances in experimental medicine and biology 819, 97-110.

12. Robinson, P. W., Green, S. J., Carter, C., Coadwell, J., Kilshaw, P. J. (2001) Studies on transcriptional regulation of the mucosal T-cell integrin alphaEbeta7 (CD103). Immunology 103, 146-54.

13. Cepek, K. L., Shaw, S. K., Parker, C. M., Russell, G. J., Morrow, J. S., Rimm, D. L., Brenner, M. B. (1994) Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature 372, 190-3.

14. Suzuki, R., Nakao, A., Kanamaru, Y., Okumura, K., Ogawa, H., Ra, C. (2002) Localization of intestinal intraepithelial T lymphocytes involves regulation of alphaEbeta7 expression by transforming growth factor-beta. Int Immunol 14, 339-45.

15. Mokrani, M., Klibi, J., Bluteau, D., Bismuth, G., Mami-Chouaib, F. (2014) Smad and NFAT pathways cooperate to induce CD103 expression in human CD8 T lymphocytes. J Immunol 192, 2471-9.

16. den Hartog, G., van Altena, C., Savelkoul, H. F., van Neerven, R. J. (2013) The mucosal factors retinoic acid and TGF-beta1 induce phenotypically and functionally distinct dendritic cell types. Int Arch Allergy Immunol 162, 225-36.

17. Iliev, I. D., Spadoni, I., Mileti, E., Matteoli, G., Sonzogni, A., Sampietro, G. M., Foschi, D., Caprioli, F., Viale, G., Rescigno, M. (2009) Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut 58, 1481-9.

18. Kim, M. J., Jeong, E. K., Kwon, E. Y., Joo, J. Y., Lee, J. Y., Choi, J. (2014) Human CD103(+) dendritic cells promote the differentiation of Porphyromonas gingivalis heat shock protein peptide-specific regulatory T cells. J Periodontal Implant Sci 44, 235-41.

19. Riedl, E., Stockl, J., Majdic, O., Scheinecker, C., Rappersberger, K., Knapp, W., Strobl, H. (2000) Functional involvement of E-cadherin in TGF-beta 1-induced cell cluster formation of in vitro developing human Langerhans-type dendritic cells. J Immunol 165, 1381-6.

20. Bakdash, G., Vogelpoel, L. T., van Capel, T. M., Kapsenberg, M. L., de Jong, E. C. (2014) Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. Mucosal Immunol.

21. Bimczok, D., Grams, J. M., Stahl, R. D., Waites, K. B., Smythies, L. E., Smith, P. D. (2011) Stromal regulation of human gastric dendritic cells restricts the Th1 response to Helicobacter pylori. Gastroenterology 141, 929-38.

22. Bimczok, D., Clements, R. H., Waites, K. B., Novak, L., Eckhoff, D. E., Mannon, P. J., Smith, P. D., Smythies, L. E. (2010) Human primary gastric dendritic cells induce a Th1 response to H. pylori. Mucosal Immunol 3, 260-9.

23. Bimczok, D., Smythies, L. E., Waites, K. B., Grams, J. M., Stahl, R. D., Mannon, P. J., Peter, S., Wilcox, C. M., Harris, P. R., Das, S., Ernst, P. B., Smith, P. D. (2013) Helicobacter pylori infection inhibits phagocyte clearance of apoptotic gastric epithelial cells. J Immunol 190, 6626-34.

24. Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

25. Wagner, M., Han, B., Jessell, T. M. (1992) Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. Development 116, 55-66.

26. Ohoka, Y., Yokota-Nakatsuma, A., Maeda, N., Takeuchi, H., Iwata, M. (2014) Retinoic acid and GM-CSF coordinately induce retinal dehydrogenase 2 (RALDH2) expression through cooperation between the RAR/RXR complex and Sp1 in dendritic cells. PLoS One 9, e96512.

27. Agace, W. W. and Persson, E. K. (2012) How vitamin A metabolizing dendritic cells are generated in the gut mucosa. Trends Immunol 33, 42-8.

28. Molenaar, R., Knippenberg, M., Goverse, G., Olivier, B. J., de Vos, A. F., O'Toole, T., Mebius, R. E. (2011) Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. J Immunol 186, 1934-42.

29. Napoli, J. L. (1986) Quantification of physiological levels of retinoic acid. Methods in enzymology 123, 112-24.

30. Lay, M. K., Atmar, R. L., Guix, S., Bharadwaj, U., He, H., Neill, F. H., Sastry, K. J., Yao, Q., Estes, M. K. (2010) Norwalk virus does not replicate in human macrophages or dendritic cells derived from the peripheral blood of susceptible humans. Virology 406, 1-11.

31. Balmer, J. E. and Blomhoff, R. (2002) Gene expression regulation by retinoic acid. J Lipid Res 43, 1773-808.

32. Yoshizawa, M., Miyazaki, H., Kojima, S. (1998) Retinoids potentiate transforming growth factor-beta activity in bovine endothelial cells through up-regulating the expression of transforming growth factor-beta receptors. J Cell Physiol 176, 565-73.

33. Gandhi, R., Anderson, D. E., Weiner, H. L. (2007) Cutting Edge: Immature human dendritic cells express latency-associated peptide and inhibit T cell activation in a TGF-beta-dependent manner. J Immunol 178, 4017-21.

34. Kang, J. S., Liu, C., Derynck, R. (2009) New regulatory mechanisms of TGF-beta receptor function. Trends Cell Biol. 19, 385-394.

35. Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., Hill, C. S. (2002) SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 62, 65-74.

36. Jin, C. J., Hong, C. Y., Takei, M., Chung, S. Y., Park, J. S., Pham, T. N., Choi, S. J., Nam, J. H., Chung, I. J., Kim, H. J., Lee, J. J. (2010) All-trans retinoic acid inhibits the differentiation, maturation, and function of human monocyte-derived dendritic cells. Leukemia research 34, 513-20.

37. Kranzer, K., Eckhardt, A., Aigner, M., Knoll, G., Deml, L., Speth, C., Lehn, N., Rehli, M., Schneider-Brachert, W. (2004) Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. Infect.Immun. 72, 4416-4423.

38. Rampal, R., Awasthi, A., Ahuja, V. (2016) Retinoic acid-primed human dendritic cells inhibit Th9 cells and induce Th1/Th17 cell differentiation. J Leukoc Biol.

39. Triantafilou, M., Gamper, F. G., Lepper, P. M., Mouratis, M. A., Schumann, C., Harokopakis, E., Schifferle, R. E., Hajishengallis, G., Triantafilou, K. (2007) Lipopolysaccharides from atherosclerosis-associated bacteria antagonize TLR4, induce formation of TLR2/1/CD36 complexes in lipid rafts and trigger TLR2-induced inflammatory responses in human vascular endothelial cells. Cell Microbiol 9, 2030-9.

40. Uno, K., Kato, K., Atsumi, T., Suzuki, T., Yoshitake, J., Morita, H., Ohara, S., Kotake, Y., Shimosegawa, T., Yoshimura, T. (2007) Toll-like receptor (TLR) 2 induced through TLR4 signaling initiated by Helicobacter pylori cooperatively amplifies iNOS induction in gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 293, G1004-12.

41. Rad, R., Ballhorn, W., Voland, P., Eisenacher, K., Mages, J., Rad, L., Ferstl, R., Lang, R., Wagner, H., Schmid, R. M., Bauer, S., Prinz, C., Kirschning, C. J., Krug, A. (2009) Extra- and Intracellular Pattern Recognition Receptors Cooperate in the Recognition of Helicobacter pylori. Gastroenterology.

42. Eksteen, B., Mora, J. R., Haughton, E. L., Henderson, N. C., Turner, L. L., Villablanca, E. J., Curbishley, S. M., Aspinall, A. I., von Andrian, U. H., Adams, D. H. (2009) Gut Homing Receptors on CD8 T Cells Are Retinoic Acid Dependent and Not Maintained by Liver Dendritic or Stellate Cells. Gastroenterology.

43. Zeng, R., Bscheider, M., Lahl, K., Lee, M., Butcher, E. C. (2016) Generation and transcriptional programming of intestinal dendritic cells: essential role of retinoic acid. Mucosal Immunol 9, 183-93.

44. Bretscher, M. S. (1992) Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. EMBO J 11, 405-10.

45. Bridgewater, R. E., Norman, J. C., Caswell, P. T. (2012) Integrin trafficking at a glance. Journal of cell science 125, 3695-701.

46. Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R. S., Wojtasiak, M., Shortman, K., Carbone, F. R., Brooks, A. G., Heath, W. R. (2009) Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. Nat Immunol 10, 488-95.

47. Jenkinson, S. E., Whawell, S. A., Swales, B. M., Corps, E. M., Kilshaw, P. J., Farthing, P. M. (2011) The alphaE(CD103)beta7 integrin interacts with oral and skin keratinocytes in an E-cadherin-independent manner*. Immunology 132, 188-96.

48. Cepek, K. L., Rimm, D. L., Brenner, M. B. (1996) Expression of a candidate cadherin in T lymphocytes. Proceedings of the National Academy of Sciences of the United States of America 93, 6567-71.

49. Abram, C. L. and Lowell, C. A. (2007) The expanding role for ITAM-based signaling pathways in immune cells. Sci STKE 2007, re2.

50. Boutet, M., Gauthier, L., Leclerc, M., Gros, G., De Montpreville, V., Theret, N., Donnadieu, E., Mami-Chouaib, F. (2016) TGF-beta signaling intersects with CD103 integrin signaling to promote T lymphocyte accumulation and antitumor activity in the lung tumor microenvironment. Cancer Res.

51. Agace, W. W., Higgins, J. M., Sadasivan, B., Brenner, M. B., Parker, C. M. (2000) T-lymphocyte-epithelial-cell interactions: integrin alpha(E)(CD103)beta(7), LEEP-CAM and chemokines. Current opinion in cell biology 12, 563-8.

52. Schlickum, S., Sennefelder, H., Friedrich, M., Harms, G., Lohse, M. J., Kilshaw, P., Schon, M. P. (2008) Integrin alpha E(CD103)beta 7 influences cellular shape and motility in a ligand-dependent fashion. Blood 112, 619-25.

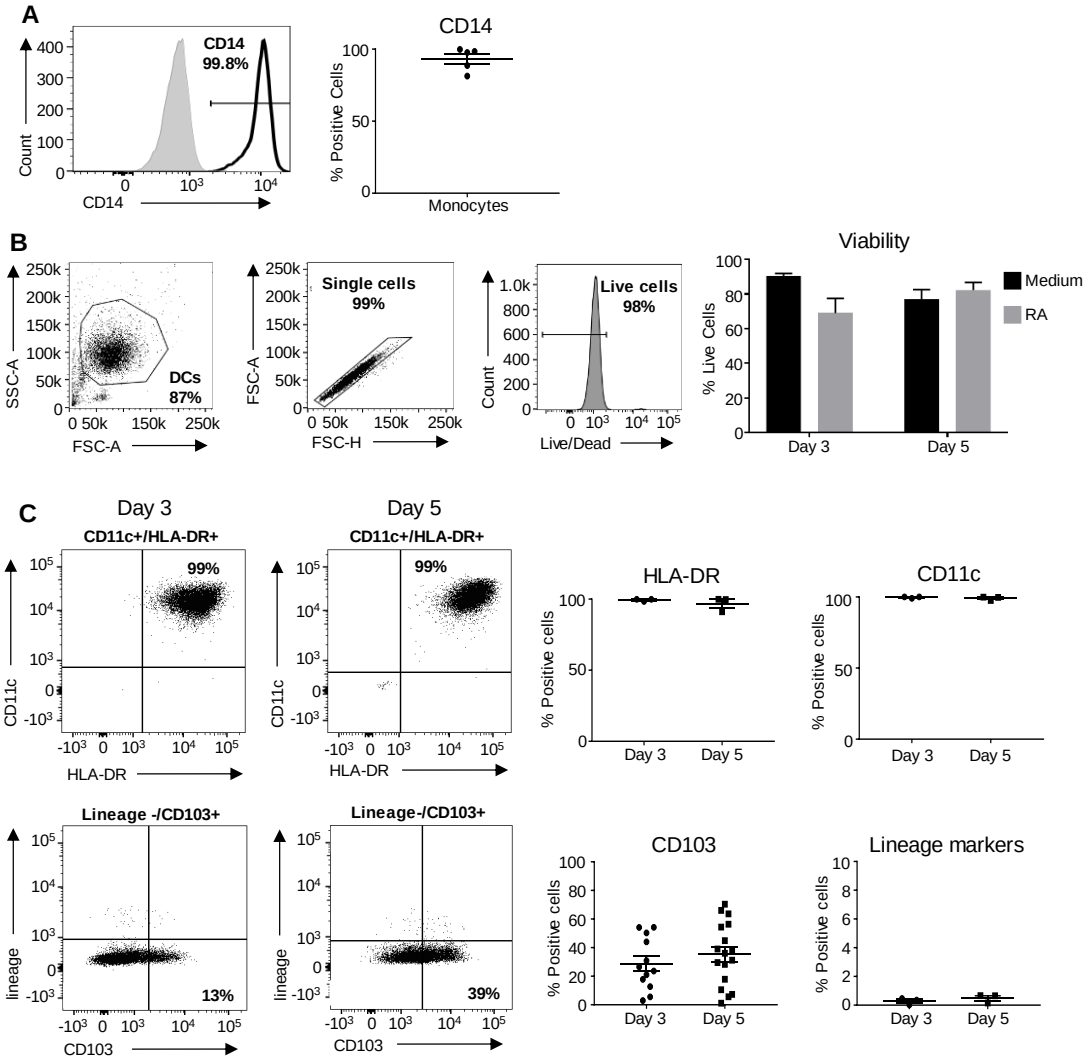
53. Saurer, L., McCullough, K. C., Summerfield, A. (2007) In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid. J Immunol. 179, 3504-3514.

54. Foitzik, K., Spexard, T., Nakamura, M., Halsner, U., Paus, R. (2005) Towards dissecting the pathogenesis of retinoid-induced hair loss: all-trans retinoic acid induces premature hair follicle regression (catagen) by upregulation of transforming growth factor-beta2 in the dermal papilla. J Invest Dermatol 124, 1119-26.

55. Moustakas, A. and Heldin, C. H. (2009) The regulation of TGFbeta signal transduction. Development 136, 3699-3714.

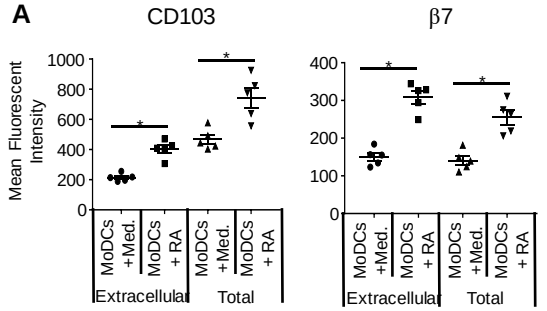
SUPPLEMENTAL FIGURES

Supplemental Figure 1:



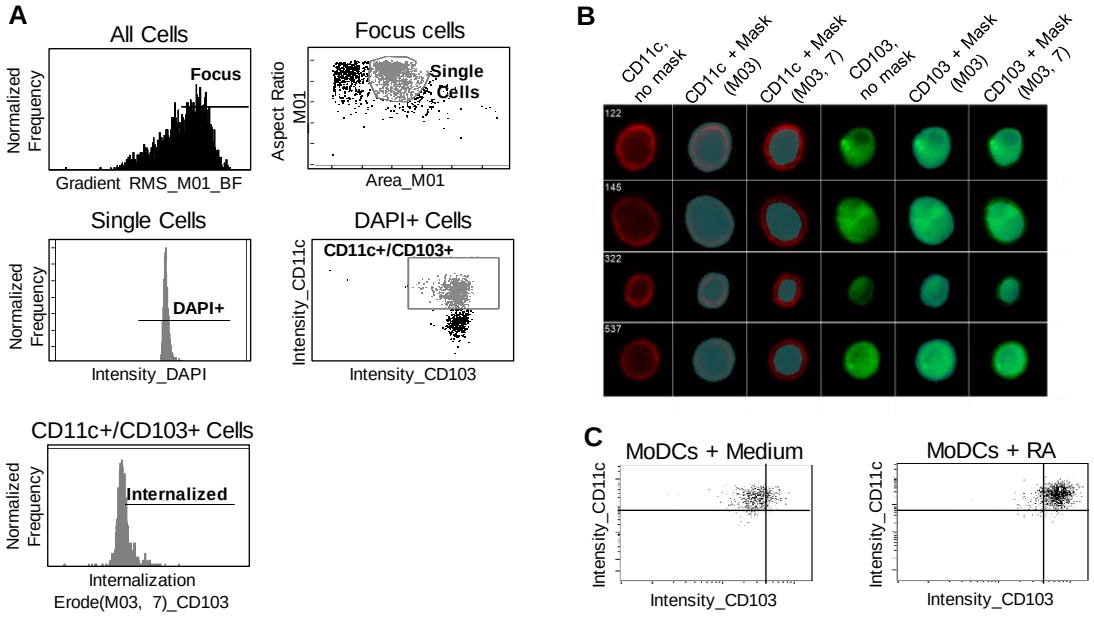
Supplemental Fig. 1: Purity, viability and phenotype of isolated monocytes and MoDCs. (A) Representative histogram of purified $CD14^{+}$ monocytes and pooled data from 5 independent experiments; (B) Gating strategy for FACS analysis of MoDCs (left) and average viability of DCs after 3 and 5 days of culture, mean ± SEM of n=4 (right); (C) CD11c, HLA-DR and CD103 expression by RA-treated MoDCs after 3 and 5 days of culture in the presence of RA, representative dot plots (left) and cumulative data from 3 or more experiments (right). Lineage cocktail contained antibodies to CD3, CD19, CD20 and CD56.

Supplemental Figure 2:



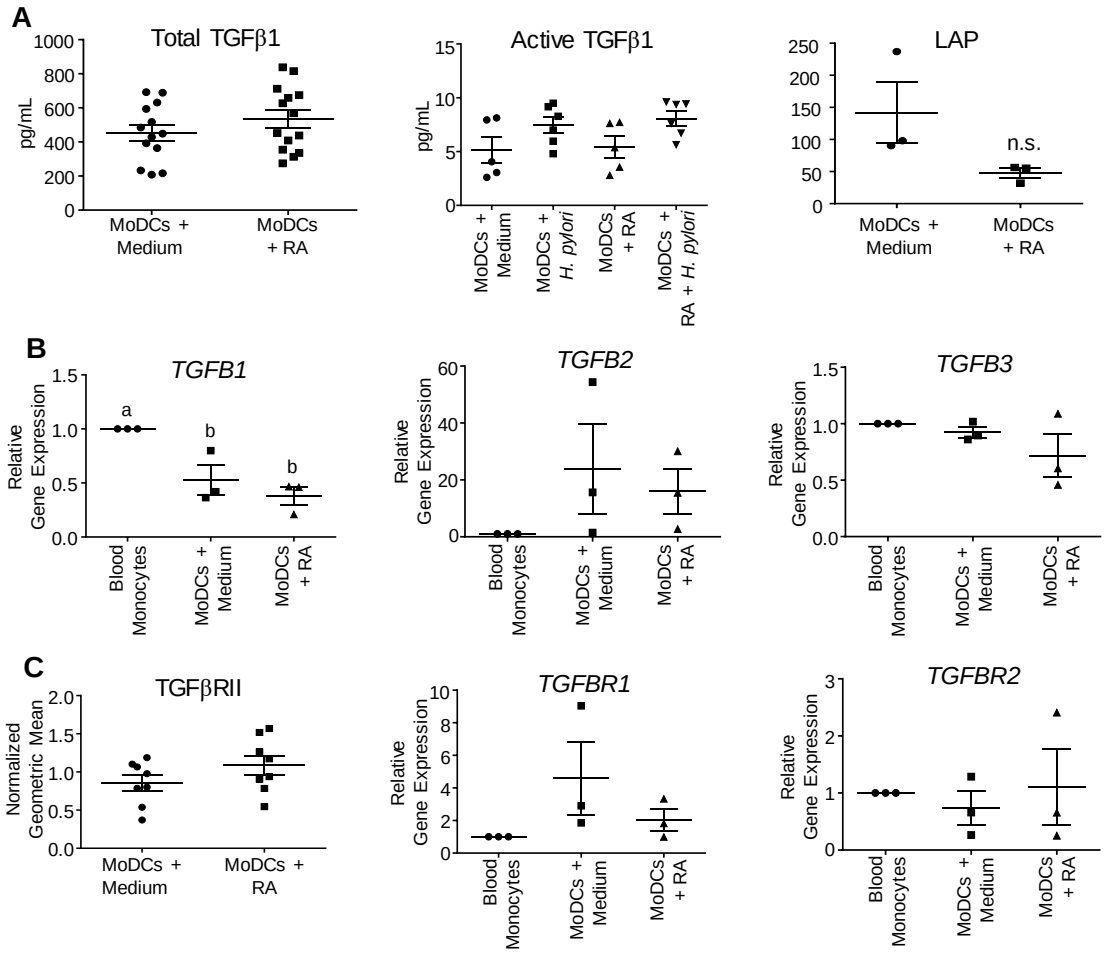
Supplemental Fig. 2. Raw geometric mean fluorescence data of CD103 and β7 expression. Raw data for extracellular and total CD103 and β7 expression by RA-treated and control MoDCs as shown in Fig. 2B.

Supplemental Figure 3:



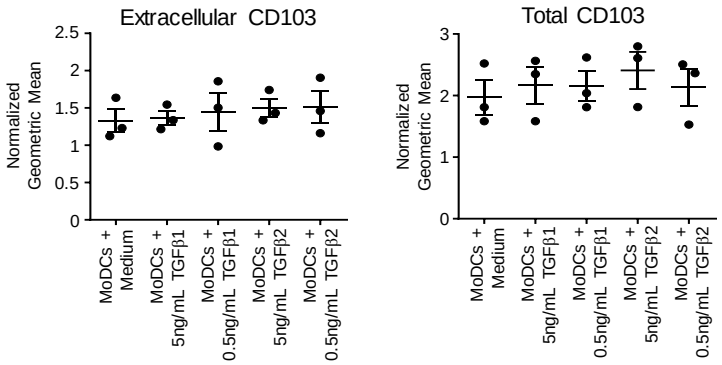
Supplemental Fig. 3: Gating strategy and masks used for ImageStream® analysis of MoDCs. (A) MoDCs were gated for brightfield gradient RMS (cells in focus), single cells, and presence of nuclear staining (DAPI-positive). Cells double positive for CD11c/CD103 were analyzed for the distribution of CD103 on the cell surface vs. in the cytoplasm. A mask for cell surface staining was created based on CD11c expression. Internalization scores were determined using the IDEAS® software; cells with an internalization score of >1 were considered to be positive for intracellular CD103 expression. Externalization scores were calculated as 100% - % internalization. (B) Representative masks based on CD11c surface staining. (C) Increased staining intensity for CD103 in RA-treated DCs.

Supplemental Figure 4:



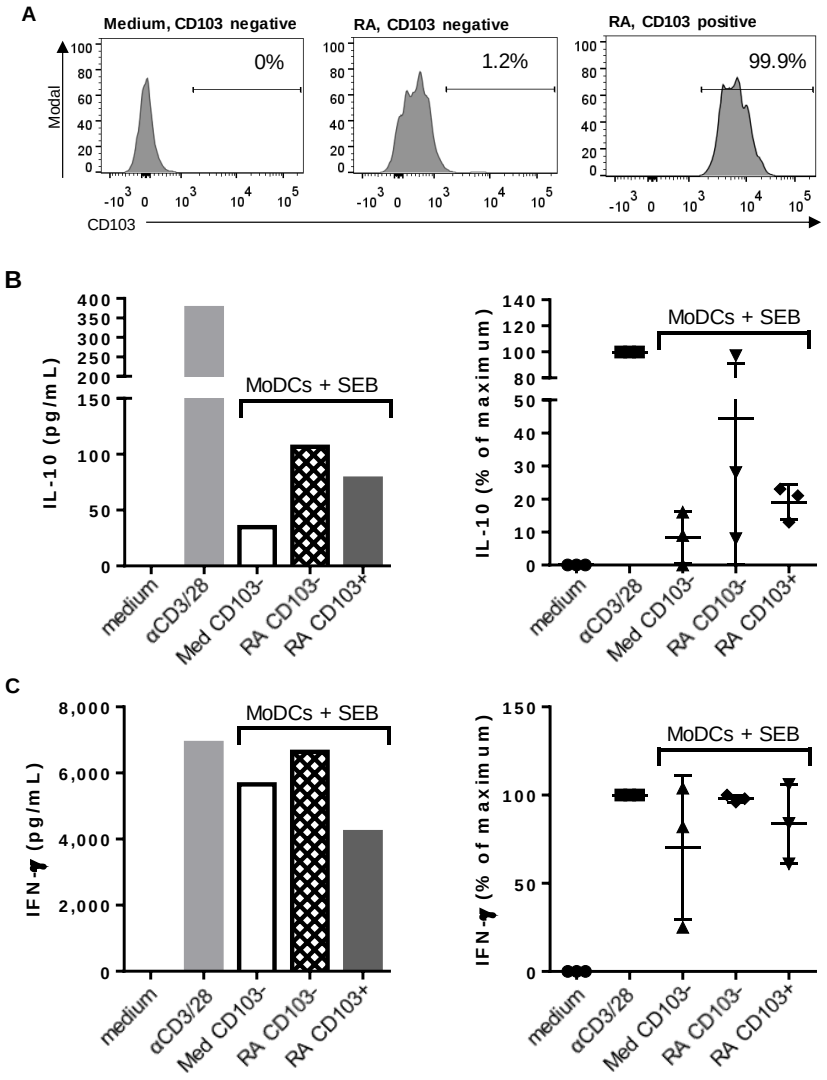
Supplemental Fig. 4. RA does not induce expression of TGF-β or TGFβ receptors in human MoDCs. (A) Supernatants of MoDCs were analyzed by ELISA for total TGFβ1 (n=13) and active TGFβ1 (n=5) after culture with or without RA and/or *H. pylori*; cell lysates of MoDCs (n=3) were analyzed for LAP. (B) Quantitative RT-PCR of fresh monocytes, untreated MoDCs or RA-treated MoDCs for *TGFB1*, *TGFB2* and *TGFB3*. Cumulative data from three independent experiments. (C) Left panel: flow cytometry analysis of TGFβRII surface expression of MoDCs treated with RA or left untreated. Cumulative data of eight independent experiments. Middle and right panels: quantitative RT-PCR of fresh monocytes, untreated MoDCs and RA-treated MoDCs for *TGFBR1* and *TGFBR2* expression. Cumulative data of three independent experiments.

Supplemental Figure 5:



Supplemental Fig. 5. Exogenous TGF-β does not significantly enhance CD103 expression. Flow cytometry analysis of extracellular and total CD103 expression of MoDCs after culture with differing concentrations of TGFβ1 or TGFβ2. Cumulative data of three independent experiments.

Supplemental Figure 6:



Supplemental Fig. 6. IL-10 and IFN-γ release by CD4 T cells co-cultured with $CD103^+$ or $CD103^-$ RA-treated MoDCs or untreated DCs. MoDCs were cultured with RA or medium alone for 3 days and were then sorted by FACS as $CD103^+$ or $CD103^-$ DCs. Due to low numbers of $CD103^+$ DCs in samples without RA-treatment, this population was excluded from the analysis. (A) Purity of sorted DC populations. (B,C) Sorted DCs were loaded with staphylococcal enterotoxin B (SEB, 1 μg/mL) for 1 h and then co-cultured with autologous naïve $CD4^+$ T cells for 4 days. Supernatants were collected and analyzed for (B) IL-10 and (C) IFN-γ by ELISA. Left panel: representative experiment; right panel: three independent experiments, concentrations normalized to cytokine production by anti CD3/CD28–treated T cells (positive control).

CHAPTER 3

CD103 (αE INTGRIN) UNDERGOES ENDOSOMAL TRAFFICKING IN HUMAN DENDRITIC CELLS, BUT DOES NOT MEDIATE EPITHELIAL ADHESION

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Planned and oversaw the experiments. Performed experiments. Analyzed data and wrote the manuscript.

Co-Author: Mandi M. Roe

Contributions: Performed experiments. Analyzed the data and wrote the manuscript.

Co-Author: Thomas A. Sebrell, Barkan Sidar, Jennifer Dankoff, Rachel VanAusdol

Contributions: Performed experiments. Provided feedback on the manuscript.

Co-Author: Lesley E. Smythies, Philip D. Smith

Contributions: Initiated the project, critically discussed the data and obtained funding.

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Contributions: Planned and oversaw the experiments. Performed experiments. Initiated the project, critically discussed the data and obtained funding. Analyzed the data and wrote the manuscript.

Manuscript Information

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Abstract

Dendritic cell (DC) expression of CD103, the α subunit of αEβ7 integrin, is thought to enable DC interactions with E-cadherin-expressing gastrointestinal epithelia for improved mucosal immunosurveillance. In the stomach, efficient DC surveillance of the epithelial barrier is crucial for the induction of immune responses to *H. pylori*, the causative agent of peptic ulcers and gastric cancer. However, gastric DCs express only low levels of surface CD103, as we previously showed. We here tested the hypothesis that intracellular pools of CD103 in human gastric DCs can be redistributed to the cell surface for engagement of epithelial cell-expressed E-cadherin to promote DC-epithelial cell adhesion. In support of our hypothesis, immunofluorescence analysis of tissue sections showed that $CD103^+$ gastric DCs were preferentially localized within the gastric epithelial layer. Flow cytometry and imaging cytometry revealed that human gastric DCs expressed intracellular CD103, corroborating our previous findings in monocyte-derived DCs (MoDCs). Using confocal microscopy, we show that CD103 was present in endosomal compartments, where CD103 partially co-localized with clathrin, early endosome antigen-1 and Rab11, suggesting that CD103 undergoes endosomal trafficking similar to β1 integrins. Dynamic expression of CD103 on human MoDCs was confirmed by internalization assay. To analyze whether DC-expressed CD103 promotes adhesion to E-cadherin, we performed adhesion and spreading assays on E-cadherin-coated glass slides. In MoDCs generated in the presence of retinoic acid, which express increased CD103, intracellular CD103 significantly redistributed towards the E-cadherin-coated glass surface. However, DCs spreading and adhesion did not differ between E-cadherin-

coated slides and slides coated with serum alone. In adhesion assays using E-cadherin-positive HT-29 cells, DC binding was significantly improved by addition of Mn^{2+} and decreased in the presence of EGTA, consistent with the dependence of integrin-based interactions on divalent cations. However, retinoic acid failed to increase DC adhesion, and a CD103 neutralizing antibody was unable to inhibit DC binding to the E-cadherin positive cells. In contrast, a blocking antibody to DC-expressed E-cadherin significantly reduced DC binding to the epithelium. Overall, these data indicate that CD103 engages in DC-epithelial cell interactions upon contact with epithelial E-cadherin, but is not a major driver of DC adhesion to gastrointestinal epithelia.

Introduction

Dendritic cells (DCs) frequently interact with the epithelial layer of the gastric mucosa, as shown in previous studies (Necchi et al., 2009; Bimczok et al., 2010; Kao et al., 2010). As professional antigen-presenting cells, DCs control the immune response to *Helicobacter pylori* (*H. pylori*) (Shiu and Blanchard, 2013), a bacterial pathogen that causes chronic gastritis, peptic ulcer disease and gastric cancer (Moss and Malfertheiner, 2007; Hitzler et al., 2011; Amieva and Peek, 2016). Specifically, the type of T cell response induced by the DCs largely determines whether *H. pylori* infection causes only mild inflammation or leads to severe inflammatory pathologies including ulcers or cancer (Harris et al., 2008; Robinson et al., 2008; Serrano et al., 2013). For those DCs that are located immediately beneath or within the gastric epithelium, their spatial interactions with the epithelial cells have important functional implications for the immune response to *H. pylori*. First, DCs that reside within the epithelial layer or extend transepithelial

dendrites have direct access to the gastric lumen for *H. pylori* antigen sampling (Rescigno et al., 2001; Necchi et al., 2009; Bimczok et al., 2010; Kao et al., 2010). Second, positioning of gastric DCs immediately below the epithelium increases the probability for pathogen capture upon epithelial barrier breach, and third, the close proximity of DCs to epithelial cells likely enhances the paracrine effects of epithelial-derived mediators that regulate DC function (Iliev et al., 2009a; Iliev et al., 2009b; Bimczok et al., 2015). In spite of the importance of DC-epithelial interactions for gastrointestinal immune responses, the molecular mechanisms of these interactions are not well defined.

Binding of DC-expressed CD103 (αEß7 integrin) to epithelial E-cadherin was proposed as a potential mechanism for DC adhesion to epithelial cells (Hadley and Higgins, 2014; Watchmaker et al., 2014; Habtezion et al., 2016). CD103, the α subunit of αEß7 integrin, is widely recognized as an important DC subset and lineage marker in humans and mice (Haniffa et al., 2013; Guilliams et al., 2014; Segura, 2016). Specifically, CD103 identifies a DC subset termed conventional DC1 that is able to cross-present exogenous antigens to CD8 T cells and that induces mucosal tolerance to commensals and dietary antigens (Scott et al., 2011; Haniffa et al., 2013). The functional role of CD103 has been extensively studied in transfected cells lines, where the A-domain of the αE (CD103) integrin subunit was shown to interact with the top surface of E-cadherin domain 1, and in intestinal intraepithelial lymphocytes (IELs), where CD103 anchors the IELs within the epithelial layer (Cepek et al., 1994; Higgins et al., 2000; Corps et al., 2001). In spite of its frequent use as a DC marker, the function of CD103 in primary human DCs has received little investigative attention. Therefore, the goal of our

study was to determine whether CD103 enables DCs in the human stomach to interact with the epithelium through E-cadherin engagement.

Notably, previous studies from our laboratory and others have shown that surface CD103 expression of gastric DCs is low compared to CD103 expression on DCs in other tissue compartments such as the small intestine (Bimczok et al., 2015; Arnold et al., 2017; Roe et al., 2017; Viladomiu et al., 2017). This low surface CD103 expression was unexpected, since gastric DCs have a tolerogenic capacity similar to that of human intestinal DCs (Bimczok et al., 2011; Bimczok et al., 2015) and also are efficient producers of retinoic acid (RA), properties generally associated with intestinal $CD103^+$ DC subsets (del Rio et al., 2010; Agace and Persson, 2012; Bimczok et al., 2015). However, we also showed that human monocyte-derived DCs express considerable amounts of CD103 in intracellular compartments (Roe et al., 2017). Other integrins including α5β1, α6β4 and αMβ2 are expressed in endosomal compartments and recirculate through the membrane to enable dynamic and tightly regulated interactions with their respective ligands (Bretscher, 1992; Bridgewater et al., 2012; Paul et al., 2015). Therefore, we hypothesized that intracellular pools of αE integrin/CD103 present in human gastric DCs can be redistributed to the cell surface for engagement of epithelial cell-expressed E-cadherin in the stomach to promote DC-epithelial cell adhesion. Interestingly, our experiments revealed that CD103 undergoes endosomal trafficking in human DCs and is engaged upon DC contact with epithelial E-cadherin, but is not the major adhesion factor that mediates epithelial cell binding.

Materials and Methods

Human Blood and Tissue Samples

Heparinized blood samples were obtained with local IRB approval from healthy adult volunteers in Birmingham, AL (IRB# X120806005), or Bozeman, MT (IRB #DB082817 and #DB092614). Gastric tissue specimens were obtained with Institutional Review Board (IRB) approval and informed consent from non-*H. pylori*-infected adult subjects undergoing elective gastric bypass surgery or sleeve gastrectomy for treatment of obesity at the University of Alabama at Birmingham (IRB# F120815005) or were provided as exempt specimens by the National Disease Research Interchange (Philadelphia, PA; IRB# DB062615-EX).

Antibodies

The following mouse anti-human monoclonal antibodies were used for flow cytometry, imaging cytometry and confocal analysis of MoDCs: HLA-DR (clone L243), CD11c (B-ly6), CD103/αE (B-Ly7,) CD3 (HIT3a), CD19 (SJ25C1), CD45 (2D1), CD56 (MY31), E-cadherin (67A4), CD49d (9F10) purchased from eBioscience, Biolegend, or Tonbo, all San Diego, CA. Endosomal compartments were labelled with rabbit anti-human clathrin (D3C6, Cell Signaling, Danvers, MA), rabbit anti-human EEA-1 (polyclonal), mouse anti-human Rab7a (Rab7-117) and rabbit anti-human Rab11 (polyclonal), all from Abcam, Cambridge, MA. The following monoclonal antibodies were used for staining of paraffin-embedded tissue sections: anti-human HLA-DR (LN-3) and anti-human CD103 (EPR4166(2)), both Abcam, Cambridge, MA. The following antibodies were used in neutralization assays: anti-human CD103 (2G5) (Beckman

Coulter, France) and anti-human E-cadherin (SHE78-7) (Thermo Fisher Scientific, Waltham, MA). Appropriate isotype-matched control antibodies were used in all experiments.

Dendritic Cells

To obtain human gastric DCs, mucosal tissue was subjected to three rounds of EDTA treatment and then digested with collagenase solution to obtain lamina propria mononuclear cells, as described previously (Bimczok et al., 2015; Roe et al., 2017). Gastric DCs were then enriched using MACS sorting for HLA-DR$^+$ cells (Miltenyi Biotec, Auburn, CA).

To generate monocyte-derived DCs (MoDCs), PBMCs were isolated using Ficoll density gradient centrifugation, and MoDCs were differentiated from MACS-isolated CD14$^+$ blood monocytes by culturing 2x10^6 monocytes per well in 24-well plates in complete medium (DMEM, 10% heat-inactivated human AB serum and antibiotics) or serum-free medium (X-Vivo 10, with HEPES and L-Glutamine) supplemented with recombinant human GM-CSF (25 ng/mL) and IL-4 (17 ng/mL), both from R&D Systems, Minneapolis, MN (Bimczok et al., 2011; Roe et al., 2017). To enhance DC CD103 expression, 100 nM retinoic acid (RA, Sigma, St. Louis, MO) was added to some MoDC cultures, as described previously (Roe et al., 2017). Cytokines and RA were replenished after 3 days, and after 5 – 6 days, non-adherent cells were harvested as MoDCs by vigorous pipetting.

Immunofluorescent Labeling of Tissues and Cells for Microscopy

We used 4 μm paraffin-embedded sections to analyze CD103 expression by human gastric DCs *in situ.* Sections were de-paraffinized and then incubated in a vegetable steamer for 30 min in pre-heated Unmasking Solution (Vector Laboratories, Burlingame, CA) for antigen retrieval. Sections were then blocked in normal goat serum and incubated in the presence of primary antibodies overnight. Species specific secondary antibodies labelled with Alexa 488 or Alexa 555 (SouthernBiotech, Birmingham, AL) were added for 30 min. Finally, nuclei were stained with DAPI, and sections were mounted in Fluoroshield (Sigma-Aldrich) and sealed with nail varnish. For microscopic analysis of MoDCs, cells were stained either directly on glass-bottom plates or chamber slides (CD103 distribution and spreading assays) or were stained in suspension and then spotted onto glass slides (endosomal markers). For intracellular labeling, DCs were permeabilized with Cytofix/Cytoperm solution (Becton Dickinson) for 20 min at 4˚C, washed with PermWash buffer (Becton Dickinson) and then were incubated with antibodies for 30 min at 4˚C. Nuclei were labeled with DRAQ5 or DAPI.

Microscopy and Image Analysis

Immunofluorescence analysis of slides was performed on an Olympus BX60 upright fluorescence microscope equipped with a DS-Ri1 digital camera and with NIS Elements software (Nikon, Melville, NY) or on an EVOS FL Cell Imaging system (Thermo Fisher Scientific). Confocal microscopy images were acquired using a Zeiss LSM 510 META system, with a 63x objective and a step size of 0.5 μm, or an inverted Leica SP5 Confocal Scanning Laser Microscope (Leica, Wetzlar, Germany) with a 20x

objective or a 63x water immersion objective with Immersol (W 2010, Zeiss, Oberkochen, Germany). Digital image analysis was performed using ImageJ 1.48v software (Schneider et al. 2012). The distribution of $CD103^{+}$ DCs in relation to the epithelium in gastric tissue sections was determined by manual counting on digital images, using NIS Elements software. Three-dimensional co-localization of red (endosomal markers) and green (CD103) voxels in confocal stacks was determined using the JaCOP plugin to calculate Mander's co-localization coefficient (Bolte and Cordelieres, 2006). Part of this procedure involved screening 16 algorithms for optimal exclusion of background staining; the Bernsen method of auto-thresholding was chosen and applied objectively to all images (Dunn et al., 2011). In tissue sections, regions of interest were set to exclude surface and glandular epithelial cells.

FACS Analysis

For Flow cytometry, cells were labeled with pre-determined optimum concentrations of antibodies at 4°C for 15 min, followed by washing in FACS Stain Buffer (BD Biosciences). For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience), and antibodies we added in the presence of BD PermWash buffer. Dead cells were labelled with LIVE/DEAD® yellow dye (Life Technologies, Carlsbad, CA). A BD LSR or LSRII was used for flow cytometry; and data were analyzed using FlowJo V10 software (Treestar, Ashland, OR). Gastric DCs were gated as live $CD45^{pos}$/$lineage^{neg}$/$HLA\text{-}DR^{high}$ cells. The lineage cocktail contained antibodies to CD3, CD19, and CD56.

Imaging Cytometry

Imaging cytometry was performed using an ImageStreamX Mark II (Amnis Corp., Seattle, WA). Cells were prepared as described for FACS analysis, with 7-AAD or DAPI used to label nuclei in fixed cells. Data were analyzed with IDEAS software v6.1 (Amnis Corp.). The following channels were recorded: Ch 1 – brightfield, Ch 2 – CD103 FITC, channel 3 – CD11c PE, and channel 5 – 7-AAD or channel 7 – DAPI. DCs were gated as focused cells based on Gradient RMS Ch 1, single cells based on Aspect Ratio and Area Ch1, and Intensity of CD11c PE in Ch 3.

Internalization Assay

Relative rates of internalization of CD103 were determined in MoDCs using a variation of the method from Chen et al. (2014). Briefly, aliquots of MoDCs generated in the presence of retinoic acid (100 nM) were chilled to 4˚C, then incubated with FITC labeled mouse anti-human CD103 for 30 minutes. Unbound antibody was then removed by washing in ice cold media. Time zero samples were left on ice, and internalization was initiated in the remaining samples by resuspending the samples in 37˚C media and incubating them at that temperature for the indicated times. At each time point, samples were quickly washed in ice cold FACS buffer and left on ice to inhibit further internalization. After washing, cells were incubated for 30 minutes with anti-mouse IgG eFluor660 to label remaining cell surface anti-CD103 antibodies. Cells were then washed again in cold FACS buffer and analyzed with a BD LSR flow cytometer. Mean fluorescent intensities were normalized to the highest value of that fluorophore in each experiment.

Adhesion and Spreading Assays

For adhesion and spreading assays with recombinant E-cadherin, glass bottom 24-well or 96-well plates were coated with goat anti-human IgG at 5 μg/mL and incubated overnight at 4ºC. Non-adherent IgG was washed off with PBS + Ca^{2+}, and Fc-tagged recombinant human E-cadherin (Acro Biosystems, Newark, DE) at 2 μg/mL or an equal volume human serum was added to wells and incubated at room temperature for 1 hour. Alternatively, wells were coated directly with 0.2 μg/mL recombinant human E-cadherin (R&D systems, Minneapolis, MN) for 1 hour. After coating, wells were washed, blocked with human serum and washed again. MoDCs were incubated for 30 minutes with an anti-human CD103 neutralizing antibody (20 μg/mL), an appropriate isotype control antibody or were left untreated and then were added to the plates at $5x10^5$/mL. After incubation for 40 min at 37˚C, non-adherent cells were gently washed off and cells were fixed with Cytofix/Cytoperm (BD biosciences, San Jose, CA). MoDCs were blocked with 10% goat serum and stained with HLA-DR FITC, a secondary IgG2a FITC to enhance signal, and DAPI to label cell nuclei. Wells were then imaged on an EVOS FL Cell imaging system and analyzed using ImageJ.

To quantify DC adhesion to gastrointestinal epithelial cells, HT-29 cells were cultured on 48 well plates for 3 days at a starting concentration of $3x10^5$ per well to obtain a 100% confluency for co-culture. MoDCs generated in medium alone or in the presence of RA were harvested and pre-treated with DC culture medium containing one of the following for 30 minutes at 37ºC: 2 mM Mn^{2+}; 1 mM EGTA; 2 mM Mn^{2+} and 1 mM EGTA; anti-human CD103 (2 – 20 μg/mL); or anti-human E-cadherin (5 μg/mL).

MoDCs were next plated with the HT-29 monolayers at $2x10^5$ cells per well and incubated for 2 hours at 37ºC, with antibodies or other additives remaining in the culture media. Following incubation, non-adherent cells were gently washed off with media, and adherent MoDCs and HT-29 cells were harvested using 0.25% trypsin/1mM EDTA (Millipore, Darmstadt, Germany). Cells were then stained with HLA-DR FITC antibody, counting beads were added, and recovered MoDCs were quantified by flow cytometry. Experiments with <5% DC adhesion were excluded from the analyses, because it was difficult to interpret minor changes in adhesion when overall adhesion levels were extremely low. Experiments with low overall adhesion correlated neither with low DC CD103 or E-cadherin expression levels nor with a specific passage number or reduced E-cadherin expression of the HT-29 cells.

To quantify CD103 expression following MoDC HT-29 co-culture, a subset of MoDCs were incubated without HT-29 cells and subjected to the same 0.25% trypsin/1mM EDTA treatment. MoDCs incubated alone and those co-cultured with HT-29 cells were then stained for CD103 expression and analyzed by flow cytometry.

Statistical Analysis

Data were analysed using GraphPad Prism 6.05. Results are presented as mean ±SEM. Differences between values were analyzed for statistical significance by the two-tailed Student's *t*-test or one- or two-way ANOVA with appropriate post hoc analysis as indicated. Differences were considered significant at $P<0.05$.

Results

Gastric Intraepithelial DCs Contain a Significant CD103-Expressing DC Subset.

Flow cytometric analyses of gastric DCs have shown that $CD103^+$ DCs are rare in both human and murine stomach (Bimczok et al., 2015; Arnold et al., 2017; Roe et al., 2017; Viladomiu et al., 2017). Here, we used immunofluorescence analysis of human gastric tissue sections to analyze CD103 expression by gastric mononuclear phagocytes in more detail. Mucosal DCs, and possibly some macrophages, were identified based on high expression of HLA-DR in conjunction with an irregular cell morphology. Notably, our previous studies showed that gastric HLA-DR^{high} cells are $CD45^+$ leukocytes that express the DC-specific transcription factor zDC, but not the intestinal macrophage marker CD13 and that do not include B cells, T cells, mast cells or NK cells (Bimczok et al., 2010; Bimczok et al., 2015). Our immunofluorescence analyses confirmed that CD103 expression by HLA-DR^{high} cells in the gastric mucosa relatively rare (Fig. 1a). Likewise, the majority of $CD103^+$ cells, likely T lymphocytes, were negative for HLA-DR expression (Fig. 1a). However, individual $CD103^+$ HLA-DR^+ DCs were detected in close association with the gastric glandular epithelium, either at intraepithelial sites or directly below the epithelium (Fig. 1b,c). We next performed a quantitative analysis of the distribution of $CD103^–$ and $CD103^+$ DC subsets in relationship to the gastric epithelium by counting DCs at intraepithelial, subepithelial (with epithelial contact), and lamina propria sites (no epithelial contact) (Fig. 1d,e). Although intraepithelial HLA-DR^+ DCs represented <2% of all gastric mucosal DCs, a proportion ($P \leq 0.05$) of these cells expressed CD103. Specifically, 46.1% of intraepithelial gastric HLA-DR^+ cells were

Figure 1

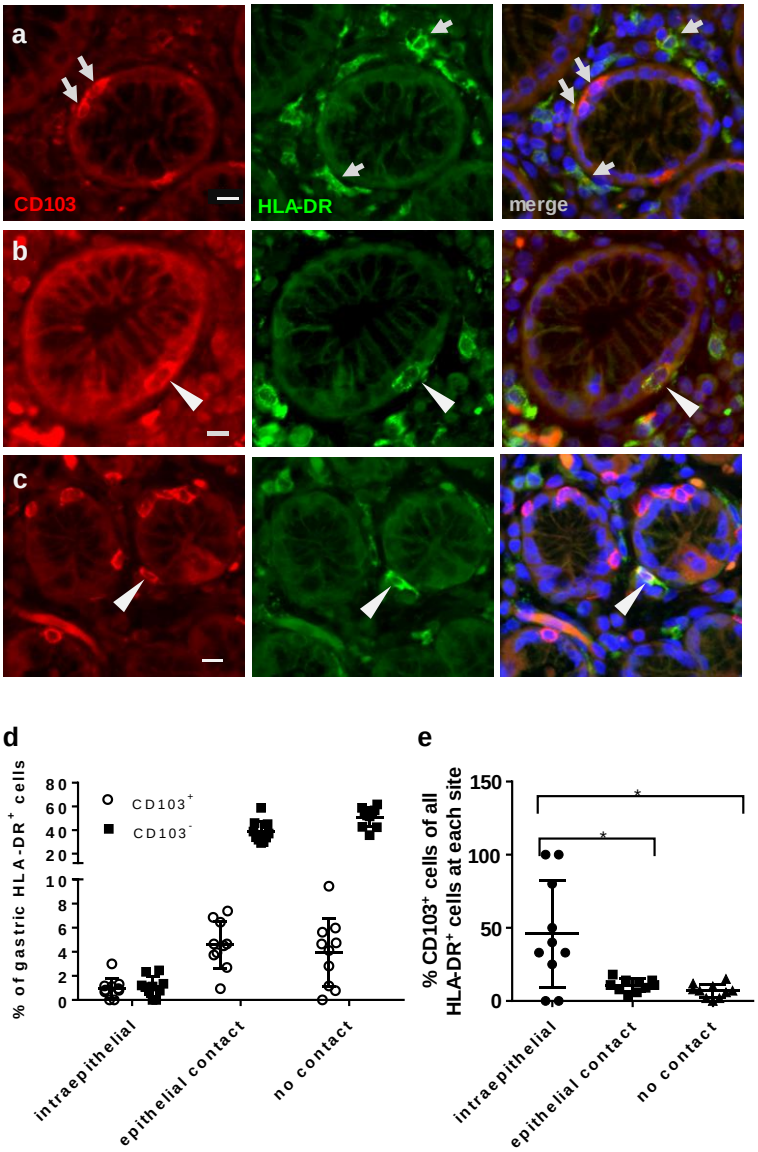

Figure 1: Distribution of CD103+ DCs in human gastric mucosa. Paraffin-embedded tissue sections from the gastric mucosa of non-H. pylori-infected human subjects was immunofluorescently labelled for HLA-DR (Alexa 488, green) and CD103 (Alexa 555, red). Nuclei were stained with DAPI. (a) High magnification single color and merged images of gastric mucosa with multiple cells positive for either CD103 or HLA-DR (arrows). (b) Occasional intraepithelial HLA-DR+ DCs and (c) subepithelial DCs with epithelial contact show staining for CD103. Arrowheads point out HLA-DR+ DCs that co-express CD103. Bar = 20 μm. (d,e) Quantitative analysis of HLA-DR+ DCs with and without CD103 expression at intraepithelial, subepithelial (with epithelial contact), and lamina propria sites (no epithelial contact). Tissue sections from 10 human subjects were analyzed. *P≤0.05, ANOVA with Tukey's post hoc test.

positive for CD103 compared to only 10.7% and 6.9% of $CD103^+$ DCs at subepithelial and lamina propria sites, respectively (Fig. 1e). These observations suggest that, in spite of an overall low expression of CD103 by human gastric DCs, CD103 might still contribute to DC interactions with the gastric epithelium in those DCs that are integrated into the epithelial cell layer.

Intracellular Expression of CD103 (αE integrin) in Human Monocyte-Derived and Gastric DCs.

Human monocyte-derived DCs (MoDCs) contain intracellular as well as surface-expressed CD103 (Roe et al., 2017). Having shown that gastric DCs express low levels of surface CD103 overall (Bimczok et al., 2015; Roe et al., 2017), but that surface CD103 expression is more frequent on gastric intraepithelial DCs (Fig. 1b,e), we hypothesized that intracellular CD103 pools may be recruited to the cell membrane to mediate binding to epithelial E-cadherin. Therefore, we next analyzed whether primary human gastric DCs also express intracellular CD103. As shown in Fig. 2a,c, significant levels of CD103 were detected in both gastric DCs and MoDCs when cells were permeabilized prior to immunolabeling, consistent with intracellular expression. In addition, intracellular CD103 expression was confirmed by imaging flow cytometry (Fig. 2d,e). In contrast, we did not observe significant intracellular expression of α4 integrin, in spite of high surface expression (Fig. 2b).

Using confocal microscopy, we detected CD103 in vesicular inclusions with a typical endosomal morphology in immature MoDCs with and without concurrent surface CD103 expression (Fig. 3a). A similar vesicular staining pattern for CD103 was also seen

Figure 2

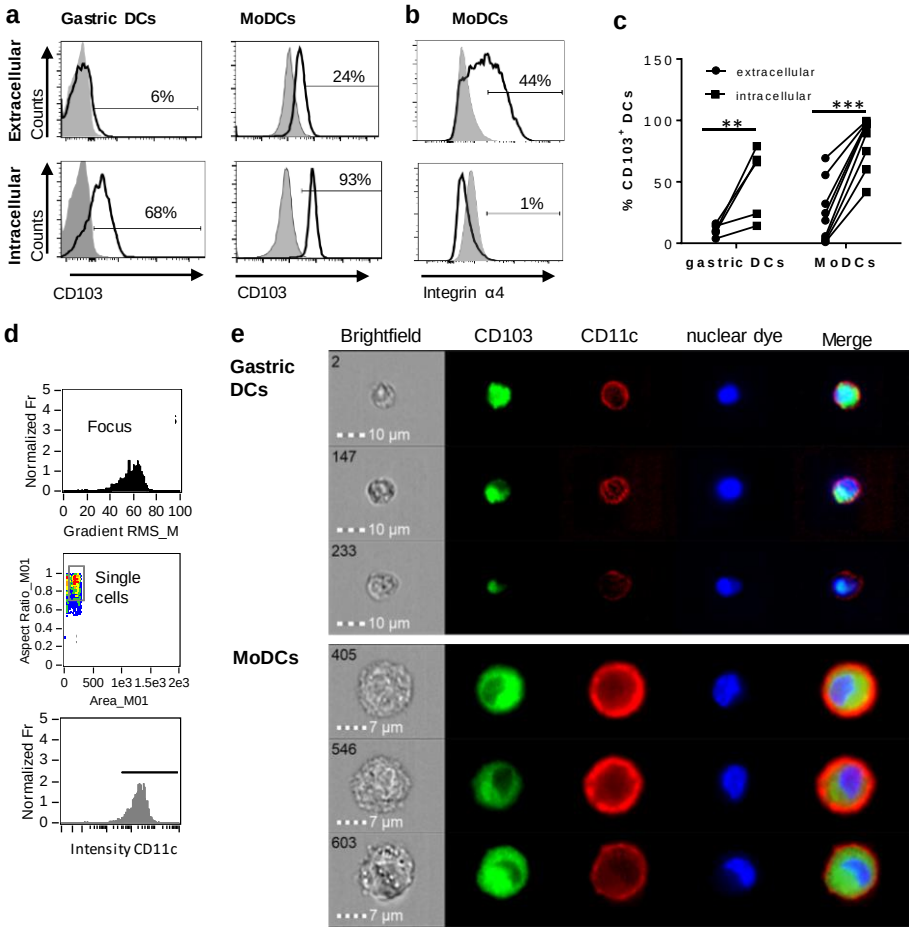

Figure 2: Human gastric and MoDCs contain intracellular CD103 pools. Human gastric DCs gated as live/ HLA-DR^{high}/CD45^{pos}/lineage^{neg} cells or MoDCs were labelled with (a) an anti-CD103 antibody or (b) an anti-α4 integrin antibody using extracellular or intracellular staining protocols. (a, b) representative histograms. and (b) individual values from 4 (gastric DCs) or 9 (MoDCs) independent experiments. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, ANOVA with Tukey's post hoc test. (**c**) Gating strategy for ImageStream imaging cytometry to select for focused, single cells with high CD11c expression. (d) Representative images from 3 independent imaging cytometry experiments show intracellular CD103 in CD11c^{+} gastric DCs and MoDCs, 40x objective. BF, bright field; nuclear dye, 7-AAD.

in DCs isolated from human gastric lamina propria (Fig. 3b). Thus, CD103 expression in endosomal compartments appears to be a common feature of human DCs.

Figure 3

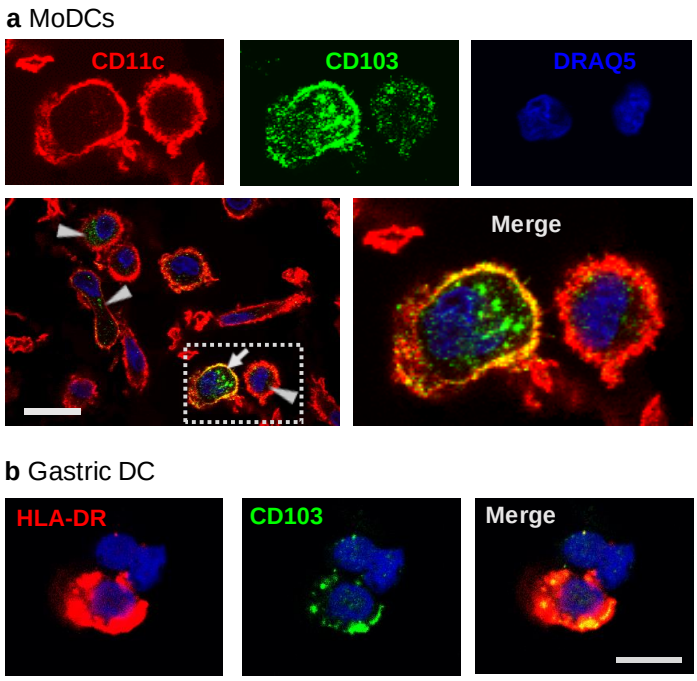

Figure 3: Confocal microscopy analysis shows endosomal expression pattern of CD103 in human monocyte-derived DCs. (a) Confocal images of human MoDCs that were permeabilized and labeled with antibodies to CD103 (Alexa488) and CD11c (Alexa555) Nuclei were labeled with DRAQ5. Arrowheads point to intracellular $CD103^+$ vesicles in DCs without surface CD103 expression, arrow indicates a DC with both surface and intracellular CD103 expression. Representative images from one of three similar experiments are shown. Bar: 20 µm. (b) Gastric lamina propria cells were permeabilized and labeled with antibodies to CD103 (Alexa488) and HLA-DR (Alexa555). Image shows a gastric $HLA\text{-}DR^+$ DC. Bar: 10 µm.

CD103 Partially Co-Localizes with Clathrin and Early, Recycling, and Late Endosomal Markers.

Previous studies have shown that integrins may undergo endosomal trafficking to allow dynamic interactions with their ligands and facilitate cell migration (Bridgewater et al., 2012; Rainero and Norman, 2013; Maritzen et al., 2015). To characterize the endosomal expression of CD103 in human DCs in more detail, we analyzed co-localization of CD103 with markers for endocytic uptake (clathrin), early endosomes

(early endosomal antigen-1, EEA-1), recycling endosomes (Rab11), and late endosomes (Rab7a) in MoDCs (Fig.4). In untreated MoDCs, 23.2 ± 1.2% of CD103 was co-localized with clathrin, corresponding to a Manders' co-localization coefficient of 0.232. Lower coefficients were detected for CD103 co-localization with Rab11 (16.7 ± 1.6%) and the late endosomal marker Rab7a (16.2 ± 1.2%), which targets endosomal cargo for lysosomal degradation (Guerra and Bucci, 2016). Only 6.6 ± 0.7% of CD103 co-localized with the early endosomal marker EEA-1. In control slides without primary antibodies, a co-localization co-efficient of 0.71 ± 0.69% was measured (data not shown). Interestingly, MoDCs treated with retinoic acid (RA) showed increased co-localization with clathrin (29.6 ± 1.3%; $P \leq 0.01$), but lower co-localization with Rab11 (12.6 ±1.1%; $P \leq 0.05$). The partial co-localization of CD103 with endosomal markers suggests that CD103 undergoes some endosomal trafficking in human DCs.

CD103 in Human MoDCs Undergoes Continuous Trafficking Through the Cell Membrane.

To functionally analyze whether DC CD103 undergoes endosomal recycling, we performed an internalization assay, as previously described by Chen et al. (2014). RA-treated MoDCs were used to achieve a high initial expression of CD103 on the DCs. DCs were then incubated for up to 40 min at 37˚C and were kept on ice at all other times to inhibit endosomal trafficking until all cells were collected, with the 0 min sample incubated on ice for the entire 40 min of the assay. As shown in Fig. 5, the level of CD103 on the cell surface as detected with the secondary reagent decreased significantly with prolonged incubation at 37˚C ($P \leq 0.01$), whereas total CD103 expression detected with the primary antibody remained constant. These observations are consistent with

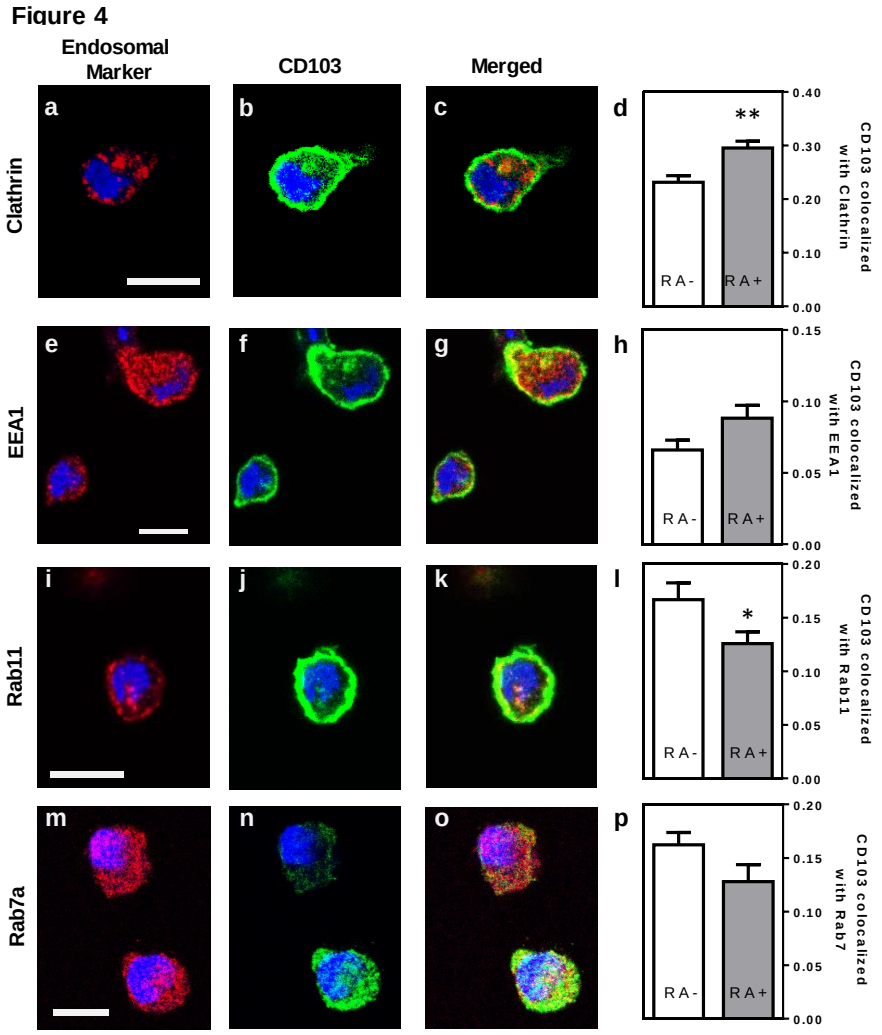

Figure 4: Partial co-localization of CD103 with endosomal markers in human MoDCs. MoDCs generated in medium alone or in the presence of retinoic acid (RA, 100 nM) were permeabilized and stained with antibodies to CD103 (green) and the following endosomal markers (red): (a-c) clathrin, (e-g) EEA-1, (i-k) Rab11, and (m-o) Rab7a. Images were obtained by confocal microscopy. Co-localization of CD103 with (d) clathrin, (h) EEA-1, (**l**) Rab11 and (p) Rab7a was calculated as the Manders' colocalization coefficient (M2) using ImageJ. Mean ± SEM of 10 – 17 confocal images obtained from 2 independent experiments. Bar = 20 μm, **P*≤0.05; ***P*≤0.01; unpaired Student's T-test.

internalization of surface expressed CD103 and indicate that CD103 undergoes endosomal recycling, as previously shown for α5β1 and α2β1 integrins (Rainero and

Norman, 2013; Chen et al., 2014). Thus, CD103 may be involved in dynamic binding of DCs to the epithelial layer in spite of low surface expression.

Figure 5

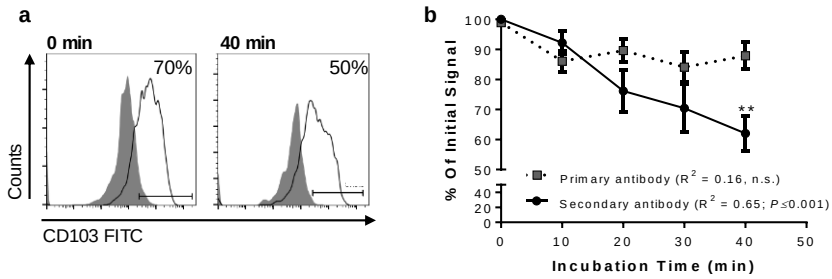

Figure 5: Internalization of surface-expressed CD103 in human MoDCs. Human MoDCs were labeled with an anti-CD103 FITC (mouse IgG1), washed, and chilled with ice-cold buffer. Time zero samples were left on ice, and internalization was initiated in the remaining samples by resuspending the samples in 37˚C media and incubating them at that temperature for the indicated times. Following incubation, cells were harvested with cold buffer and then left on ice until all samples were collected. Anti-CD103 antibody remaining on the surface of the DCs was detected with a secondary anti-mouse IgG1 antibody. (a) Representative FACS plots and (b) pooled data from 4 independent experiments show a significant decrease in surface CD103 after 40 min. 2-way ANOVA with Sidak's multiple comparisons.

Intracellular CD103 Engages in E-cadherin Binding, but does not Mediate DC Adhesion to Epithelial Cells.

Having shown that human DCs contain dynamic pools of intracellular CD103, we next asked whether intracellular CD103 may be recruited to the DC surface for E-cadherin binding. RA-treated or untreated DCs were incubated on glass-bottom plates coated with recombinant E-cadherin (Supplemental Fig. 1). The RA-treated DCs expressed increased levels of surface CD103 (Supplemental Fig. 2). We hypothesized that engagement of E-cadherin by DC CD103 would lead to an accumulation of CD103 staining close to the E-cadherin-coated glass surface (Fig. 6a). Our analysis of CD103 distribution across the vertical axis of the DCs showed a small, but significant shift in the proportion of CD103 close to the glass surface in RA-treated MoDCs in wells that were

coated with E-cadherin compared to serum alone (Fig. 6b, $P \leq 0.05$). However, this trend was not observed in MoDCs generated in the absence of RA.

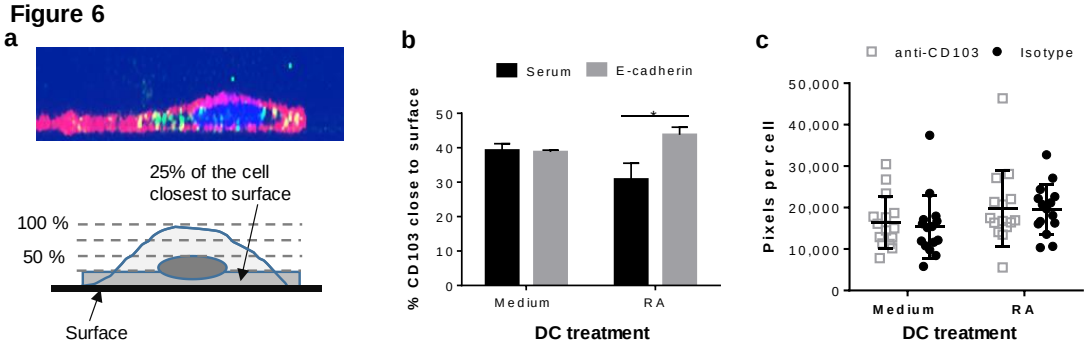

Figure 6: Adhesion of RA-treated MoDCs to E-cadherin-coated surfaces alters distribution of CD103. DC interactions with E-cadherin were analyzed by performing adhesion and spreading assays on glass slides coated with recombinant human E-cadherin. (a, b) RA-treated and untreated MoDCs were added to E-cadherin-coated slides and were incubated at 37˚C for 40 min. Cells were then fixed, permeabilized and stained for CD103 expression. Z-stack images (0.5 µm step size) of adherent MoDCs were collected by confocal microscopy and analyzed for the distribution of CD103 in relation to the glass surface. (a) Top panel: Orthogonal representation of an immunofluorescently labeled MoDC adhered to a glass surface and analyzed by z-stack confocal imaging. CD11c: red, CD103: green, DAPI nuclear stain: blue. Bottom panel: Graphical representation of the image analysis approach. (b) Summarized data from 3 independent experiments were analyzed. *$P \leq 0.05$, 1-way ANOVA with Tukey's post hoc test. (**c**) Spreading analysis of DCs on E-cadherin-coated glass slides. Glass slides were first coated with anti-human IgG antibodies and then with recombinant human E-cadherin-Fc. MoDCs generated in the presence of medium alone or 100 nM RA were pre-treated with a CD103 neutralizing antibody or an isotype control antibody for 30 min at 4˚C. Cells were then added to the slides in the presence of 2 mM Mn^{2+} and incubated at 37˚C for 40 min. Cell spreading was analyzed by fluorescence microscopy based on HLA-DR^{+} pixels per nucleus. Individual data points (n=15 areas), mean and SEM from one representative out of 3 independent experiments are shown.

On hard surfaces such as glass slides, adhesion complexes including integrin-dependent interactions influence cell spreading by enabling cells to extend actin-based lamellipodia (Vernerey and Farsad, 2014). A previous study had shown that K562 cells transfected with αE(CD103)β7 formed epithelial protrusions and migrated on E-cadherin-coated surfaces (Schlickum et al., 2008). To determine whether CD103 promotes

spreading and adhesion of human DCs on E-cadherin-positive surfaces, we used RA-treated and untreated MoDCs that were blocked with a CD103 neutralizing antibody or an isotype control antibody. As shown in Fig. 6c, RA-treated DCs showed a trend (P=0.05) for increased spreading on E-cadherin. However, DC spreading was not influenced by blocking CD103 on the DCs with a neutralizing antibody. The total number of adhered DCs also did not differ between treatments (data not shown). These results indicate that CD103 may relocate to the cell surface to engage in E-cadherin binding, but that overall DC adhesion to E-cadherin is largely independent of CD103.

Bivalent Cations Promote DC Adhesion to E-cadherin-Expressing Gastrointestinal Epithelial Cells.

To analyze the interactions between MoDCs and cell-expressed E-cadherin, we performed MoDC adhesion assays with HT-29 cells, a colonic epithelial cell line strongly positive for E-cadherin. Importantly, HT-29 cells have an atypical E-cadherin distribution that involves E-cadherin expression on the apical cell surface (Fig. 7a, b), but do not have any mutations in the E-cadherin gene *CDH1* (Efstathiou et al., 1999; Gout et al., 2004). DCs were incubated on top of HT-29 monolayers, and adherent cells were recovered by trypsinization after 2h (Fig. 7c). On average, 14.4 ± 3.7% of MoDCs were recovered from the cultures as adherent cells (Fig. 7d). To determine whether integrins including αE integrin (CD103) are involved in DC adhesion to the HT-29 cells, we added 1 mM manganese (Mn^{2+}), a strong activator of integrins that promotes ligand binding (Ye et al., 2012; Zhang and Chen, 2012). Both in RA-treated and untreated MoDCs, addition of Mn^{2+} significantly increased adhesion to HT-29 cells ($P \leq 0.001$ and $P \leq 0.05$, respectively). We also added EGTA, which inactivates bivalent cations such as Ca^{2+},

Mg^{2+} and Mn^{2+} that are involved in integrin- and E-cadherin-dependent interactions. EGTA significantly decreased the number of both and RA-treated and untreated DCs that were recovered from the co-cultures ($P \leq 0.05$ and $P \leq 0.01$, respectively). Although there was a trend for increased adhesion in RA-treated MoDCs, RA had no significant effect on DC binding to HT-29 cells, similar to our observations from the spreading analysis (Fig. 6). Interestingly, co-culture with the HT-29 slightly increased surface expression of CD103 on the MoDCs (Fig. 7e**,** $P = 0.056$).

Neutralization of DC CD103 does not Inhibit
Adhesion to E-cadherin Expressing Epithelial Cells.

To specifically assess the involvement of CD103 in the interactions between DCs and epithelial cells, RA-treated MoDCs were blocked with a CD103 neutralizing antibody prior to adding the cells to the HT-29 monolayer in the presence of Mn^{2+}. To avoid loss of blocking activity due to CD103 internalization, excess antibody was left in the cell culture medium during the adhesion assays. However, no decrease in DC adhesion to the HT-29 cells was seen with a wide range of antibody concentrations (Fig. 7f).

Homotypic E-cadherin Interactions may be Involved in DC
Binding to E-cadherin on Gastrointestinal Epithelial Cells.

To form adherens junctions, E-cadherin undergoes homotypic interactions with E-cadherin expressed on other cells (van Roy and Berx, 2008), and DCs have been shown

Figure 7

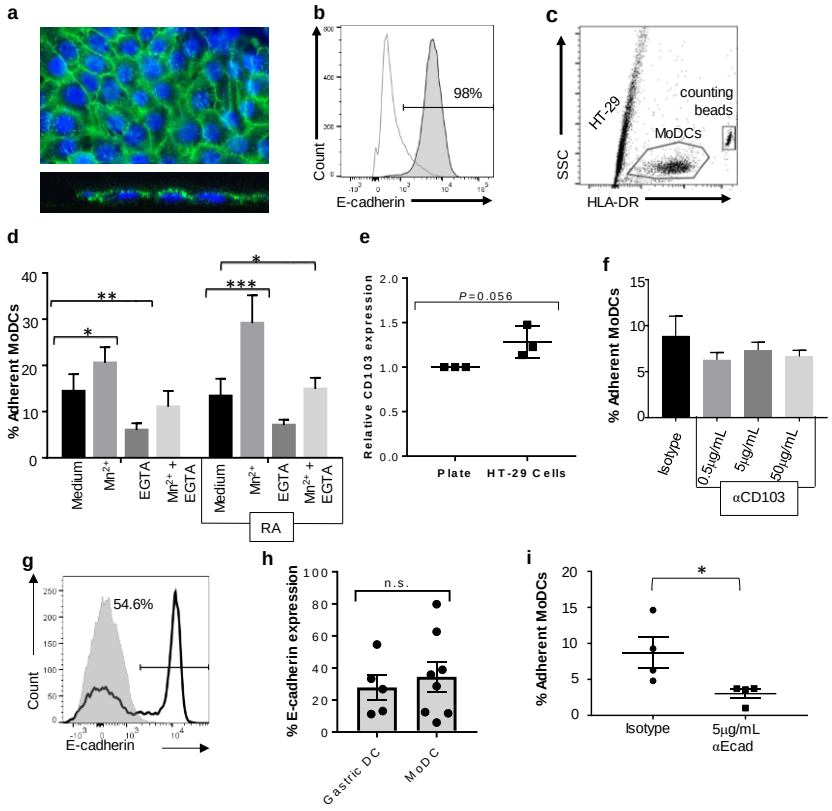

Figure 7: CD103 is not a major mediator of DC adhesion to E-cadherin-expressing epithelial cells. To analyze MoDC binding to E-cadherin expressed by epithelial cells, MoDCs were added to confluent monolayers of HT-29 cells for 2 h. Non-adherent cells were then removed by gentle washing, the remaining cells were collected by trypsinization, and the number of adherent DCs was determined using counting beads and HLA-DR-labeling of the DCs. (a) Confocal analysis of an HT-29 monolayer shows E-cadherin expression on the luminal surface. Top panel: maximum Z-projection; bottom panel: orthogonal view. Nuclei are labeled with DRAQ5 (blue). (b) Representative FACS histogram of E-cadherin expression on HT-29 cells. Grey line: isotype control; dark grey filled: anti-E-cadherin. (c) Gating strategy for counting adherent MoDCs following co-culture with HT-29 cells. (d) Percentage of MoDCs adherent to HT-29 cells in the presence of Mn^{2+}, EGTA, or Mn^{2+} + EGTA (n=6). RA indicates that cells were derived in the presence of 100 nM RA. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; 2-way ANOVA with Dunnett's multiple comparisons. (e)_Normalized CD103 expression on MoDCs after recovery from HT-29 co-cultures (with Mn^{2+}). Data from 3 independent experiments, paired, one-tailed Student's *t* test. (f) MoDCs were incubated with an isotype control antibody or the indicated concentrations of anti-CD103 antibody before and during co-culture with HT-29 cells, and the number of adherent DCs was determined. N=3, *$P \leq 0.05$; unpaired, two-tailed T-test. (g, h) Representative histogram and pooled data for E-cadherin expression on human gastric (n=5) and MoDCs (n=8). Continued on next page.

Figure 7 continued: (i) MoDCs were treated with anti-E-cadherin antibody before and during co-culture with HT-29 cells, and the number of adherent DCs was determined. N=4, *$P \leq 0.05$; unpaired, two-tailed Student's *t* test.

to express E-cadherin in previous studies (Jakob et al., 1999; Riedl et al., 2000; Van den Bossche et al., 2012). Our FACS analysis revealed that subsets of both human gastric DCs and MoDCs expressed E-cadherin (Fig. 7g,h), independent of RA treatment (Supplemental Fig. 2). Thus, E-cadherin-E-cadherin interactions may contribute to DC-epithelial cell interactions. Indeed, pre-treatment of the MoDCs with an E-cadherin neutralizing antibody significantly decreased DC adhesion to HT-29 monolayers (Fig. 7i). However, when we compared MoDC adhesion to HT-29 cells with adhesion to AGS cells, which lack E-cadherin expression, we found that a significantly higher number of DCs adhered to the AGS cells than the HT-29 cells (Supplemental Fig. 3, $P \leq 0.01$). Interestingly, binding to AGS cells decreased when DCs were generated in the presence of RA. Overall, these data suggest that both E-cadherin-dependent and integrin-dependent mechanisms contribute to DC binding to the gastrointestinal epithelium, but that CD103-E-cadherin interactions are only minor contributors.

Discussion

CD103 (αE integrin) is widely used as a maker for DC subsets in humans and mice (del Rio et al., 2010), but the functional role of CD103 for the DCs has attracted little investigative attention. We here sought to elucidate whether CD103 could mediate DC-epithelial cell interactions in the human gastric mucosa. A number of previous reports had speculated that CD103 might mediate adhesion of gastrointestinal DCs to E-

cadherin expressed in the epithelial layer (Hadley and Higgins, 2014; Watchmaker et al., 2014; Habtezion et al., 2016), similar to the mechanism shown for the retention of intraepithelial lymphocytes within the epithelial compartment (Cepek et al., 1994; Schon et al., 1999). Studies from our laboratory have shown that DCs in the gastric mucosa are exposed to RA generated by gastric epithelial cells, and that RA induces CD103 expression in human MoDCs (Bimczok et al., 2015; Roe et al., 2017). Our results from the present study suggest that CD103 is engaged upon binding of primary DCs to gastrointestinal epithelium, but is not a major mediator of adhesion.

One specific consideration when investigating CD103 in human gastric DCs was that less than 10% of the DCs expressed CD103 on their surface, as we have previously shown (Bimczok et al., 2015; Roe et al., 2017). However, based on the detection of intracellular CD103 (αE integrin), we hypothesized that these intracellular pools could be recruited to the cell surface for dynamic interactions with their ligands. Thus, integrins α5β1, α6β4, αMβ1 and α4β1 are continuously recycled through endosomal pathways during cell migration (Paul et al., 2015). However, not all integrins participate in the endocytotic cycle, and some integrins are recycled at only low rates (Bretscher, 1992). Indeed, we here confirmed that α4 integrin, which, like αE integrin, pairs with β7 integrin, was not expressed intracellularly. Our report is the first to demonstrate that CD103 in human DCs is expressed in endosomal compartments and recirculates through the cell membrane, suggesting that αE integrin recycling occurs in human DCs. It has been proposed that motile cells, such as DCs performing immunosurveillance functions, may require more trafficking of integrins and therefore contain a higher intracellular

proportion (Lobert et al., 2010). We demonstrated that 40% of surface CD103 was internalized in less than 1 hour. Moreover, 23 – 30% of CD103 co-localized with clathrin, consistent with the established role of clathrin in the endocytic recycling of integrin-mediated adhesions (Ezratty et al., 2009). A lower percentage of CD103 co-localized with the early endosome antigen 1 (EEA-1, 7%) and Rab11 (17%), a marker for long-loop endosomal recycling. Notably, previous publications have similarly reported co-localization co-efficients between 5% and 30% for integrins and endosomal markers in cells that were not specifically treated to enhance endosomal trafficking. Thus, Ezratty et al. (2009) reported that 20 – 25% of β1 integrin co-localized with Rab5. In a publication by the Goldenring group, a Manders' co-localization co-efficient of >0.2 (>20%) for co-localization of Rab25 with β1 and α5 integrins was considered high (Krishnan et al., 2013). Gu et al. (2011) from the Brenner lab analyzed co-localization of β3 integrin with endosomal markers and found between 2 – 8 % of co-localization with EEA-1, Rab4, 5 and 11 at baseline, with increased co-localization upon PDGF-stimulated micropinocytosis. Khandelwal (2010) used a functional endocytosis assay with fluorescently labeled cargo and detected co-localization co-efficients of 10% and 20% for the endosomal cargo with EEA-1 and Rab11, respectively, and Karjalainen (2008) analyzed co-localization of α2 integrin with caveolin and detected 5 – 10% of co-localization at baseline. Therefore, we consider our observed co-localization of CD103 with the endosomal markers to be biologically relevant. Co-localization of CD103/ integrin αE with clathrin, EEA1, and Rab11 suggests that integrin αE undergoes canonical trafficking similar to α5β1 integrin (De Franceschi et al., 2015). However, the

fact that CD103 also was co-localized with the late endosomal marker Rab7a may indicate that a proportion of intracellular CD103 is targeted for lysosomal degradation rather than recycling to the cell surface. Notably, when added together, less than 60% of all CD103 co-localized with any endosomal marker, suggesting that a significant proportion of CD103 is present at sites that are not endosomal compartments. These might represent newly synthesized CD103 molecules in the endoplasmic reticulum or in the Golgi apparatus. Interestingly, RA treatment of the MoDCs led to increased co-localization with clathrin and EEA-1, but decreased co-localization with Rab11 and Rab7a. Thus, RA seems to both upregulate CD103 expression (den Hartog et al., 2013; Roe et al., 2017) and alter its trafficking. Overall, our results suggest that, even on cells with low surface CD103 expression, CD103 may be recruited from endosomal pools for dynamic binding to epithelial E-cadherin or other ligands.

In support of a role for CD103 in human DC-epithelial interactions, we here showed significantly increased expression of CD103 on human HLA-DR$^+$ DCs that were integrated into the gastric epithelial layer. Moreover, Z-stack analysis of MoDCs bound to E-cadherin-coated glass slides showed significant re-distribution of DC-expressed CD103 to the E-cadherin positive interface, albeit only in RA-treated cells. In addition, *in vitro* adhesion assays to E-cadherin-expressing HT-29 cells revealed a dependence on bivalent cations including manganese, consistent with an integrin-dependent mechanism (Zhang and Chen, 2012). Conversely, when MoDCs were treated with a CD103 neutralizing antibody, adhesion to E-cadherin-positive HT-29 cells or to E-cadherin-coated glass slides was unaffected, arguing against a major role of CD103 in mediating

DC binding to the gastrointestinal epithelium. Notably, while internalization of CD103 with bound blocking antibody may have decreased the efficiency of the neutralization, this would not be expected to completely abrogate functional activity of the blocking antibody, especially since a high antibody concentration (5μg/mL) was used and additional antibody was present in the culture medium during the assay. Also, RA treatment, which increases MoDC CD103 expression, did not significantly influence MoDC binding to E-cadherin-expressing HT-29 cells or spreading on E-cadherin-coated glass surface, corroborating the results from the antibody neutralization experiments. Thus, adhesion of human DCs to gastrointestinal epithelia does not appear to be driven by CD103-E-cadherin interactions. Notably, we used high expression of HLA-DR to detect DCs in human gastric tissue sections, since no other more specific general DC marker has been identified for human stomach (Bimczok et al., 2010; Bimczok et al., 2011). In the murine gastric mucosa, CX_3CR1^+ $CD103^-$macrophages and $CD103^-$ DCs were able to sample *H. pylori* bacteria, whereas bacterial uptake by $CD103^+$ DCs could not be detected (Arnold et al., 2017), which also does not support our original hypothesis that CD103 positions gastric mononuclear cells at the epithelial interface for luminal *H. pylori* uptake. Along the same lines, an earlier report that investigated intraepithelial DCs in murine small intestine, the spatial relationship of murine intestinal DCs with the epithelium was not altered in CD103 knockout mice (Farache et al., 2013), whereas the number of IELs was significantly reduced in these animals (Taraszka et al., 2000). Together, these observations indicate that there are functional differences between T cell and DC-expressed CD103.

Our adhesion experiments did show that antibody inhibition of DC-expressed E-cadherin significantly suppressed DC binding to HT-29 cells, suggesting a role for homotypic E-cadherin-E-cadherin interactions. Thus, our results corroborate previous studies that showed Langerhans cells and other DCs of the skin and female genital tract interact with the epithelium through E-cadherin-E-cadherin binding (Tang et al., 1993; Jakob et al., 1999; Hubert et al., 2005). Notably, although homotypic E-cadherin interactions are calcium dependent, it appears that calcium can be replaced by the bivalent transitional elements manganese (Mn^{2+}) and cadmium (Cd^{2+}) (Lacaz-Vieira, 1997), which would explain the observed increase in adhesion in the presence of Mn^{2+}. The significant decrease in MoDC HT-29 adhesion in the presence of EGTA is consistent with either homotypic E-cadherin-E-cadherin interactions, which are calcium-dependent (Nagafuchi and Takeichi, 1988), or heterotypic E-cadherin interactions with αE integrin, which is activated by manganese (Mn^{2+}) (Ni et al., 1998). Since E-cadherin is widely expressed on mucosal epithelial cells, it is a likely candidate for mediating the retention of motile immune cells at the epithelial barrier, and additional heterotypic E-cadherin ligands with expression on DCs including killer cell lectin-like receptor G1 (KLRG1) have been identified (Cepek et al., 1994; Ito et al., 2006; Banh et al., 2009; Van den Bossche et al., 2012). Surprisingly, MoDC adhesion to E-cadherin negative AGS cells was significantly higher than adhesion to HT-29 cells. These results suggest that DC adhesion to the gastrointestinal epithelium may involve additional molecular interactions independent of CD103 or E-cadherin, such as tight junction proteins (Rescigno et al., 2001; Cavarelli et al., 2013) and other integrins. Notably, all myeloid cells including DCs

express β2 integrin (CD18). CD18 forms heterodimers with CD11a, CD11b, and CD11c (Schittenhelm et al., 2017) and contributes to cell-cell contact formation by binding to intracellular adhesion molecules (ICAMs), which may be expressed on gastric and intestinal epithelial cells (Crowe et al., 1995; Mane and Muro, 2012). Notably, adhesion of DCs to epithelial cells via β2 integrins would be consistent with our experimental observations that showed increased binding in the presence of Mn^{2+} and decreased binding in the presence of EGTA. Whether β2 integrins are major mediators of DC adhesion to the human gastric epithelium will be a subject of future studies.

If the interactions between DC-expressed CD103 and epithelial cells that we observed in human gastric tissue section do not result in strong adhesion, one might question the relevance of these interactions. However, engagement of CD103 by epithelial E-cadherin may lead to outside-in signaling through the cytoplasmic portion of the integrin (Shen et al., 2012). Previous studies have shown that in cytotoxic T cells, engagement of E-cadherin by CD103 triggers the phosphorylation of PLCγ1 and ERK1/2 (Le Floc'h et al., 2011), and engagement of CD103 by an anti-CD103 antibody can enhance T cell proliferation (Russell et al., 1994). Therefore, interactions between DC CD103 and epithelial E-cadherin could regulate certain DC functional characteristics through the activation of intracellular signaling cascades.

In summary, our study has provided novel insights in the regulation and function of CD103 (αE integrin) in human DCs. We show that, like other integrins in motile cells, CD103 undergoes endosomal trafficking, which likely enables dynamic interactions between CD103 and its ligands. Our results also corroborate previous reports (Farache et

al., 2013) that CD103 is not essential for the retention of DCs at gastrointestinal epithelial sites. The mechanisms by which CD103 on DCs in the human gastrointestinal tract interact with epithelial cells may be more subtle than simple adhesive interactions and requires further experimental exploration.

Ethics Statement

This study was carried out in accordance with the recommendations of the U.S. Department of Health and Human Services' Policy for Protection of Human Research Subjects (45 CFR 46) with written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Boards of Montana State University and the University of Alabama at Birmingham.

Author Contributions

DB and SS planned and oversaw the experiments. DB, SS, MR, BS, JD, TS, and RV performed the experiments. DB, PS, and LS initiated the project, critically discussed the data and obtained funding. DB, SS, and MR analyzed the data. MR, SS, and DB wrote the manuscript. All authors provided feedback on the manuscript.

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References Cited

Agace, W.W., and Persson, E.K. (2012). How vitamin A metabolizing dendritic cells are generated in the gut mucosa. *Trends Immunol* 33**,** 42-48.

Amieva, M., and Peek, R.M., Jr. (2016). Pathobiology of Helicobacter pylori-Induced Gastric Cancer. *Gastroenterology* 150**,** 64-78.

Arnold, I.C., Zhang, X., Urban, S., Artola-Boran, M., Manz, M.G., Ottemann, K.M., and Muller, A. (2017). NLRP3 Controls the Development of Gastrointestinal CD11b(+) Dendritic Cells in the Steady State and during Chronic Bacterial Infection. *Cell Rep* 21**,** 3860-3872.

Banh, C., Fugere, C., and Brossay, L. (2009). Immunoregulatory functions of KLRG1 cadherin interactions are dependent on forward and reverse signaling. *Blood* 114**,** 5299-5306.

Bimczok, D., Clements, R.H., Waites, K.B., Novak, L., Eckhoff, D.E., Mannon, P.J., Smith, P.D., and Smythies, L.E. (2010). Human primary gastric dendritic cells induce a Th1 response to H. pylori. *Mucosal Immunol* 3**,** 260-269.

Bimczok, D., Grams, J.M., Stahl, R.D., Waites, K.B., Smythies, L.E., and Smith, P.D. (2011). Stromal regulation of human gastric dendritic cells restricts the Th1 response to Helicobacter pylori. *Gastroenterology* 141**,** 929-938.

Bimczok, D., Kao, J.Y., Zhang, M., Cochrun, S., Mannon, P., Peter, S., Wilcox, C.M., Monkemuller, K.E., Harris, P.R., Grams, J.M., Stahl, R.D., Smith, P.D., and Smythies, L.E. (2015). Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells. *Mucosal Immunol* 8**,** 533-544.

Bolte, S., and Cordelieres, F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc.* 224**,** 213-232.

Bretscher, M.S. (1992). Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. *EMBO J* 11**,** 405-410.

Bridgewater, R.E., Norman, J.C., and Caswell, P.T. (2012). Integrin trafficking at a glance. *J Cell Sci* 125**,** 3695-3701.

Cavarelli, M., Foglieni, C., Rescigno, M., and Scarlatti, G. (2013). R5 HIV-1 envelope attracts dendritic cells to cross the human intestinal epithelium and sample luminal virions via engagement of the CCR5. *EMBO Mol Med* 5**,** 776-794.

Cepek, K.L., Shaw, S.K., Parker, C.M., Russell, G.J., Morrow, J.S., Rimm, D.L., and Brenner, M.B. (1994). Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* 372**,** 190-193.

Chen, P.W., Luo, R., Jian, X., and Randazzo, P.A. (2014). The Arf6 GTPase-activating proteins ARAP2 and ACAP1 define distinct endosomal compartments that regulate integrin alpha5beta1 traffic. *J Biol Chem* 289**,** 30237-30248.

Corps, E., Carter, C., Karecla, P., Ahrens, T., Evans, P., and Kilshaw, P. (2001). Recognition of E-cadherin by integrin alpha(E)beta(7): requirement for cadherin dimerization and implications for cadherin and integrin function. *J Biol Chem* 276**,** 30862-30870.

Crowe, S.E., Alvarez, L., Dytoc, M., Hunt, R.H., Muller, M., Sherman, P., Patel, J., Jin, Y., and Ernst, P.B. (1995). Expression of interleukin 8 and CD54 by human gastric epithelium after Helicobacter pylori infection in vitro. *Gastroenterology* 108**,** 65-74.

De Franceschi, N., Hamidi, H., Alanko, J., Sahgal, P., and Ivaska, J. (2015). Integrin traffic - the update. *J Cell Sci* 128**,** 839-852.

Del Rio, M.L., Bernhardt, G., Rodriguez-Barbosa, J.I., and Forster, R. (2010). Development and functional specialization of CD103+ dendritic cells. *Immunol Rev* 234**,** 268-281.

Den Hartog, G., Van Altena, C., Savelkoul, H.F., and Van Neerven, R.J. (2013). The mucosal factors retinoic acid and TGF-beta1 induce phenotypically and functionally distinct dendritic cell types. *Int Arch Allergy Immunol* 162**,** 225-236.

Dunn, K.W., Kamocka, M.M., and Mcdonald, J.H. (2011). A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol* 300**,** C723-742.

Efstathiou, J.A., Liu, D., Wheeler, J.M., Kim, H.C., Beck, N.E., Ilyas, M., Karayiannakis, A.J., Mortensen, N.J., Kmiot, W., Playford, R.J., Pignatelli, M., and Bodmer, W.F. (1999). Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells. *Proc Natl Acad Sci U S A* 96**,** 2316-2321.

Ezratty, E.J., Bertaux, C., Marcantonio, E.E., and Gundersen, G.G. (2009). Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. *J Cell Biol* 187**,** 733-747.

Farache, J., Koren, I., Milo, I., Gurevich, I., Kim, K.W., Zigmond, E., Furtado, G.C., Lira, S.A., and Shakhar, G. (2013). Luminal bacteria recruit CD103+ dendritic

cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity* 38**,** 581-595.

Gout, S., Marie, C., Laine, M., Tavernier, G., Block, M.R., and Jacquier-Sarlin, M. (2004). Early enterocytic differentiation of HT-29 cells: biochemical changes and strength increases of adherens junctions. *Exp Cell Res* 299**,** 498-510.

Gu, Z., Noss, E.H., Hsu, V.W., and Brenner, M.B. (2011). Integrins traffic rapidly via circular dorsal ruffles and macropinocytosis during stimulated cell migration. *J Cell Biol* 193**,** 61-70.

Guerra, F., and Bucci, C. (2016). Multiple Roles of the Small GTPase Rab7. *Cells* 5**,** E34.

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B.U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol* 14**,** 571-578.

Habtezion, A., Nguyen, L.P., Hadeiba, H., and Butcher, E.C. (2016). Leukocyte Trafficking to the Small Intestine and Colon. *Gastroenterology* 150**,** 340-354.

Hadley, G.A., and Higgins, J.M. (2014). Integrin alphaEbeta7: molecular features and functional significance in the immune system. *Adv Exp Med Biol* 819**,** 97-110.

Haniffa, M., Collin, M., and Ginhoux, F. (2013). Ontogeny and functional specialization of dendritic cells in human and mouse. *Adv Immunol* 120**,** 1-49.

Harris, P.R., Wright, S.W., Serrano, C., Riera, F., Duarte, I., Torres, J., Pena, A., Rollan, A., Viviani, P., Guiraldes, E., Schmitz, J.M., Lorenz, R.G., Novak, L., Smythies, L.E., and Smith, P.D. (2008). Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology* 134**,** 491-499.

Higgins, J.M., Cernadas, M., Tan, K., Irie, A., Wang, J., Takada, Y., and Brenner, M.B. (2000). The role of alpha and beta chains in ligand recognition by beta 7 integrins. *J Biol Chem* 275**,** 25652-25664.

Hitzler, I., Oertli, M., Becher, B., Agger, E.M., and Muller, A. (2011). Dendritic cells prevent rather than promote immunity conferred by a helicobacter vaccine using a mycobacterial adjuvant. *Gastroenterology* 141**,** 186-196, 196 e181.

Hubert, P., Caberg, J.H., Gilles, C., Bousarghin, L., Franzen-Detrooz, E., Boniver, J., and Delvenne, P. (2005). E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in cervical human papillomavirus-associated (pre)neoplastic lesions. *J Pathol* 206**,** 346-355.

Iliev, I.D., Mileti, E., Matteoli, G., Chieppa, M., and Rescigno, M. (2009a). Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol* 2**,** 340-350.

Iliev, I.D., Spadoni, I., Mileti, E., Matteoli, G., Sonzogni, A., Sampietro, G.M., Foschi, D., Caprioli, F., Viale, G., and Rescigno, M. (2009b). Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. *Gut* 58**,** 1481-1489.

Ito, M., Maruyama, T., Saito, N., Koganei, S., Yamamoto, K., and Matsumoto, N. (2006). Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. *J Exp Med* 203**,** 289-295.

Jakob, T., Brown, M.J., and Udey, M.C. (1999). Characterization of E-cadherin-containing junctions involving skin-derived dendritic cells. *J Invest Dermatol* 112**,** 102-108.

Kao, J.Y., Zhang, M., Miller, M.J., Mills, J.C., Wang, B., Liu, M., Eaton, K.A., Zou, W., Berndt, B.E., Cole, T.S., Takeuchi, T., Owyang, S.Y., and Luther, J. (2010). Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. *Gastroenterology* 138**,** 1046-1054.

Karjalainen, M., Kakkonen, E., Upla, P., Paloranta, H., Kankaanpaa, P., Liberali, P., Renkema, G.H., Hyypia, T., Heino, J., and Marjomaki, V. (2008). A Raft-derived, Pak1-regulated entry participates in alpha2beta1 integrin-dependent sorting to caveosomes. *Mol Biol Cell* 19**,** 2857-2869.

Khandelwal, P., Ruiz, W.G., and Apodaca, G. (2010). Compensatory endocytosis in bladder umbrella cells occurs through an integrin-regulated and RhoA- and dynamin-dependent pathway. *EMBO J* 29**,** 1961-1975.

Krishnan, M., Lapierre, L.A., Knowles, B.C., and Goldenring, J.R. (2013). Rab25 regulates integrin expression in polarized colonic epithelial cells. *Mol Biol Cell* 24**,** 818-831.

Lacaz-Vieira, F. (1997). Calcium site specificity. Early Ca2+-related tight junction events. *J Gen Physiol* 110**,** 727-740.

Le Floc'h, A., Jalil, A., Franciszkiewicz, K., Validire, P., Vergnon, I., and Mami-Chouaib, F. (2011). Minimal engagement of CD103 on cytotoxic T lymphocytes with an E-cadherin-Fc molecule triggers lytic granule polarization via a phospholipase Cgamma-dependent pathway. *Cancer Res* 71**,** 328-338.

Lobert, V.H., Brech, A., Pedersen, N.M., Wesche, J., Oppelt, A., Malerod, L., and Stenmark, H. (2010). Ubiquitination of alpha 5 beta 1 integrin controls fibroblast migration through lysosomal degradation of fibronectin-integrin complexes. *Dev Cell* 19**,** 148-159.

Mane, V., and Muro, S. (2012). Biodistribution and endocytosis of ICAM-1-targeting antibodies versus nanocarriers in the gastrointestinal tract in mice. *Int J Nanomedicine* 7**,** 4223-4237.

Maritzen, T., Schachtner, H., and Legler, D.F. (2015). On the move: endocytic trafficking in cell migration. *Cell Mol Life Sci*.

Moss, S.F., and Malfertheiner, P. (2007). Helicobacter and gastric malignancies. *Helicobacter.* 12 Suppl 1**,** 23-30.

Nagafuchi, A., and Takeichi, M. (1988). Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO J* 7**,** 3679-3684.

Necchi, V., Manca, R., Ricci, V., and Solcia, E. (2009). Evidence for transepithelial dendritic cells in human H. pylori active gastritis. *Helicobacter* 14**,** 208-222.

Ni, H., Li, A., Simonsen, N., and Wilkins, J.A. (1998). Integrin activation by dithiothreitol or Mn2+ induces a ligand-occupied conformation and exposure of a novel NH2-terminal regulatory site on the beta1 integrin chain. *J Biol Chem* 273**,** 7981-7987.

Paul, N.R., Jacquemet, G., and Caswell, P.T. (2015). Endocytic Trafficking of Integrins in Cell Migration. *Curr Biol* 25**,** R1092-1105.

Rainero, E., and Norman, J.C. (2013). Late endosomal and lysosomal trafficking during integrin-mediated cell migration and invasion: cell matrix receptors are trafficked through the late endosomal pathway in a way that dictates how cells migrate. *Bioessays* 35**,** 523-532.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., and Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2**,** 361-367.

Riedl, E., Stockl, J., Majdic, O., Scheinecker, C., Rappersberger, K., Knapp, W., and Strobl, H. (2000). Functional involvement of E-cadherin in TGF-beta 1-induced cell cluster formation of in vitro developing human Langerhans-type dendritic cells. *J Immunol* 165**,** 1381-1386.

Robinson, K., Kenefeck, R., Pidgeon, E.L., Shakib, S., Patel, S., Polson, R.J., Zaitoun, A.M., and Atherton, J.C. (2008). Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut* 57**,** 1375-1385.

Roe, M.M., Swain, S., Sebrell, T.A., Sewell, M.A., Collins, M.M., Perrino, B.A., Smith, P.D., Smythies, L.E., and Bimczok, D. (2017). Differential regulation of CD103 (alphaE integrin) expression in human dendritic cells by retinoic acid and Toll-like receptor ligands. *J Leukoc Biol* 101**,** 1169-1180.

Russell, G.J., Parker, C.M., Cepek, K.L., Mandelbrot, D.A., Sood, A., Mizoguchi, E., Ebert, E.C., Brenner, M.B., and Bhan, A.K. (1994). Distinct structural and functional epitopes of the alpha E beta 7 integrin. *Eur J Immunol* 24**,** 2832-2841.

Schittenhelm, L., Hilkens, C.M., and Morrison, V.L. (2017). beta2 Integrins As Regulators of Dendritic Cell, Monocyte, and Macrophage Function. *Front Immunol* 8**,** 1866.

Schlickum, S., Sennefelder, H., Friedrich, M., Harms, G., Lohse, M.J., Kilshaw, P., and Schon, M.P. (2008). Integrin alpha E(CD103)beta 7 influences cellular shape and motility in a ligand-dependent fashion. *Blood* 112**,** 619-625.

Schon, M.P., Arya, A., Murphy, E.A., Adams, C.M., Strauch, U.G., Agace, W.W., Marsal, J., Donohue, J.P., Her, H., Beier, D.R., Olson, S., Lefrancois, L., Brenner, M.B., Grusby, M.J., and Parker, C.M. (1999). Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J Immunol* 162**,** 6641-6649.

Scott, C.L., Aumeunier, A.M., and Mowat, A.M. (2011). Intestinal CD103+ dendritic cells: master regulators of tolerance? *Trends Immunol* 32**,** 412-419.

Segura, E. (2016). Review of Mouse and Human Dendritic Cell Subsets. *Methods Mol Biol* 1423**,** 3-15.

Serrano, C., Wright, S.W., Bimczok, D., Shaffer, C.L., Cover, T.L., Venegas, A., Salazar, M.G., Smythies, L.E., Harris, P.R., and Smith, P.D. (2013). Downregulated Th17 responses are associated with reduced gastritis in Helicobacter pylori-infected children. *Mucosal Immunol*.

Shen, B., Delaney, M.K., and Du, X. (2012). Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Curr Opin Cell Biol* 24**,** 600-606.

Shiu, J., and Blanchard, T.G. (2013). Dendritic cell function in the host response to Helicobacter pylori infection of the gastric mucosa. *Pathog Dis* 67**,** 46-53.

Tang, A., Amagai, M., Granger, L.G., Stanley, J.R., and Udey, M.C. (1993). Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* 361**,** 82-85.

Taraszka, K.S., Higgins, J.M., Tan, K., Mandelbrot, D.A., Wang, J.H., and Brenner, M.B. (2000). Molecular basis for leukocyte integrin alpha(E)beta(7) adhesion to epithelial (E)-cadherin. *J Exp Med* 191**,** 1555-1567.

Van Den Bossche, J., Malissen, B., Mantovani, A., De Baetselier, P., and Van Ginderachter, J.A. (2012). Regulation and function of the E-cadherin/catenin complex in cells of the monocyte-macrophage lineage and DCs. *Blood* 119**,** 1623-1633.

Van Roy, F., and Berx, G. (2008). The cell-cell adhesion molecule E-cadherin. *Cell Mol Life Sci* 65**,** 3756-3788.

Vernerey, F.J., and Farsad, M. (2014). A mathematical model of the coupled mechanisms of cell adhesion, contraction and spreading. *J Math Biol* 68**,** 989-1022.

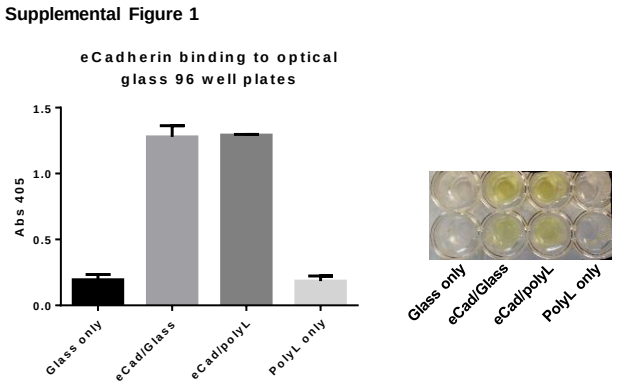
Viladomiu, M., Bassaganya-Riera, J., Tubau-Juni, N., Kronsteiner, B., Leber, A., Philipson, C.W., Zoccoli-Rodriguez, V., and Hontecillas, R. (2017). Cooperation of Gastric Mononuclear Phagocytes with Helicobacter pylori during Colonization. *J Immunol* 198**,** 3195-3204.

Watchmaker, P.B., Lahl, K., Lee, M., Baumjohann, D., Morton, J., Kim, S.J., Zeng, R., Dent, A., Ansel, K.M., Diamond, B., Hadeiba, H., and Butcher, E.C. (2014). Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. *Nat Immunol* 15**,** 98-108.

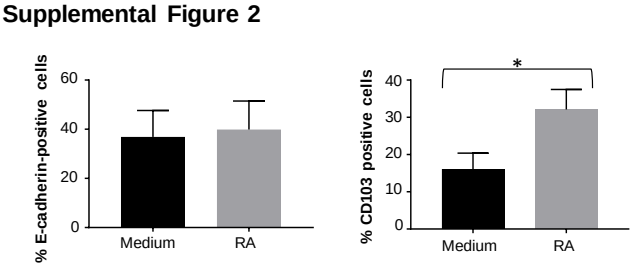
Ye, F., Kim, C., and Ginsberg, M.H. (2012). Reconstruction of integrin activation. *Blood* 119**,** 26-33.

Zhang, K., and Chen, J. (2012). The regulation of integrin function by divalent cations. *Cell Adh Migr* 6**,** 20-29.

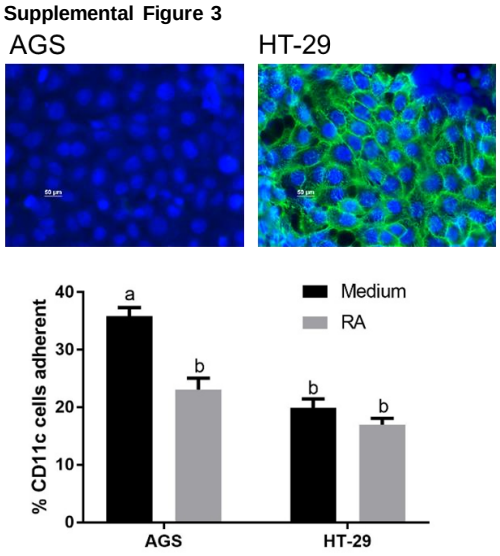
SUPPLEMENTAL FIGURES



Supplemental Figure 1: Coating of glass surfaces with rh E-cadherin. Untreated or poly-L lysine-coated wells of a glass bottom 96 well plate were incubated with recombinant human E-cadherin (1 µg/mL) in PBS for 60 min. Following a blocking step with 10 % human serum, and several washes, E-cadherin bound to the plate was detected using an anti-E-cadherin antibody and visualized using an alkaline phosphatase detection system. Left panel: Mean absorption (405 nm) ± SEM of duplicate wells. Right panel: Plate image.



Supplemental Figure 2: Retinoic acid significantly increases expression of CD103, but not E-cadherin in human MoDCs. MoDCs were treated with 100nM RA and surface expression of E-cadherin (left panel, n=12) and CD103 (right panel, n=13) were analyzed with flow cytometry. *$P \leq 0.05$; unpaired, two-tailed T-test.



Supplemental Figure 3: Strong adhesion of MoDCs to E-cadherin negative AGS cells. Top panel: Confocal analysis of an AGS and an HT-29 monolayer shows surface E-cadherin expression (green) by the HT-29, but not by AGS cells. Bottom panel: RA-treated or untreated MoDCs were added to confluent monolayers of AGS or HT-29 cells for 2 h. Non-adherent cells were then removed by gentle washing, the remaining cells were collected by trypsinization, and the number of adherent DCs was determined using counting beads and CD11c-labeling of the DCs. Mean ± SEM of 3 independent experiments. Data were analyzed by ANOVA with Tukey's post hoc test. a, b: different letters indicate significantly different values ($P \leq 0.05$).

CHAPTER 4

A NOVEL ROLE OF P38 MAPK SIGNALING AND NFAT IN THE RA-INDUCED EXPRESSION OF CD103 IN HUMAN DENDRITIC CELLS

Contribution of Authors and Co-Authors

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Manuscript Information

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Summary

Retinoic acid (RA) is an active derivative of vitamin A and a key regulator of immune cell function. In dendritic cells (DCs), RA drives the expression of CD103 (integrin αE), a functionally relevant mucosal DC subset marker. However, the molecular pathway leading to the expression of CD103 in DCs has not been elucidated. We here demonstrate a novel role of p38 MAPK signaling and NFAT in the RA-induced expression of CD103 in human DCs. Induction of CD103 by RA was specific to myeloid cells, as both DCs and macrophages, but not lymphocytes, upregulated CD103 in response to RA. We showed that RA-induced induction of CD103 was largely mediated by RARα, since only treatment with a RARα agonist but not RARβ and RARγ agonists significantly increased CD103 and integrin β7 expression. Additionally, RA-induced upregulation of CD103 was abrogated in DCs treated with RARα siRNA. Western blot analysis of RA-treated DCs showed a significant upregulation of p38 MAPK phosphorylation, whereas DCs cultured with RA and a p38 MAPK inhibitor had a significantly reduced expression of CD103 compared with DCs cultured with RA only. These data indicate that p38 MAPK is involved in RA-driven CD103 regulation. We explored a role of NFAT as a candidate transcription factor downstream of p38 MAPK and demonstrated that an NFAT inhibitor significantly decreased CD103 expression in RA-treated MoDCs. In summary, these findings suggest that the RA-induced expression of CD103 is mediated through RARα and p38 MAPK signaling and is dependent on NFAT.

Introduction

Retinoic acid (RA) is an active derivative of vitamin A and plays substantial roles in many biological systems and signaling networks. RA is important in embryonic development, development and maintenance of eye health, and is a major regulator of the immune system (1). Although the incidence of Vitamin A deficiency (VAD) in the US is rare, VAD can be a problem in developing countries (1). VAD can lead to many ill health consequences, including blindness and immune deficiency (1). VAD has been linked to an increase in morbidity to diarrheal disease and measles (2, 3).Within the immune system, RA plays important roles in the homing of immune cells to mucosal sites (4-6), induction of regulation that maintains homeostasis (7, 8), the induction of IgA secreting B cells (9, 10), and establishment of dendritic cell (DC) populations(4, 5).

RA is essential in the establishment and maintenance of the DC populations within the intestinal mucosa (4, 5, 7). DCs are professional antigen presenting cells that mediate the activation of adaptive immune responses. VAD has been shown to reduce cDC2 populations (4, 7), suggesting that RA is required for the maintenance of this cell population. Intestinal cDC2s are a population of DCs that express CD11b, are dependent on IRF4 for development, and have the capacity to activate both regulatory T cells and inflammatory T cell responses (8, 11-13). Multiple studies have shown that the development of these cDC2 populations is dependent on RA signaling through RA receptor alpha (RARα) (4, 7).

RA exhibits pleotropic effects through genomic and non-genomic signaling pathways (14-17). Genomic RA signaling includes the transcription of genes by direct

binding of RARs to RA response elements (RARE) (18-20). Additionally, when RA is not present, RARs bound to DNA can act as corepressors interfering with histone modifications (19, 21). Recent findings suggest that RA treatment can induce swift chromatin remodeling through conformational changes of RARs (20, 22). Although RARs are generally considered nuclear transcription factors that directly regulate gene expression, recent studies have demonstrated that RA can also elicit non-genomic effects, including the phosphorylation of signaling proteins including p38 MAPK and ERK1 (15, 17, 23, 24).

Analyses from our laboratory and others have demonstrated that RA drives the expression of CD103 (αE integrin) on human DCs (25, 26). In murine models, $CD103^+$ DCs sample luminal antigens and migrate to the lymph node to present antigen to T cells (27), indicating that $CD103^+$ DCs play an important role in the immune response to infection. $CD103^+$ DCs have also been associated with the maintenance of homeostasis in mucosal sites (8) through the capacity to biosynthesize RA and in turn supply RA to other cell types (26, 28, 29). This has been well established in the interactions between $CD103^+$ DCs and regulatory T cells within the lymph nodes (25, 30).

Currently, the signaling pathway involved in RA-dependent CD103 and β7 expression remains unknown. TGF-β can induce the expression of CD103 on T cells (31), but does not have the same effect on DC CD103 expression (25, 26). Interestingly, when TGF-β signaling is impaired, the RA-induced effect on CD103 expression is diminished (26). This suggests that the RA and TGF-β signaling pathways may be intertwined in the RA-induced expression of CD103.

We here have investigated the molecular signaling pathways that drive RA-dependent expression of CD103 and integrin β7 in human DCs. Our experiments showed that the RA-induced expression of CD103 was dependent on signaling through RARα and p38 MAPK. Additionally, inhibition of NFAT diminishes the expression of CD103 in DCs in the presence of RA, pointing to NFAT as the downstream target of p38 MAPK in RAR-dependent signaling. These observations suggest that RA may impact CD103 expression through non-genomic pathways that involve p38 MAPK signaling and NFAT activation.

Materials and Methods

Human Donors

Whole blood was obtained from healthy, adult volunteers with approved consent. Collection of human blood samples was approved by Montana State University's Institutional Review Board (IRB), protocol #DB082817.

MoDC Isolation and Culture

$CD14^+$ monocytes were isolated from heparinized whole blood by centrifugation and MACS sorting, as previously shown (26). Monocytes were cultured in X-vivo media (Lonza, Basel, Switzerland) supplemented with 100 U/l penicillin, 100 µg/l streptomycin, 50 µg/ml gentamycin, 5mM Hepes, and 2 mM L. glutamine (all Hyclone, Logan, UT, USA) with 25 ng/ml rhGM-CSF and 7 ng/ml rhIL-4 (R&D Systems, Minneapolis, MN, USA) or 25 ng/ml rhM-CSF (PeproTech, Rocky Hill, NJ, USA). Aliquots of all monocyte samples were tested for TNFα secretion after 24 hours. Monocytes with

spontaneous TNFα secretion were considered pre-activated and thus excluded from the analyses. Retinoic acid (RA, 100 nM) (Sigma-Aldrich, St. Louis, MO, USA) and/or other chemical inhibitors/agonists were added to the MoDC cultures as specified. All RA-treated cells were handled under red light to prevent RA degradation.

Reagents and Antibodies

MoDCs were cultured for 3 days with 10 μM RARα (AM80), 1-10 μM RARβ (CD2314), or 1-10 μM RARγ (CD437) agonists (all Tocris Bioscience, Bristol, UK) or RAR antagonists, 1-50 nM BMS195614 (Tocris Bioscience) and 60 nM R041-5253 (Sigma-Aldrich). MoDCs were harvested and analyzed by FACS for CD103 and integrin β7 expression. The p38 MAPK inhibitor, SB202190, (Sigma-Aldrich) was added to MoDCs at a concentration of 10 μM following 3 days of culture. MoDCs were incubated at 37ºC for 1 hour before addition of RA. After 24 hours, MoDCs were harvested for FACS and RT-qPCR analysis of CD103 and integrin β7 expression. MoDCs were cultured for 3 days prior to the addition of 10-50 μM NFAT peptide inhibitor (Tocris Bioscience) for 1 hour at 37ºC before the addition of RA. MoDCs were harvested after 24 hours for FACS analysis of CD103 expression. The following antibodies were used for flow cytometry: CD103 (eBioscience, Inc., San Diego, CA, USA), integrin β7 (eBioscience, Inc.), CD3 (BD biosciences, San Jose, CA, USA), CD4 (Tonbo Biosciences, San Diego, CA, USA), and phosflow SMAD2/3 (BD biosciences). The following antibodies were used for western blot analysis: p38 MAPK, pThr180/182 p38 MAPK, and GAPDH (all Cell Signaling, Danvers, MA, USA).

RARα siRNA

siRNA specific to RARα was used to inhibit RARα expression in MoDCs. MoDCs were differentiated for three days before treatment. MoDCs were resuspended in BTX electroporation solution (BTX, Holliston, MA, USA) and 5 μg RARα siRNA or control scramble siRNA (Santa Cruz Biotechnology, Dallas, TX, USA). MoDCs were electroporated using program U002 on Nucleofector™ 2b device, (Lonza) for eukaryotic cells. MoDCs were immediately added to pre-warmed media and incubated for 24 hours before the addition of RA. Following 24 hours of culture with RA, MoDCs were harvested for reverse transcriptase-qPCR (RT-qPCR) analysis of RARα, CD103, and integrin β7 expression.

Western Blot

MoDCs cultured in the presence of RA for 3 days were harvested for western blot into RIPA lysis buffer for 30 minutes at 4ºC, then centrifuged at 10,000g for 15 minutes. Supernatant was recovered and mixed with running buffer (Bio-Rad Laboratories, Hercules, CA, USA). Protein was quantified with a BCA assay following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Equal quantities of protein, 1-3μg per lane, were run on a 10% SDS-PAGE gel for 1 hour at 140 V. Protein was transferred to a PVDF membrane (Bio-Rad Laboratories) for 40 minutes at 100 V. Membranes were blocked with TBST, 5% BSA, sodium fluoride, proteinase inhibitors, and phosphatase inhibitors (both Thermo Fisher Scientific) . Membranes were incubated overnight with the primary antibody, washed, and then incubated with the secondary antibody conjugated with horseradish peroxidase for 1 hour at room

temperature. Membranes were developed with SuperSignal™ West Pico PLUS (Thermo Fisher Scientific) per manufacturer's specifications and then were read on a FluorChemR system (Protein Simple, San Jose, CA, USA).

RT-qPCR

RNA extraction from MoDCs was performed with a Directzol kit (Zymo Research, Irvine, CA, USA) as per manufacturer's specifications. RNA was quantified using a Nanodrop1000 (Thermo Fisher Scientific). An iScript™ cDNA synthesis kit (Bio-Rad Laboratories) was used to generate cDNA. TaqMan mastermix and primer/probes (Thermo Fisher Scientific) for each gene of interest and GAPDH housekeeping gene were used for qPCR, as previously described (26). Samples were amplified on a Lightcycler®96 (Roche Holding AG, Basel, Switzerland). Samples were amplified by the following protocol: 1 cycle for UNG activation at 50ºC for 2 minutes. 1 cycle for hot start at 95ºC for 10 minutes. 40 cycles of amplification as follows: 95ºC for 15 seconds, 60ºC for 1 minute. Fluorescence was measured after each of these cycles. Data analysis was performed using the Pfaffl method (32).

Flow Cytometry

MoDCs were stained with the antibodies listed above. Isotype controls for each antibody were used to control for non-specific binding. We used a live/dead yellow fixable stain for viability (Invitrogen, Carlsbad, CA, USA). Following antibody staining, MoDCs were fixed with Cytofix (BD Biosciences) and run on an LSR II or an LSR Fortessa Flow Cytometer (BD Biosciences). FACS data were analyzed using FlowJo X software (Tree Star, Ashland, OR, USA). MoDCs were gated based upon size, single

cells, and live cells. For intracellular staining of phosflow SMAD2/3, MoDCs were cultured for 3 days, harvested, and counted. Cells were allowed to rest for 2 hours prior to addition of RA, 5 ng TGF-β1 (R&D Systems), or TGF-β1 and 50 μM TGF-βRII inhibitor, SB431542 (Tocris Bioscience) for 30 minutes. Pre-warmed cytofix (BD Biosciences) was added to cells and incubated at 37ºC for 10 minutes. Cells were washed and resuspended in Perm Buffer III (Fisher Scientific) for 30 minutes at 4ºC. Cells were stained with antibody or isotype.

Statistics

Data were analyzed using Graphpad Prism 8.2 (GraphPad Software, San Diego, CA, USA). Results are presented as means ± SEM. Differences between values were analyzed for statistical significance by the 2-tailed Student's *t* test or ANOVA with Tukey's post hoc test. Differences were considered significant at $P < 0.05$.

Results

The RA-Induced Expression of CD103 is Conserved in the Human Mononuclear Phagocyte Compartment.

Our previous research has demonstrated that RA and not TGF-β1 or TGF-β2 is the driver of CD103 expression in human MoDCs (26), whereas previous studies have shown that TGF-β2 induces CD103 expression on T cells (31). We here compared the effect of RA on the expression of CD103 in human DCs, macrophages, and T cells. MACS $CD14^+$ sorted monocytes were treated with RA and either M-CSF (MoM) to induce differentiation of the monocytes to a macrophage phenotype or GM-CSF and IL-4 (MoDC) to induce differentiation of the monocytes to a DC phenotype. Interestingly,

monocyte-derived macrophages (MoMs) cultured with RA showed enhanced upregulation of CD103 expression compared with MoDCs (Fig. 1A,B). We cultured monocyte-depleted PBMCs with RA, TGF-β1, or TGF-β2. Following 24 hours in culture, the cells were harvested and stained for T cell markers and CD103. Gating strategies were used to identify $CD4^+$ and $CD8^+$ T cell populations (Fig. 1C). We here demonstrate that $CD8^+$ T cells showed minimal increased expression of CD103 in response to both TGF-β and RA (Fig. 1E,F). In contrast, gated $CD4^+$ T cells showed no change in CD103 expression in response to any of the treatments (Fig. 1D,F). Taken together, these data suggest that the RA-induced expression of CD103 is conserved in the mononuclear phagocyte compartment in humans.

RA-Induced Expression of CD103 is Mediated Through RARα in Human MoDCs.

RA signaling can be mediated through different forms of retinoic acid receptors (RARs), RARα. RARβ, or RARγ, all of which form heterodimers with the retinoid X receptor (RXR) (bastein). In order to elucidate the RA signaling pathway involved in RA-induced CD103 expression, we used agonists and antagonists of the different RAR forms. Treatment of MoDCs with AM80, a RARα-specific agonist, induced a similar CD103 protein expression as treatment with 100 nM of RA, with a significantly increased CD103 expression as compared with MoDCs cultured in media alone (Fig. 2A,B). CD103 (integrin αE) generally forms heterodimers with integrin β7, and we have previously shown that CD103 and integrin β7 have similar expression patterns when MoDCs were cultured with RA (26). Consistent with our earlier observations, integrin β7 expression

followed a similar increase in CD103 expression when MoDCs were cultured with RA or AM80 (Fig. 2C).

Figure 1

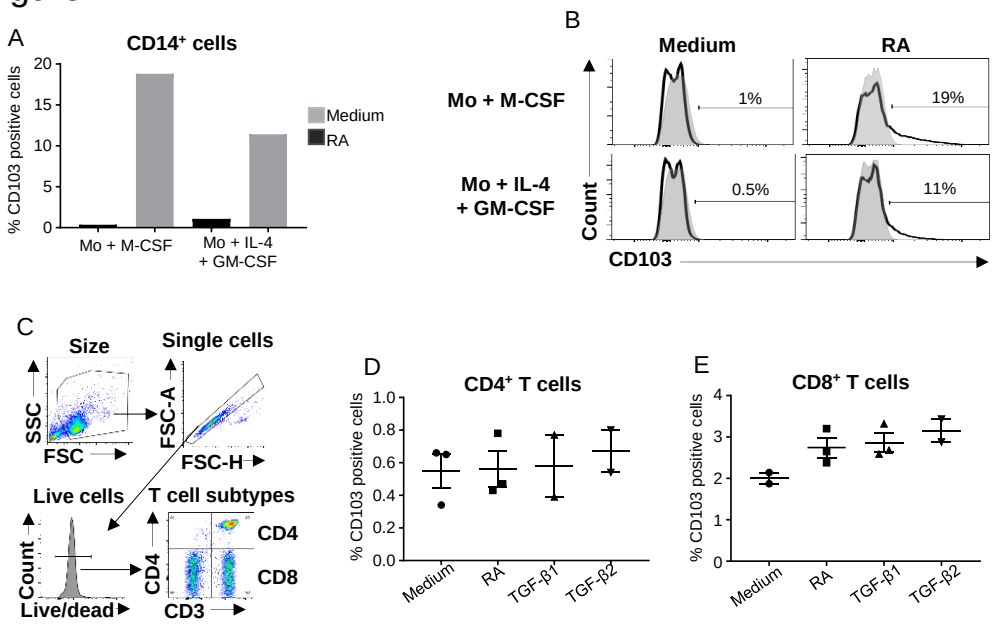

Figure 1: The RA-induced expression of CD103 is conserved in the human mononuclear phagocyte compartment. A) $CD14^+$ monocytes (Mo) were cultured with RA and either M-CSF to differentiate to macrophages or were cultured with GM-CSF and IL-4 to differentiate to DCs for 24 hours. CD103 expression was measured by FACS analysis (n=1). B) Representative FACS histogram of CD103 expression in $CD14^+$ Mos. Isotype control-grey shaded, CD103 Ab-black solid line. C-F) Flow-through from CD14 MACS was cultured with or without RA, TGF-β1, or TGF-β2 for 24 hours. C) FACS gating strategy to identify $CD4^+$ and $CD8^+$ T cells. D-E) CD103 expression was analyzed by FACS for $CD4^+$ T cells (D) and $CD8^+$ T cells (E) (n=3 for medium and RA treatment, n=2 for TGF-β1 and TGF-β2 treatment). F) Representative FACS histogram of CD103 expression in T cells cultured with RA, TGF-β1, or TGF-β2. Isotype control-grey shaded, CD103 Ab-black solid line.

We next used agonists of RARβ and RARγ to evaluate any role these two RARs had in the RA-induced expression of CD103. MoDCs cultured with CD2314, a RARβ-specific agonist, showed a minimal increase in CD103 (Fig. 2D,F). MoDCs cultured with CD437, a RARγ-specific agonist, had a slight increase in CD103 expression, but not to

the extent of MoDCs cultured with RA (Fig. 2D,F). Conversely, the expression of integrin β7 was not increased in the presence of the RARβ and RARγ agonists (Fig. 2E).

In order to confirm the involvement of RARα in the RA-induced expression of DC CD103, we also used siRNA specific to RARα to block RARα signaling in MoDCs. Notably, experiments with two different RARα inhibitors led to a paradoxical increase in CD103 expression (Suppl. Fig. 1). Therefore, MoDCs were differentiated for 3 days and then treated with RARα siRNA or a non-specific scramble siRNA. RA was added to the MoDCs 24 hours following siRNA treatment and harvested 24 hours after RA treatment. RARα siRNA reduced the *RARA* mRNA as expected (Fig. 2G). MoDCs treated with RARα siRNA and RA showed a significant decrease in *ITGAE* (gene name of CD103) mRNA expression compared with MoDCs treated with RA and scramble siRNA (Fig. 2H). In addition, this same pattern was observed in the *ITGB7* (gene name of integrin β7) mRNA, with a decrease in *ITGB7* expression with MoDCs treated with RARα siRNA and RA compared with scramble siRNA and RA (Fig. 2I). These data suggest that the RA-induced expression of CD103 in human DCs is mediated through RARα signaling.

TGF-β1 but not RA Drives Phosphorylation of SMAD2/3 in Human MoDCs.

Our lab has previously shown that blocking the TGF-β signaling pathway disrupts the RA-induced increase in CD103 expression in DCs (26). However, the addition of TGF-β1 or TGF-β2 did not have an effect on CD103 expression (26). Previous studies have demonstrated that there can be interdependency between RA and TGF-β signaling (26, 33). We here evaluated whether RA could induce the phosphorylation of SMAD2/3 in DCs, since SMAD2/3 activation is the major signaling pathway downstream of the

Figure 2

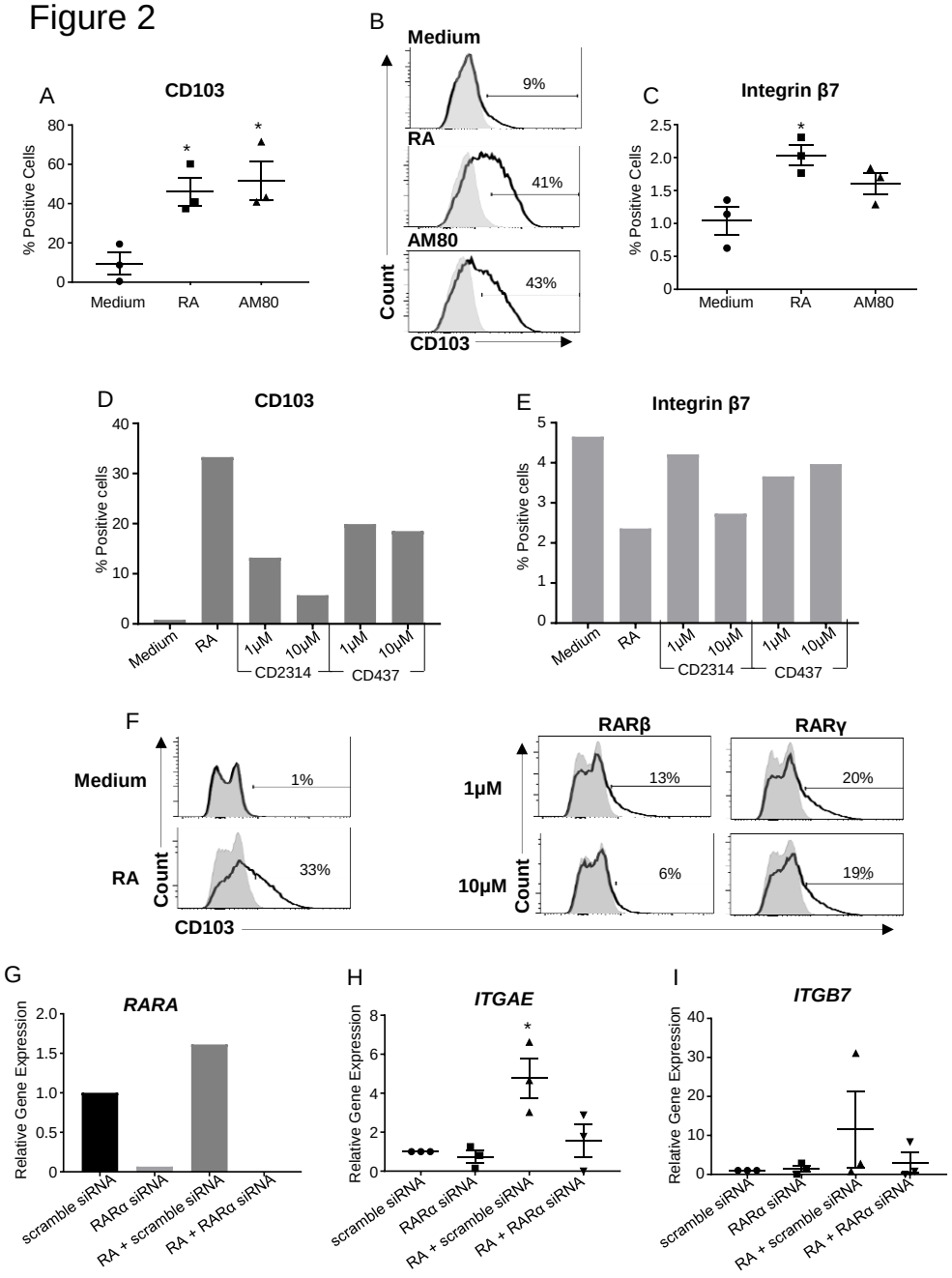

Figure 2: RARα mediates RA-induced expression of CD103 in human MoDCs. A-C) MoDCs were cultured with RA or AM80 (RARα agonist) for 3 days. CD103 (A) and integrin β7 (C) expression was analyzed by FACS analysis. B) FACS histograms of representative experiments showing the difference in CD103 expression in MoDCs without treatment (top), cultured with RA (middle), or cultured with AM80 (bottom). Isotype control-grey shaded, CD103 Ab-black solid line. D-E) MoDCs were treated with RA or 1μM or 10μM of CD2314 (RARβ agonist) or CD437 (RARγ agonist) for 3 days. CD103 (D) and integrin β7 (E) expression (continued on next page)

was measured by FACS (n=1). F) Representative FACS histograms of MoDCs cultured with RA, and two different concentrations of CD2314 and CD437. Isotype control-grey shaded, CD103 Ab-black solid line. G-I) MoDCs differentiated for 3 days before treatment of siRNA. RA was added 24 hours after siRNA treatment and MoDCs were harvested 24 hours later. G) Representative RT-qPCR showing the knockdown of *RARA* mRNA of MoDCs treated with scramble or RARα siRNA and RA. G-H) Relative gene expression of *ITGAE* (G) and *ITGB7* (H) of MoDCs treated with scramble or RARα siRNA and RA. RT-qPCR analyzed using the Pfaffl method (32) and normalized to GAPDH. * reveals statistical significance from media only control. ANOVA, $p < 0.05$

TGF-β receptor. MoDCs were cultured with RA, TGF-β1 or TGF-β2 and the TGF-β signaling inhibitor, SB431542. As expected, there was a significant increase in the phosphorylation of SMAD2/3 when MoDCs were cultured with TGF-β1, which was blocked upon the addition of SB431542 (Fig. 3A,B). Interestingly, we found no increase in the phosphorylation of SMAD2/3 when MoDCs were cultured in the presence of RA (Fig. 3A,B). This suggests that RA signaling in human DCs interacts with TGF-β signaling, but is independent of the SMAD pathway.

<u>RA Increases the Phosphorylation of p38 MAPK in Human MoDCs.</u>

A thorough review of the literature revealed that p38 mitogen activated protein kinase (MAPK) is involved in both TGF-β and RA signaling pathways. Importantly, Yu *et al*. demonstrated that TGF-β induced p38 MAPK signaling is independent of SMAD2/3 activation. In addition, multiple recent studies have established that RA can increase the phosphorylation of p38 MAPK in multiple cell types (14, 15, 17, 23, 24). Based upon these observations and our current and previous (26) data that demonstrated involvement of TGF-β pathways, but not SMAD-signaling, we investigated whether p38 MAPK plays a role in the RA-induced expression of CD103 on DCs.

Figure 3

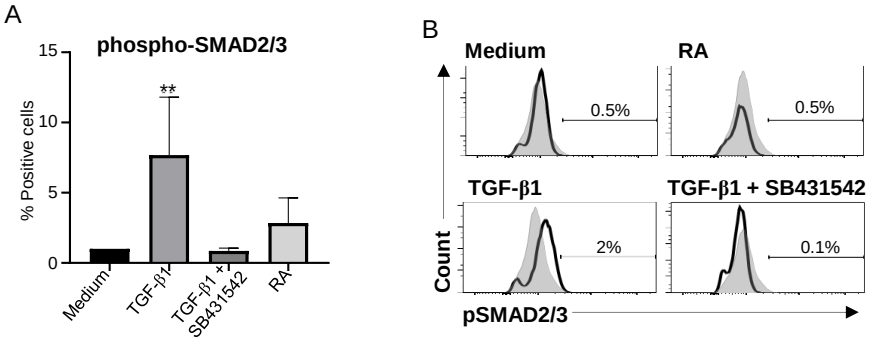

Figure 3: TGF-β1 but not RA drives phosphorylation of SMAD2/3 in human DCs. A) MoDCs were cultured with RA, TGF-β1, or TGF-β1 with TGF-β1 inhibitor, SB431542 for 3 days. Phosphorylation of SMAD2/3 was measured by FACS. B) Representative FACS histograms of MoDCs cultured with RA, TGF-β1, or TGF-β1 and SB431542. ** represents statistical significance from the medium only cells. One-way ANOVA, $p<0.05$.

MoDCs cultured with RA for 3 days were harvested for western blot analysis to investigate the phosphorylation of p38 MAPK. Interestingly, we found that MoDCs cultured with RA demonstrated significantly increased phosphorylation of p38 MAPK compared with MoDCs cultured with medium alone (Fig. 4A,B). These data demonstrate that p38 MAPK can be activated by RA signaling in human DCs.

RA-Induced Expression of CD103 in Human DCs is Dependent on p38 MAPK Signaling

We next determined whether p38 MAPK activation is involved in the RA-induced expression of CD103 and integrin β7. To that end, MoDCs were cultured with RA and the p38 MAPK inhibitor SB202190. When MoDCs were cultured with RA, we found the expected significant increase in *ITGAE* mRNA (Fig. 4C). However, MoDCs cultured with both RA and the p38 MAPK inhibitor showed *ITGAE* mRNA levels similar to that of baseline (Fig. 4C). Similarly, MoDCs cultured with RA had the expected significant

increase in CD103 protein expression above MoDCs cultured with media alone (Fig. 4D,E). However, when MoDCs were cultured with RA and the p38 MAPK inhibitor, RA-induced CD103 protein expression was abrogated (Fig. 4D,E). This is demonstrated well in the representative FACS histograms (Fig. 4E). Similarly, integrin β7 expression in MoDCs followed the same pattern of expression, although no statistical difference was established (Fig. 4F). These data suggest that RA-induced expression of CD103 and integrin β7 in human DCs is dependent on p38 MAPK signaling.

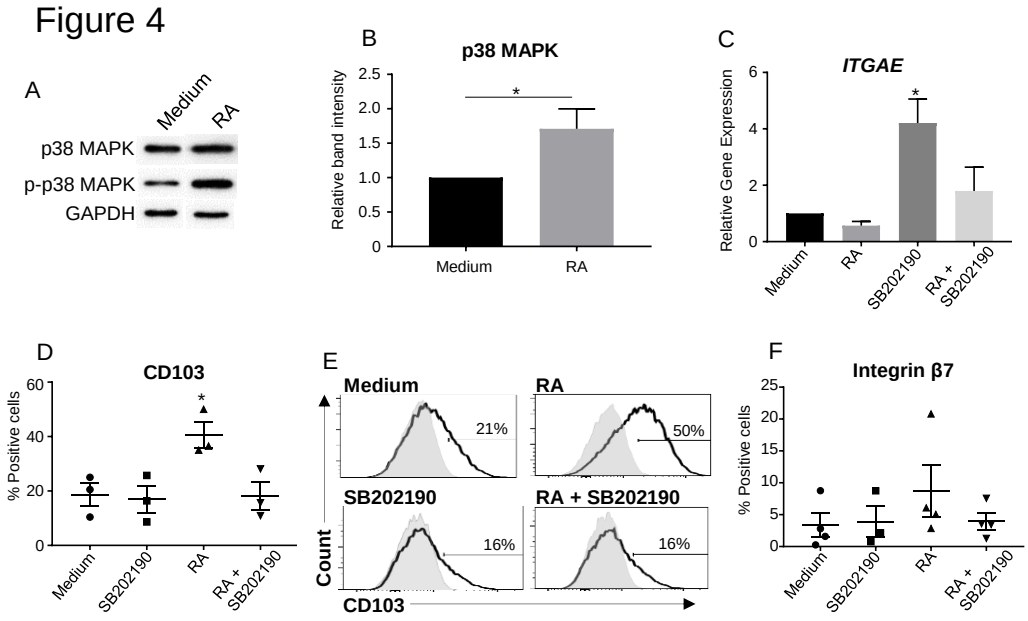

Figure 4: RA-induced CD103 expression in MoDCs is dependent on p38 MAPK signaling. A) Western blot of MoDCs cultured with or without RA for 3 days. B) Band intensity of protein normalized to GAPDH and phosphorylated protein (Thr180/Tyr182) relative to total p38 MAPK protein. C-F) MoDCs were differentiated for 3 days. RA and the p38 MAPK inhibitor, SB202190 was added and MoDCs were harvested after 24 hours. C) Relative gene expression of MoDCs cultured with RA and SB202190. Gene expression analyzed using the Pfaffl method (32). D) CD103 expression was measured with FACS analysis. E) Representative FACS histogram of MoDCs cultured in medium (top, left), SB202190 (bottom, left), RA (top, right), and RA + SB202190 (bottom, right). Isotype control-grey shaded, CD103 Ab-black solid line. F) Integrin β7 expression of MoDCs cultured with RA and SB202190 measured by FACS analysis. * represents statistical significance compared to untreated cells (medium). One-way ANOVA for RT-qPCR and FACS and unpaired T-test for western blot, $p<0.05$.

NFAT is an Important Component of the Signaling
Pathway of RA-Induced CD103 Expression in MoDCs.

Previous studies have found that p38 MAPK can activate the transcription factor nuclear factor of activated T cells (NFAT) (34, 35). In addition, NFAT has been shown to interact with the enhancer region of CD103 in T cells (36). Based on these studies, we next asked whether NFAT activation might contribute to the induction of CD103 expression downstream of RARα and p38 MAPK in human DCs. MoDCs were cultured with RA and increasing concentrations of an NFAT peptide inhibitor. There was a dose dependent decrease in the expression of CD103 in MoDCs cultured with RA and the NFAT peptide inhibitor compared to MoDCs cultured in the presence of RA alone (Fig. 5A,B). These data indicate that NFAT is involved in the RA-induced expression of CD103 downstream of RARα and p38 MAPK in human DCs.

Figure 5

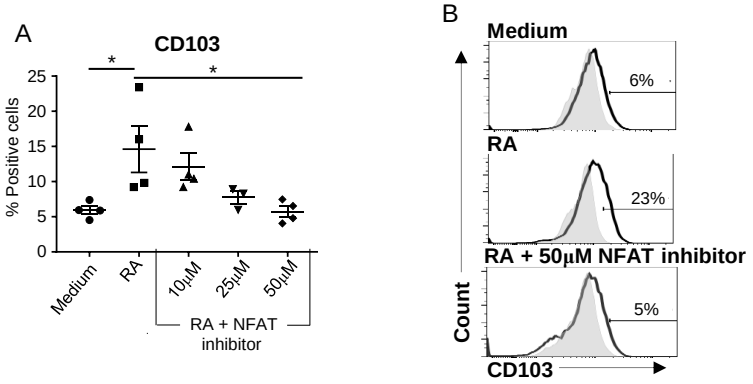

Figure 5: NFAT is an important component of the signaling pathway of RA-induced CD103 expression in MoDCs. A) MoDCs were differentiated for 3 days. RA and NFAT inhibitor were then added and MoDCs were harvested 24 hours later. CD103 expression was analyzed by FACS. B) Representative FACS histogram of MoDCs cultured in medium (top), treated with RA (middle), or RA + 50μM NFAT inhibitor (bottom). Isotype control-grey filled in, CD103 Ab-black solid lines. * indicates statistical significance. One-way ANOVA, $p<0.05$.

Discussion

Vitamin A is a key dietary micronutrient involved in the regulation of growth and development, vision and mucosal integrity, and that promotes anti-inflammatory immune responses (37). In the mammalian immune system, most effects of vitamin A are mediated by its major metabolite, retinoic acid (RA) (38). In a previous study that investigated the effect of RA on human DCs, we showed that RA drives the expression of CD103 (αE integrin) and integrin β7 (26). However, the molecular signaling pathway that regulates CD103 and integrin β7 expression remains unknown. RA is involved in the establishment and maintenance of dendritic cell populations within the intestinal mucosa (4, 5, 7). Specifically, RA has an essential role in the development of pre-mucosal DCs that give rise to intestinal DCs (5). The development of the cDC2 population in the small intestinal and mesenteric lymph nodes (MLN) is dependent on RA signaling through RARα (4, 7). It has been well established in human and mouse studies that RA increases the expression of Aldh1a2 in DCs (28, 29). Additionally, murine $CD103^+$ MLN DCs have higher expression of Aldh1a2 than $CD103^-$ DCs (37). This subpopulation of $CD103^+$ cDC2s have been found to be important in the induction of T regulatory cells (12) and gut homing molecules on T cells (12, 39). Taken together, these multiple relations between RA production, CD103 expression, and tolerogenic DC function in the gut, we here sought to define the regulatory pathways that control these interactions in more detail.

TGF-β induces CD103 expression in T cells, but is unable to enhance CD103 expression in human DCs. Conversely, our experiments revealed that RA-driven

expression of CD103 is conserved within the mononuclear phagocyte (MNP) compartment. Monocytes cultured with cytokines to differentiate into either a macrophage or DC phenotype showed an increase in the expression of CD103 upon culture with RA. In contrast, we saw a small increase in CD103 expression in RA-treated $CD8^+$ T cells and no effect in $CD4^+$ T cells. These current findings give further evidence to a differential pathway of CD103 regulation between immune cell types.

To elucidate the signaling pathway involved in RA-induced expression of CD103, we investigated the RA receptor (RAR) involved. RA can interact with three different nuclear receptors, RARα, RARβ, and RARγ, to induce gene expression upon RA stimulation (21). We here established that RA-induced CD103 and integrin β7 expression is predominantly RARα-dependent. MoDCs cultured with RA or the RARα agonist, AM80, had a 5-fold increase in CD103 expression, and to a lesser extent integrin β7. In contrast, agonists specific for RARβ and RARγ only caused a minor increase in CD103 expression, and no change in integrin β7 expression. Additionally, we confirmed the importance of RARα in RA-mediated CD103 expression through siRNA knockdown of RARα signaling, which led to the abrogation of RA-induced CD103 and integrin β7 expression.

RA can modify gene expression through two distinct signaling mechanisms. Classical genomic RA effects involve the binding of RAR to target DNA as transcription factors, which can either repress or induce transcription of target genes (14-17, 40). Non-genomic RA effects involve the activation of kinase cascades in the cytoplasm, which can indirectly impact gene expression through alternate pathways (14, 15, 17, 23, 24). In our

investigation to understand the RA-induced signaling that leads to CD103 expression in DCs, we looked to identify any non-genomic RA involvement.

We have previously shown that in the presence of RA, TGF-β signaling plays a role in the expression of CD103. Inhibition of the TGF-β receptor II (TGF-βRII) blocked RA-induced upregulation of CD103 in human DCs (26). Murine DC-specific knockouts of TGF-βR had fewer $CD103^+$ DCs and in turn a decrease in $Foxp3^+$ T regulatory cells (41). In human adenocarcinoma cells, RA-induced expression of VE-cadherin was abrogated when TGF-βR was inhibited (33). Interestingly, our experiments showed that RA-induced CD103 expression was independent of SMAD2/3 activation, the major signaling pathway downstream of the TGF-βR. P38 MAPK is a known component of the TGF-β signaling, and p38 MAPK activation by TGF-β is independent of SMAD signaling (42). Therefore, we hypothesized that p38 MAPK signaling may also be involved in RA-dependent CD103 expression.

Therefore, to evaluate the role of p38 MAPK in the RA-induced expression of CD103, we used an inhibitor of p38 MAPK signaling. This experiment confirmed that CD103 and integrin β7 expression in MoDCs was dependent on p38 MAPK signaling. There was a 2-fold decrease of CD103 and integrin β7 expression in MoDCs cultured with RA and a p38 MAPK inhibitor compared with MoDCs cultured with RA only. The role of p38 MAPK in RA signaling has previously been demonstrated in human osteoblasts and mouse embryonic fibroblasts (17, 23). Specifically, the additive effect of RA and TGF-β on VEGF expression in osteoblasts was highly dependent on the RA-induced p38 MAPK signaling (23). Additionally, in murine embryonic fibroblasts, RA-

driven expression of Cyp26A1 was abrogated when p38 MAPK signaling was blocked (17). Thus, the results from our investigations with human DCs corroborate previous reports that have demonstrated the importance of p38 MAPK in RA signaling.

We next sought to evaluate the downstream mediators of p38 MAPK signaling. Multiple studies have shown that p38 MAPK can have direct interactions with the immune cell transcription factor NFAT (34, 35). Furthermore, investigations by Mokrani *et al*. revealed that NFAT interacts with the enhancer region of CD103 in T cells (36). Our results point to a role for NFAT in CD103 regulation downstream of RARα and p38 MAPK. We here showed that increasing concentrations of NFAT inhibitor prevented RA-induced upregulation of DC CD103 expression in a dose-dependent manner. This suggests that NFAT may be a key downstream mediator that connects RARα-p38 MAPK signaling to DC CD103 expression.

Figure 6

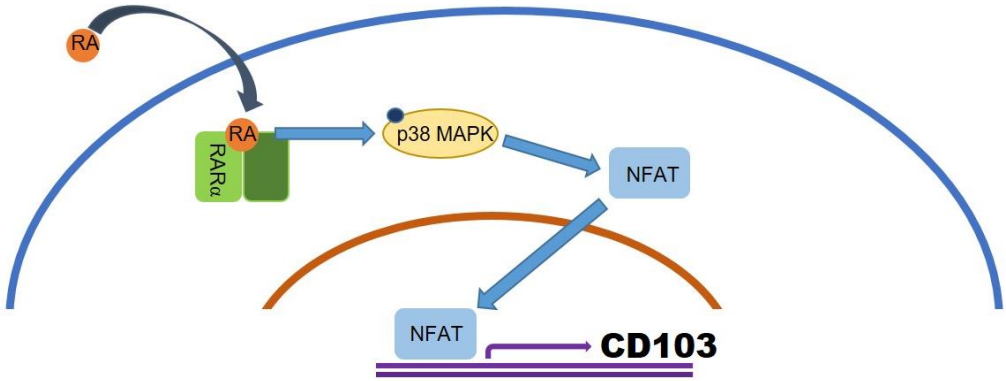

Figure 6: Graphical representation of the putative signaling pathway of RA-induced expression of CD103 in human MoDCs.

In summary, we here have elucidated a putative signaling pathway involved in the RA-induced expression of CD103 in human DCs (Fig. 5). Our data show that expression of CD103 is dependent on RARα and p38 MAPK signaling and NFAT. Importantly, this

effect is cell specific for MNPs, since T cells did not respond in the same way. Taking in the impacts of RA in the maintenance of immune homeostasis in the gut and especially DC development, these data evaluate other signaling molecules involved in the RA-induced pathway of CD103. A lack of sufficient nutritional RA has global health implications directly related to DC function. This study demonstrates novel signaling molecules in the RA-dependent expression of CD103 and integrin β7.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

References Cited

1. Sommer A. Vitamin A deficiency, child health, and survival. Nutrition. 1997;13(5):484-5. doi: 10.1016/s0899-9007(97)00013-0. PubMed PMID: 9225350.

2. Stevens GA, Bennett JE, Hennocq Q, Lu Y, De-Regil LM, Rogers L, Danaei G, Li G, White RA, Flaxman SR, Oehrle SP, Finucane MM, Guerrero R, Bhutta ZA, Then-Paulino A, Fawzi W, Black RE, Ezzati M. Trends and mortality effects of vitamin A deficiency in children in 138 low-income and middle-income countries between 1991 and 2013: a pooled analysis of population-based surveys. Lancet Glob Health. 2015;3(9):e528-36. doi: 10.1016/S2214-109X(15)00039-X. PubMed PMID: 26275329.

3. Vlasova AN, Chattha KS, Kandasamy S, Siegismund CS, Saif LJ. Prenatally acquired vitamin A deficiency alters innate immune responses to human rotavirus in a gnotobiotic pig model. J Immunol. 2013;190(9):4742-53. doi: 10.4049/jimmunol.1203575. PubMed PMID: 23536630; PMCID: PMC3633669.

4. Zeng R, Bscheider M, Lahl K, Lee M, Butcher EC. Generation and transcriptional programming of intestinal dendritic cells: essential role of retinoic acid. Mucosal Immunol. 2016;9(1):183-93. doi: 10.1038/mi.2015.50. PubMed PMID: 26129652; PMCID: PMC4698111.

5. Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, Butcher EC. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. Mucosal Immunol. 2013;6(4):847-56. doi: 10.1038/mi.2012.123. PubMed PMID: 23235743; PMCID: PMC3612556.

6. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004;21(4):527-38. doi: 10.1016/j.immuni.2004.08.011. PubMed PMID: 15485630.

7. Klebanoff CA, Spencer SP, Torabi-Parizi P, Grainger JR, Roychoudhuri R, Ji Y, Sukumar M, Muranski P, Scott CD, Hall JA, Ferreyra GA, Leonardi AJ, Borman ZA, Wang J, Palmer DC, Wilhelm C, Cai R, Sun J, Napoli JL, Danner RL, Gattinoni L, Belkaid Y, Restifo NP. Retinoic acid controls the homeostasis of pre-cDC-derived splenic and intestinal dendritic cells. J Exp Med. 2013;210(10):1961-76. doi: 10.1084/jem.20122508. PubMed PMID: 23999499; PMCID: PMC3782040.

8. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007;204(8):1757-64. doi: 10.1084/jem.20070590. PubMed PMID: 17620361; PMCID: PMC2118683.

9. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science. 2006;314(5802):1157-60. doi: 10.1126/science.1132742. PubMed PMID: 17110582.

10. Watanabe K, Sugai M, Nambu Y, Osato M, Hayashi T, Kawaguchi M, Komori T, Ito Y, Shimizu A. Requirement for Runx proteins in IgA class switching acting downstream of TGF-beta 1 and retinoic acid signaling. J Immunol. 2010;184(6):2785-92. doi: 10.4049/jimmunol.0901823. PubMed PMID: 20142360.

11. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, Ho AW, See P, Shin A, Wasan PS, Hoeffel G, Malleret B, Heiseke A, Chew S, Jardine L, Purvis HA, Hilkens CM, Tam J, Poidinger M, Stanley ER, Krug AB, Renia L, Sivasankar B, Ng LG, Collin M, Ricciardi-Castagnoli P, Honda K, Haniffa M, Ginhoux F. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. Immunity. 2013;38(5):970-83. doi: 10.1016/j.immuni.2013.04.011. PubMed PMID: 23706669; PMCID: PMC3666057.

12. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, Segura E, Tussiwand R, Yona S. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol. 2014;14(8):571-8. doi: 10.1038/nri3712. PubMed PMID: 25033907; PMCID: PMC4638219.

13. Sittig SP, Bakdash G, Weiden J, Skold AE, Tel J, Figdor CG, de Vries IJ, Schreibelt G. A Comparative Study of the T Cell Stimulatory and Polarizing Capacity of Human Primary Blood Dendritic Cell Subsets. Mediators Inflamm. 2016;2016:3605643. doi: 10.1155/2016/3605643. PubMed PMID: 27057096; PMCID: PMC4761397.

14. Canon E, Cosgaya JM, Scsucova S, Aranda A. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell. 2004;15(12):5583-92. doi: 10.1091/mbc.e04-05-0439. PubMed PMID: 15371543; PMCID: PMC532036.

15. Piskunov A, Rochette-Egly C. A retinoic acid receptor RARalpha pool present in membrane lipid rafts forms complexes with G protein alphaQ to activate p38MAPK. Oncogene. 2012;31(28):3333-45. doi: 10.1038/onc.2011.499. PubMed PMID: 22056876.

16. Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res. 2002;43(11):1773-808. doi: 10.1194/jlr.r100015-jlr200. PubMed PMID: 12401878.

17. Bruck N, Vitoux D, Ferry C, Duong V, Bauer A, de The H, Rochette-Egly C. A coordinated phosphorylation cascade initiated by p38MAPK/MSK1 directs RARalpha to target promoters. EMBO J. 2009;28(1):34-47. doi: 10.1038/emboj.2008.256. PubMed PMID: 19078967; PMCID: PMC2633082.

18. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. Science. 2001;294(5548):1866-70. doi: 10.1126/science.294.5548.1866. PubMed PMID: 11729302.

19. Dilworth FJ, Chambon P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene. 2001;20(24):3047-54. doi: 10.1038/sj.onc.1204329. PubMed PMID: 11420720.

20. Brelivet Y, Rochel N, Moras D. Structural analysis of nuclear receptors: from isolated domains to integral proteins. Mol Cell Endocrinol. 2012;348(2):466-73. doi: 10.1016/j.mce.2011.08.015. PubMed PMID: 21888944.

21. Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene. 2004;328:1-16. doi: 10.1016/j.gene.2003.12.005. PubMed PMID: 15019979.

22. Osz J, Brelivet Y, Peluso-Iltis C, Cura V, Eiler S, Ruff M, Bourguet W, Rochel N, Moras D. Structural basis for a molecular allosteric control mechanism of cofactor binding to nuclear receptors. Proc Natl Acad Sci U S A. 2012;109(10):E588-94. doi: 10.1073/pnas.1118192109. PubMed PMID: 22355136; PMCID: PMC3309777.

23. Tokuda H, Hatakeyama D, Akamatsu S, Tanabe K, Yoshida M, Shibata T, Kozawa O. Involvement of MAP kinases in TGF-beta-stimulated vascular endothelial growth factor synthesis in osteoblasts. Arch Biochem Biophys. 2003;415(1):117-25. doi: 10.1016/s0003-9861(03)00225-x. PubMed PMID: 12801520.

24. Alsayed Y, Uddin S, Mahmud N, Lekmine F, Kalvakolanu DV, Minucci S, Bokoch G, Platanias LC. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to all-trans-retinoic acid. J Biol Chem. 2001;276(6):4012-9. doi: 10.1074/jbc.M007431200. PubMed PMID: 11060298.

25. Iliev ID, Spadoni I, Mileti E, Matteoli G, Sonzogni A, Sampietro GM, Foschi D, Caprioli F, Viale G, Rescigno M. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut. 2009;58(11):1481-9. doi: 10.1136/gut.2008.175166. PubMed PMID: 19570762.

26. Roe MM, Swain S, Sebrell TA, Sewell MA, Collins MM, Perrino BA, Smith PD, Smythies LE, Bimczok D. Differential regulation of CD103 (alphaE integrin) expression in human dendritic cells by retinoic acid and Toll-like receptor ligands. J Leukoc Biol. 2017;101(5):1169-80. doi: 10.1189/jlb.1MA0316-131R. PubMed PMID: 28087652; PMCID: PMC5380378.

27. Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, Furtado GC, Lira SA, Shakhar G. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity. 2013;38(3):581-95. doi: 10.1016/j.immuni.2013.01.009. PubMed PMID: 23395676; PMCID: PMC4115273.

28. Bimczok D, Kao JY, Zhang M, Cochrun S, Mannon P, Peter S, Wilcox CM, Monkemuller KE, Harris PR, Grams JM, Stahl RD, Smith PD, Smythies LE. Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells. Mucosal Immunol. 2015;8(3):533-44. doi: 10.1038/mi.2014.86. PubMed PMID: 25249167; PMCID: PMC4372513.

29. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, Mebius RE. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. J Immunol. 2011;186(4):1934-42. doi: 10.4049/jimmunol.1001672. PubMed PMID: 21220692.

30. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, Belkaid Y, Mathis D, Benoist C. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity. 2008;29(5):758-70. doi: 10.1016/j.immuni.2008.09.018. PubMed PMID: 19006694; PMCID: PMC3140207.

31. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature. 1994;372(6502):190-3. doi: 10.1038/372190a0. PubMed PMID: 7969453.

32. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45. doi: 10.1093/nar/29.9.e45. PubMed PMID: 11328886; PMCID: PMC55695.

33. Prahalad P, Dakshanamurthy S, Ressom H, Byers SW. Retinoic acid mediates regulation of network formation by COUP-TFII and VE-cadherin expression by TGFbeta receptor kinase in breast cancer cells. PLoS One. 2010;5(4):e10023. doi: 10.1371/journal.pone.0010023. PubMed PMID: 20386594; PMCID: PMC2850308.

34. Wu CC, Hsu SC, Shih HM, Lai MZ. Nuclear factor of activated T cells c is a target of p38 mitogen-activated protein kinase in T cells. Mol Cell Biol. 2003;23(18):6442-54. doi: 10.1128/mcb.23.18.6442-6454.2003. PubMed PMID: 12944472; PMCID: PMC193716.

35. Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S, Hisatake K, Nogi Y. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. J Biol Chem. 2004;279(44):45969-79. doi: 10.1074/jbc.M408795200. PubMed PMID: 15304486.

36. Mokrani M, Klibi J, Bluteau D, Bismuth G, Mami-Chouaib F. Smad and NFAT pathways cooperate to induce CD103 expression in human CD8 T lymphocytes. J Immunol. 2014;192(5):2471-9. doi: 10.4049/jimmunol.1302192. PubMed PMID: 24477908.

37. Huang Z, Liu Y, Qi G, Brand D, Zheng SG. Role of Vitamin A in the Immune System. J Clin Med. 2018;7(9). doi: 10.3390/jcm7090258. PubMed PMID: 30200565; PMCID: PMC6162863.

38. Larange A, Cheroutre H. Retinoic Acid and Retinoic Acid Receptors as Pleiotropic Modulators of the Immune System. Annu Rev Immunol. 2016;34:369-94. doi: 10.1146/annurev-immunol-041015-055427. PubMed PMID: 27168242.

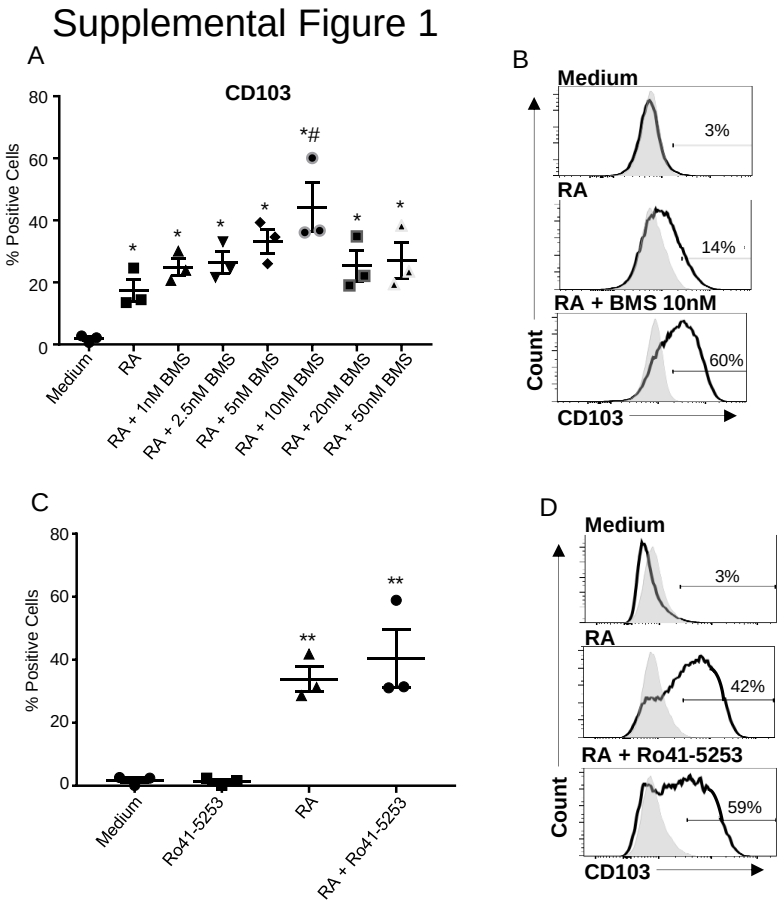
39. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med. 2008;205(9):2139-49. doi: 10.1084/jem.20080414. PubMed PMID: 18710932; PMCID: PMC2526207.

40. Manicassamy S, Pulendran B. Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin Immunol. 2009;21(1):22-7. doi: 10.1016/j.smim.2008.07.007. PubMed PMID: 18778953; PMCID: PMC2642591.

41. Bain CC, Montgomery J, Scott CL, Kel JM, Girard-Madoux MJH, Martens L, Zangerle-Murray TFP, Ober-Blobaum J, Lindenbergh-Kortleve D, Samsom JN, Henri S, Lawrence T, Saeys Y, Malissen B, Dalod M, Clausen BE, Mowat AM. TGFbetaR signalling controls CD103(+)CD11b(+) dendritic cell development in the intestine. Nat Commun. 2017;8(1):620. doi: 10.1038/s41467-017-00658-6. PubMed PMID: 28931816; PMCID: PMC5607002.

42. Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J. 2002;21(14):3749-59. doi: 10.1093/emboj/cdf366. PubMed PMID: 12110587; PMCID: PMC126112.

SUPPLEMENTAL FIGURES



Supplemental Figure 1: Inhibitors of RARα increase the expression of CD103 in MoDCs. A) MoDCs were cultured with RA and increasing concentrations of RARα inhibitor, BMS195614 (BMS) as indicated. CD103 expression was analyzed using FACS analysis. B) Representative FACS histogram of MoDCs cultured in medium only (top), cultured with RA only (middle), or cultured with RA and BMS195614 (bottom). Isotype: filled in, grey. CD103 Ab: black solid line. C) MoDCS were cultured with RA and RARα inhibitor, Ro41-5253, as indicated and CD103 expression was analyzed by FACS. D) Representative FACS histograms of MoDCs cultured with media only (top), RA only (middle), or RA and Ro41-5253 (bottom). Isotype: filled in, grey. CD103 Ab: solid black line. * indicates statistical significance from medium only control ($p < 0.05$). ** indicates statistical significance from medium only control ($p < 0.01$). *# indicates statistical significance from medium only and RA only treated MoDCs ($p < 0.05$). Statistics analyzed using ANOVA.

CHAPTER 5

DISCUSSION

This dissertation work sought to investigate the regulation and function of human DC CD103. We hypothesized that RA modulates the regulation and function of CD103 on human DCs. We asked the following question sot evaluate this hypothesis: 1) What are the molecular mechanisms driving the expression of CD103 on human DCs? 2) What is the function of CD103 on human DCs?

CD103 on human DCs is used as a marker to distinguish functional mucosal DC subsets. $CD103^+$ DCs have a role in the induction of immune tolerance through priming of Treg cells in the presence of TGF-β and RA (1-3). In addition, $CD103^+$ DCs are involved in the uptake, transport, and presentation of bacterial antigen (4-6). $CD103^+$ DCs are important in the biosynthesis of RA (RA) (7, 8), which is driven by exposure to RA through a positive feedback mechanism (7, 9). RA is a metabolite of vitamin A and is an important modulator of immune function (10). Despite the broad use of CD103 as a marker of functional DC subsets, few studies have evaluated either the regulation of CD103 expression or the functional aspects of CD103 for DCs.

The major findings of this dissertation research are: 1) RA drives the expression of CD103 in human DCs mediated through RARα and p38 MAPK signaling and NFAT. 2) CD103 is found intracellularly in human gastric and monocyte-derived DCs and undergoes endosomal recycling. 3) DC CD103 interaction with Ecad is not the main driver of DC-epithelial cell adhesion.

We demonstrate that RA drives the expression of CD103 and integrin β7 in human DCs (11) (Chap. 2). The addition of TGF-β1 or TGF-β2 to DCs did not affect the expression of CD103, as it has been demonstrated in T cells (12) (Chap. 2). However, inhibition of the TGF-β signaling pathway in the presence of RA decreases the expression of CD103 in DCs (11), indicating that the RA signaling interacts with TGF-β signaling in DCs, as previously suggested by other studies (13) (Chap. 2). Additionally, we found that RA increased the phosphorylation of p38 MAPK. Based on these data and literature, we evaluated whether p38 MAPK and NFAT are essential components of the RA-induced signaling pathway of CD103 expression. Indeed, we found that inhibiting either p38 MAPK signaling or NFAT blocked the RA-induced expression of CD103 in human DCs (Chap. 4). This data also suggests that at least some part of the RA signaling involved in CD103 expression is through non-genomic signaling. Additionally, the stimulation of DCs with TLR ligands abrogated the RA-induced expression of CD103 (Chap. 2). These data indicate that signaling through TLR interferes with the RA-p38 MAPK-NFAT pathway outlined here. This could be through negative regulators of p38 MAPK signaling, such as MSK5 or WIP1 (14, 15). Our proposed signaling pathway involved in DC CD103 expression is shown in Figure 1. Further studies are needed to understand how the interplay between TLR and RA signaling effect the expression of CD103 in human DCs.

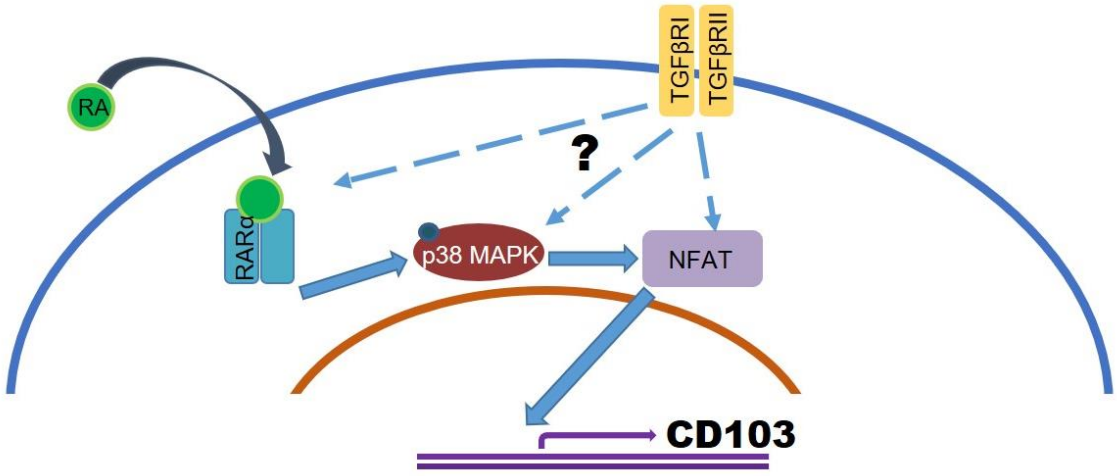


Figure 1: Graphical representation of putative RA-induced regulation of CD103 expression in human DCs.

We hypothesized that interactions between CD103 on DCs and Ecad on epithelial cells anchors DCs to the epithelium. It has been previously reported that CD103 has this role in IELs (12, 16-18). Consistent with these studies, we found that a high proportion of $CD103^{+}$ DCs in gastric tissue samples were located within the epithelial layer. These findings supported our hypothesis that CD103 mediates DC binding to the epithelium. However, this hypothesis did not hold up when DC-epithelial cell interactions were investigated using a DC-epithelial cell co-culture model. Inhibition of CD103 on DCs did not have an effect on the percentage of DCs that adhered to the epithelial layer. However, when Ecad on DCs was blocked, there was a significant decrease in adherent DCs (Chap. 3). These findings suggest that Ecad-Ecad interactions play a major role in DC-epithelial cell adhesion under these conditions. DC CD103 interactions with epithelial cell Ecad may be a way to induce signaling in the DCs, as has been suggested in other studies in T cells (19-21).

We established that CD103 was present within intracellular pockets in both primary gastric and monocyte-derived human DCs (Chap. 3). Multiple integrins, such as α5β1 and α6β4, have been shown to undergo endosomal recycling (22-26). We evaluated whether CD103 on DCs may also be undergoing endosomal recycling. We found that CD103 co-localized with clathrin, which has been shown to be important for disassembly of integrin-based focal adhesion of migrating cells (23, 27). Additionally, CD103 co-localized with early endosome antigen 1 (EEA-1) and Rab11, endosomal recycling markers (28, 29). We also demonstrated the active internalization of CD103 in human DCs (Chap. 3). These data suggest that CD103 undergoes endosomal recycling, possibly to be readily available for ligand binding.

We evaluated another proposed functional aspect of CD103 on human DCs. $CD103^+$ DCs have been attributed to driving a Treg response in the presence of TGF-β and RA (1). We found that CD103 is dispensable for inducing the secretion of IL-10 and the expression of the gut-homing molecule α4β7 by naïve T cells cultured with RA-treated DCs (Chap. 2). These data suggest that RA, and not the expression of CD103 on DCs is the driver of IL-10 secretion and α4β7 expression. These findings are consistent with a study demonstrating that human $CD103^+$ and $CD103^-$ DCs induced α4β7 expression on T cells (30). However, this same study and others have demonstrated that in the mouse model, CD103 is essential in the induction of T cell α4β7 and CCR9 expression (30, 31). However, when CD103 was knocked out in a murine model, α4β7 and CCR9 expression was unaffected compared with wild type mice (30). There may be more cell intrinsic changes occurring upon DC development and redundant pathways that

lead to the induction of Treg cells and the expression of α4β7 and CCR9. Some of our findings suggest this may be the case. RA readily drives an increase of CD103 expression in monocyte-derived DCs, however, after isolating primary gastric DCs from human tissue, RA did not increase the expression of CD103 on these cells (11) (Chap. 2). Taken together, these data suggest other, yet unknown factors are involved in $CD103^+$ DC attributed immune function. In addition, the differences of immune system function between mice and human is not fully understood.

In summary, we here have unraveled molecular signaling components of the RA-induced expression of CD103 on human DCs. The extent of vitamin A deficiency and the burden on the health of children in developing countries has recently been given more attention (32). Mortalities to infectious disease, such as diarrheal diseases and measles, are increased when the availability of vitamin A is diminished (32, 33). Jones *et al*. revealed a correlation with neutralizing antibody to influenza and vitamin A availability, suggesting that vitamin A deficiency decreases influenza specific antibodies (33). Additionally, the lack of vitamin A in children is detrimental to immune response to pathogens, but also a deficiency in utero can have impacts on the neonate's ability to mount a sufficient immune response to diarrheal infections (34). Vitamin A has been evaluated for the use against infectious agents since 1928 (35). In addition to infectious disease implications, retinoids have been evaluated for the treatment of multiple sclerosis, leukemia, and in combination with cancer treatments (36-38). Further knowledge of the way in which RA signals in the cells and the multiple impacts that retinoids have on

different cell types could help to improve these treatments or help to better understand off-target side effects.

Future Directions

Future research into the RA-induced expression of CD103 would include identifying other signaling molecules involved. Identification of these signaling proteins can be obtained by the use of a phospho-protein array with MoDCs cultured with or without RA. As RA has multiple functions within the cell, the target proteins would then need to be evaluated individually to determine a role in the RA-induced expression of CD103. A phospho-protein array could also be utilized to analyze MoDCs cultured with RA and stimulated with TLR agonists to identify the signaling pathways involved in CD103 downregulation in response to TLR engagement. These studies may lead to a better understanding of how TLR agonists can lead to the abrogation of RA-induced expression of CD103. Additional transcription factors involved in CD103 expression can also be identified. Performing ChIP PCR experiments, pulling down NFAT to determine if NFAT has increased interactions with the enhancer region of CD103 culturing MoDCs with RA would be a place to start. Additional transcription factors would be identified by mass spectrometry of proteins pulled down with NFAT when MoDCs are treated with RA.

Future research to evaluate the function of CD103 on human DCs would be to look at transcriptomics of DCs following binding of CD103 and E-cadherin. These experiments would identify any outside-in signaling that may be emerging upon binding

of DCs to the epithelium. These target transcripts could then be evaluated individually in the context of interactions of MoDCs with epithelial cells.

References Cited

1. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007;204(8):1757-64. doi: 10.1084/jem.20070590. PubMed PMID: 17620361; PMCID: PMC2118683.

2. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, Belkaid Y, Mathis D, Benoist C. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity. 2008;29(5):758-70. doi: 10.1016/j.immuni.2008.09.018. PubMed PMID: 19006694; PMCID: PMC3140207.

3. Cassani B, Villablanca EJ, Quintana FJ, Love PE, Lacy-Hulbert A, Blaner WS, Sparwasser T, Snapper SB, Weiner HL, Mora JR. Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. Gastroenterology. 2011;141(6):2109-18. doi: 10.1053/j.gastro.2011.09.015. PubMed PMID: 21925467; PMCID: PMC3222333.

4. Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, Furtado GC, Lira SA, Shakhar G. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity. 2013;38(3):581-95. doi: 10.1016/j.immuni.2013.01.009. PubMed PMID: 23395676; PMCID: PMC4115273.

5. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2001;2(4):361-7. doi: 10.1038/86373. PubMed PMID: 11276208.

6. Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, Pabst O. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med. 2009;206(13):3101-14. doi: 10.1084/jem.20091925. PubMed PMID: 20008524; PMCID: PMC2806467.

7. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, Mebius RE. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. J Immunol. 2011;186(4):1934-42. doi: 10.4049/jimmunol.1001672. PubMed PMID: 21220692.

8. Agace WW, Persson EK. How vitamin A metabolizing dendritic cells are generated in the gut mucosa. Trends Immunol. 2012;33(1):42-8. doi: 10.1016/j.it.2011.10.001. PubMed PMID: 22079120.

9. Bimczok D, Kao JY, Zhang M, Cochrun S, Mannon P, Peter S, Wilcox CM, Monkemuller KE, Harris PR, Grams JM, Stahl RD, Smith PD, Smythies LE. Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells. Mucosal Immunol. 2015;8(3):533-44. doi: 10.1038/mi.2014.86. PubMed PMID: 25249167; PMCID: PMC4372513.

10. Czarnewski P, Das S, Parigi SM, Villablanca EJ. Retinoic Acid and Its Role in Modulating Intestinal Innate Immunity. Nutrients. 2017;9(1). doi: 10.3390/nu9010068. PubMed PMID: 28098786; PMCID: PMC5295112.

11. Roe MM, Swain S, Sebrell TA, Sewell MA, Collins MM, Perrino BA, Smith PD, Smythies LE, Bimczok D. Differential regulation of CD103 (alphaE integrin) expression in human dendritic cells by retinoic acid and Toll-like receptor ligands. J Leukoc Biol. 2017;101(5):1169-80. doi: 10.1189/jlb.1MA0316-131R. PubMed PMID: 28087652; PMCID: PMC5380378.

12. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature. 1994;372(6502):190-3. doi: 10.1038/372190a0. PubMed PMID: 7969453.

13. Prahalad P, Dakshanamurthy S, Ressom H, Byers SW. Retinoic acid mediates regulation of network formation by COUP-TFII and VE-cadherin expression by TGFbeta receptor kinase in breast cancer cells. PLoS One. 2010;5(4):e10023. doi: 10.1371/journal.pone.0010023. PubMed PMID: 20386594; PMCID: PMC2850308.

14. Chew J, Biswas S, Shreeram S, Humaidi M, Wong ET, Dhillion MK, Teo H, Hazra A, Fang CC, Lopez-Collazo E, Bulavin DV, Tergaonkar V. WIP1 phosphatase is a negative regulator of NF-kappaB signalling. Nat Cell Biol. 2009;11(5):659-66. doi: 10.1038/ncb1873. PubMed PMID: 19377466.

15. Qian F, Deng J, Cheng N, Welch EJ, Zhang Y, Malik AB, Flavell RA, Dong C, Ye RD. A non-redundant role for MKP5 in limiting ROS production and preventing LPS-induced vascular injury. EMBO J. 2009;28(19):2896-907. doi: 10.1038/emboj.2009.234. PubMed PMID: 19696743; PMCID: PMC2760111.

16. Corps E, Carter C, Karecla P, Ahrens T, Evans P, Kilshaw P. Recognition of E-cadherin by integrin alpha(E)beta(7): requirement for cadherin dimerization and implications for cadherin and integrin function. J Biol Chem. 2001;276(33):30862-70. doi: 10.1074/jbc.M101712200. PubMed PMID: 11413131.

17. Higgins JM, Cernadas M, Tan K, Irie A, Wang J, Takada Y, Brenner MB. The role of alpha and beta chains in ligand recognition by beta 7 integrins. J Biol Chem. 2000;275(33):25652-64. doi: 10.1074/jbc.M001228200. PubMed PMID: 10837471.

18. Russell GJ, Parker CM, Cepek KL, Mandelbrot DA, Sood A, Mizoguchi E, Ebert EC, Brenner MB, Bhan AK. Distinct structural and functional epitopes of the alpha E beta 7 integrin. Eur J Immunol. 1994;24(11):2832-41. doi: 10.1002/eji.1830241138. PubMed PMID: 7525307.

19. Le Floc'h A, Jalil A, Franciszkiewicz K, Validire P, Vergnon I, Mami-Chouaib F. Minimal engagement of CD103 on cytotoxic T lymphocytes with an E-cadherin-Fc molecule triggers lytic granule polarization via a phospholipase Cgamma-dependent pathway. Cancer Res. 2011;71(2):328-38. doi: 10.1158/0008-5472.CAN-10-2457. PubMed PMID: 21224355.

20. Le Floc'h A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, Chouaib S, Mami-Chouaib F. Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. J Exp Med. 2007;204(3):559-70. doi: 10.1084/jem.20061524. PubMed PMID: 17325197; PMCID: PMC2137907.

21. Shen B, Delaney MK, Du X. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. Curr Opin Cell Biol. 2012;24(5):600-6. doi: 10.1016/j.ceb.2012.08.011. PubMed PMID: 22980731; PMCID: PMC3479359.

22. Bretscher MS. Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. EMBO J. 1992;11(2):405-10. PubMed PMID: 1531629; PMCID: PMC556468.

23. Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. J Cell Biol. 2009;187(5):733-47. doi: 10.1083/jcb.200904054. PubMed PMID: 19951918; PMCID: PMC2806590.

24. Roberts MS, Woods AJ, Dale TC, Van Der Sluijs P, Norman JC. Protein kinase B/Akt acts via glycogen synthase kinase 3 to regulate recycling of alpha v beta 3 and alpha 5 beta 1 integrins. Mol Cell Biol. 2004;24(4):1505-15. doi: 10.1128/mcb.24.4.1505-1515.2004. PubMed PMID: 14749368; PMCID: PMC344170.

25. Arjonen A, Alanko J, Veltel S, Ivaska J. Distinct recycling of active and inactive beta1 integrins. Traffic. 2012;13(4):610-25. doi: 10.1111/j.1600-0854.2012.01327.x. PubMed PMID: 22222055; PMCID: PMC3531618.

26. Sonnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol. 2000;149(4):901-14. doi: 10.1083/jcb.149.4.901. PubMed PMID: 10811830; PMCID: PMC2174575.

27. Chao WT, Kunz J. Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. FEBS Lett. 2009;583(8):1337-43. doi: 10.1016/j.febslet.2009.03.037. PubMed PMID: 19306879; PMCID: PMC2801759.

28. Rubino M, Miaczynska M, Lippe R, Zerial M. Selective membrane recruitment of EEA1 suggests a role in directional transport of clathrin-coated vesicles to early endosomes. J Biol Chem. 2000;275(6):3745-8. doi: 10.1074/jbc.275.6.3745. PubMed PMID: 10660521.

29. Ullrich O, Reinsch S, Urbe S, Zerial M, Parton RG. Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol. 1996;135(4):913-24. doi: 10.1083/jcb.135.4.913. PubMed PMID: 8922376; PMCID: PMC2133374.

30. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med. 2008;205(9):2139-49. doi: 10.1084/jem.20080414. PubMed PMID: 18710932; PMCID: PMC2526207.

31. Jaensson-Gyllenback E, Kotarsky K, Zapata F, Persson EK, Gundersen TE, Blomhoff R, Agace WW. Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. Mucosal Immunol. 2011;4(4):438-47. doi: 10.1038/mi.2010.91. PubMed PMID: 21289617; PMCID: PMC3130189.

32. Stevens GA, Bennett JE, Hennocq Q, Lu Y, De-Regil LM, Rogers L, Danaei G, Li G, White RA, Flaxman SR, Oehrle SP, Finucane MM, Guerrero R, Bhutta ZA, Then-Paulino A, Fawzi W, Black RE, Ezzati M. Trends and mortality effects of vitamin A deficiency in children in 138 low-income and middle-income countries between 1991 and 2013: a pooled analysis of population-based surveys. Lancet Glob Health. 2015;3(9):e528-36. doi: 10.1016/S2214-109X(15)00039-X. PubMed PMID: 26275329.

33. Jones BG, Oshansky CM, Bajracharya R, Tang L, Sun Y, Wong SS, Webby R, Thomas PG, Hurwitz JL. Retinol binding protein and vitamin D associations with serum antibody isotypes, serum influenza virus-specific neutralizing activities and airway cytokine profiles. Clin Exp Immunol. 2016;183(2):239-47. doi: 10.1111/cei.12718. PubMed PMID: 26425827; PMCID: PMC4711169.

34. Vlasova AN, Chattha KS, Kandasamy S, Siegismund CS, Saif LJ. Prenatally acquired vitamin A deficiency alters innate immune responses to human rotavirus in a gnotobiotic pig model. J Immunol. 2013;190(9):4742-53. doi: 10.4049/jimmunol.1203575. PubMed PMID: 23536630; PMCID: PMC3633669.

35. Green HN, Mellanby E. Vitamin a as an Anti-Infective Agent. Br Med J. 1928;2(3537):691-6. doi: 10.1136/bmj.2.3537.691. PubMed PMID: 20774205; PMCID: PMC2456524.

36. Niles RM. Signaling pathways in retinoid chemoprevention and treatment of cancer. Mutat Res. 2004;555(1-2):81-96. doi: 10.1016/j.mrfmmm.2004.05.020. PubMed PMID: 15476854.

37. Racke MK, Burnett D, Pak SH, Albert PS, Cannella B, Raine CS, McFarlin DE, Scott DE. Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. J Immunol. 1995;154(1):450-8. PubMed PMID: 7527821.

38. Tobita T, Takeshita A, Kitamura K, Ohnishi K, Yanagi M, Hiraoka A, Karasuno T, Takeuchi M, Miyawaki S, Ueda R, Naoe T, Ohno R. Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid. Blood. 1997;90(3):967-73. PubMed PMID: 9242525.

REFERENCES CITED

Abram, C. L. and Lowell, C. A. (2007) The expanding role for ITAM-based signaling pathways in immune cells. Sci STKE 2007, re2.

Adler HS, Kubsch S, Graulich E, Ludwig S, Knop J, Steinbrink K. Activation of MAP kinase p38 is critical for the cell-cycle-controlled suppressor function of regulatory T cells. Blood. 2007;109(10):4351-9. doi: 10.1182/blood-2006-09-047563. PubMed PMID: 17244678.

Agace WW, Persson EK. How vitamin A metabolizing dendritic cells are generated in the gut mucosa. Trends Immunol. 2012;33(1):42-8. doi: 10.1016/j.it.2011.10.001. PubMed PMID: 22079120.

Agace, W. W., Higgins, J. M., Sadasivan, B., Brenner, M. B., Parker, C. M. (2000) T-lymphocyte-epithelial-cell interactions: integrin alpha(E)(CD103)beta(7), LEEP-CAM and chemokines. Current opinion in cell biology 12, 563-8.

Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, Pulendran B. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J Immunol. 2003;171(10):4984-9. doi: 10.4049/jimmunol.171.10.4984. PubMed PMID: 14607893.

Alsayed Y, Uddin S, Mahmud N, Lekmine F, Kalvakolanu DV, Minucci S, Bokoch G, Platanias LC. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to all-trans-retinoic acid. J Biol Chem. 2001;276(6):4012-9. doi: 10.1074/jbc.M007431200. PubMed PMID: 11060298.

Amieva, M., and Peek, R.M., Jr. (2016). Pathobiology of Helicobacter pylori-Induced Gastric Cancer. *Gastroenterology* 150**,** 64-78.

Arjonen A, Alanko J, Veltel S, Ivaska J. Distinct recycling of active and inactive beta1 integrins. Traffic. 2012;13(4):610-25. doi: 10.1111/j.1600-0854.2012.01327.x. PubMed PMID: 22222055; PMCID: PMC3531618.

Arnold, I.C., Zhang, X., Urban, S., Artola-Boran, M., Manz, M.G., Ottemann, K.M., and Muller, A. (2017). NLRP3 Controls the Development of Gastrointestinal CD11b(+) Dendritic Cells in the Steady State and during Chronic Bacterial Infection. *Cell Rep* 21**,** 3860-3872.

Arrighi JF, Rebsamen M, Rousset F, Kindler V, Hauser C. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. J Immunol. 2001;166(6):3837-45. doi: 10.4049/jimmunol.166.6.3837. PubMed PMID: 11238627.

Atherton JC, Peek RM, Jr., Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. Gastroenterology. 1997;112(1):92-9. doi: 10.1016/s0016-5085(97)70223-3. PubMed PMID: 8978347.

Bain CC, Montgomery J, Scott CL, Kel JM, Girard-Madoux MJH, Martens L, Zangerle-Murray TFP, Ober-Blobaum J, Lindenbergh-Kortleve D, Samsom JN, Henri S, Lawrence T, Saeys Y, Malissen B, Dalod M, Clausen BE, Mowat AM. TGFbetaR signalling controls CD103(+)CD11b(+) dendritic cell development in the intestine. Nat Commun. 2017;8(1):620. doi: 10.1038/s41467-017-00658-6. PubMed PMID: 28931816; PMCID: PMC5607002.

Bakdash G, Vogelpoel LT, van Capel TM, Kapsenberg ML, de Jong EC. Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. Mucosal Immunol. 2015;8(2):265-78. doi: 10.1038/mi.2014.64. PubMed PMID: 25027601.

Balmer JE, Blomhoff R. Gene expression regulation by retinoic acid. J Lipid Res. 2002;43(11):1773-808. doi: 10.1194/jlr.r100015-jlr200. PubMed PMID: 12401878.

Banh, C., Fugere, C., and Brossay, L. (2009). Immunoregulatory functions of KLRG1 cadherin interactions are dependent on forward and reverse signaling. *Blood* 114**,** 5299-5306.

Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene. 2004;328:1-16. doi: 10.1016/j.gene.2003.12.005. PubMed PMID: 15019979.

Bedoui, S., Whitney, P. G., Waithman, J., Eidsmo, L., Wakim, L., Caminschi, I., Allan, R. S., Wojtasiak, M., Shortman, K., Carbone, F. R., Brooks, A. G., Heath, W. R. (2009) Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. Nat Immunol 10, 488-95.

Bimczok D, Kao JY, Zhang M, Cochrun S, Mannon P, Peter S, Wilcox CM, Monkemuller KE, Harris PR, Grams JM, Stahl RD, Smith PD, Smythies LE. Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells. Mucosal Immunol. 2015;8(3):533-44. doi: 10.1038/mi.2014.86. PubMed PMID: 25249167; PMCID: PMC4372513.

Bimczok, D., Clements, R. H., Waites, K. B., Novak, L., Eckhoff, D. E., Mannon, P. J., Smith, P. D., Smythies, L. E. (2010) Human primary gastric dendritic cells induce a Th1 response to H. pylori. Mucosal Immunol 3, 260-9.

Bimczok, D., Grams, J. M., Stahl, R. D., Waites, K. B., Smythies, L. E., Smith, P. D. (2011) Stromal regulation of human gastric dendritic cells restricts the Th1 response to Helicobacter pylori. Gastroenterology 141, 929-38.

Bimczok, D., Smythies, L. E., Waites, K. B., Grams, J. M., Stahl, R. D., Mannon, P. J., Peter, S., Wilcox, C. M., Harris, P. R., Das, S., Ernst, P. B., Smith, P. D. (2013) Helicobacter pylori infection inhibits phagocyte clearance of apoptotic gastric epithelial cells. J Immunol 190, 6626-34.

Bolte, S., and Cordelieres, F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc.* 224**,** 213-232.

Boutet, M., Gauthier, L., Leclerc, M., Gros, G., De Montpreville, V., Theret, N., Donnadieu, E., Mami-Chouaib, F. (2016) TGF-beta signaling intersects with CD103 integrin signaling to promote T lymphocyte accumulation and antitumor activity in the lung tumor microenvironment. Cancer Res.

Brelivet Y, Rochel N, Moras D. Structural analysis of nuclear receptors: from isolated domains to integral proteins. Mol Cell Endocrinol. 2012;348(2):466-73. doi: 10.1016/j.mce.2011.08.015. PubMed PMID: 21888944.

Bretscher MS. Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. EMBO J. 1992;11(2):405-10. PubMed PMID: 1531629; PMCID: PMC556468.

Bridgewater, R. E., Norman, J. C., Caswell, P. T. (2012) Integrin trafficking at a glance. Journal of cell science 125, 3695-701.

Bruck N, Vitoux D, Ferry C, Duong V, Bauer A, de The H, Rochette-Egly C. A coordinated phosphorylation cascade initiated by p38MAPK/MSK1 directs RARalpha to target promoters. EMBO J. 2009;28(1):34-47. doi: 10.1038/emboj.2008.256. PubMed PMID: 19078967; PMCID: PMC2633082.

Canon E, Cosgaya JM, Scsucova S, Aranda A. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell. 2004;15(12):5583-92. doi: 10.1091/mbc.e04-05-0439. PubMed PMID: 15371543; PMCID: PMC532036.

Cassani B, Villablanca EJ, Quintana FJ, Love PE, Lacy-Hulbert A, Blaner WS, Sparwasser T, Snapper SB, Weiner HL, Mora JR. Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. Gastroenterology. 2011;141(6):2109-18. doi: 10.1053/j.gastro.2011.09.015. PubMed PMID: 21925467; PMCID: PMC3222333.

Cavarelli, M., Foglieni, C., Rescigno, M., and Scarlatti, G. (2013). R5 HIV-1 envelope attracts dendritic cells to cross the human intestinal epithelium and sample luminal virions via engagement of the CCR5. *EMBO Mol Med* 5**,** 776-794.

Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the

alpha E beta 7 integrin. Nature. 1994;372(6502):190-3. doi: 10.1038/372190a0. PubMed PMID: 7969453.

Cepek, K. L., Rimm, D. L., Brenner, M. B. (1996) Expression of a candidate cadherin in T lymphocytes. Proceedings of the National Academy of Sciences of the United States of America 93, 6567-71.

Chao WT, Kunz J. Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. FEBS Lett. 2009;583(8):1337-43. doi: 10.1016/j.febslet.2009.03.037. PubMed PMID: 19306879; PMCID: PMC2801759.

Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. Science. 2001;294(5548):1866-70. doi: 10.1126/science.294.5548.1866. PubMed PMID: 11729302.

Chen, P.W., Luo, R., Jian, X., and Randazzo, P.A. (2014). The Arf6 GTPase-activating proteins ARAP2 and ACAP1 define distinct endosomal compartments that regulate integrin alpha5beta1 traffic. *J Biol Chem* 289**,** 30237-30248.

Chew J, Biswas S, Shreeram S, Humaidi M, Wong ET, Dhillion MK, Teo H, Hazra A, Fang CC, Lopez-Collazo E, Bulavin DV, Tergaonkar V. WIP1 phosphatase is a negative regulator of NF-kappaB signalling. Nat Cell Biol. 2009;11(5):659-66. doi: 10.1038/ncb1873. PubMed PMID: 19377466.

Cohn L, Chatterjee B, Esselborn F, Smed-Sorensen A, Nakamura N, Chalouni C, Lee BC, Vandlen R, Keler T, Lauer P, Brockstedt D, Mellman I, Delamarre L. Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation. J Exp Med. 2013;210(5):1049-63. doi: 10.1084/jem.20121251. PubMed PMID: 23569326; PMCID: PMC3646496.

Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology. 2018;154(1):3-20. doi: 10.1111/imm.12888. PubMed PMID: 29313948; PMCID: PMC5904714.

Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med. 2007;204(8):1757-64. doi: 10.1084/jem.20070590. PubMed PMID: 17620361; PMCID: PMC2118683.

Corps E, Carter C, Karecla P, Ahrens T, Evans P, Kilshaw P. Recognition of E-cadherin by integrin alpha(E)beta(7): requirement for cadherin dimerization and implications for cadherin and integrin function. J Biol Chem. 2001;276(33):30862-70. doi: 10.1074/jbc.M101712200. PubMed PMID: 11413131.

Crowe, S.E., Alvarez, L., Dytoc, M., Hunt, R.H., Muller, M., Sherman, P., Patel, J., Jin, Y., and Ernst, P.B. (1995). Expression of interleukin 8 and CD54 by human gastric epithelium after Helicobacter pylori infection in vitro. *Gastroenterology* 108**,** 65-74.

Czarnewski P, Das S, Parigi SM, Villablanca EJ. Retinoic Acid and Its Role in Modulating Intestinal Innate Immunity. Nutrients. 2017;9(1). doi: 10.3390/nu9010068. PubMed PMID: 28098786; PMCID: PMC5295112.

De Franceschi, N., Hamidi, H., Alanko, J., Sahgal, P., and Ivaska, J. (2015). Integrin traffic - the update. *J Cell Sci* 128**,** 839-852.

de Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA, Yazdanbakhsh M, Kapsenberg ML. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. J Immunol. 2002;168(4):1704-9. doi: 10.4049/jimmunol.168.4.1704. PubMed PMID: 11823500.

del Rio, M. L., Bernhardt, G., Rodriguez-Barbosa, J. I., Forster, R. (2010) Development and functional specialization of CD103+ dendritic cells. Immunol Rev 234, 268-81.

den Hartog, G., van Altena, C., Savelkoul, H. F., van Neerven, R. J. (2013) The mucosal factors retinoic acid and TGF-beta1 induce phenotypically and functionally distinct dendritic cell types. Int Arch Allergy Immunol 162, 225-36.

Dilworth FJ, Chambon P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene. 2001;20(24):3047-54. doi: 10.1038/sj.onc.1204329. PubMed PMID: 11420720.

Ding SZ, Torok AM, Smith MF, Jr., Goldberg JB. Toll-like receptor 2-mediated gene expression in epithelial cells during Helicobacter pylori infection. Helicobacter. 2005;10(3):193-204. doi: 10.1111/j.1523-5378.2005.00311.x. PubMed PMID: 15904477.

Dunn, K.W., Kamocka, M.M., and Mcdonald, J.H. (2011). A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol* 300**,** C723-742.

Edwards AD, Manickasingham SP, Sporri R, Diebold SS, Schulz O, Sher A, Kaisho T, Akira S, Reis e Sousa C. Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. J Immunol. 2002;169(7):3652-60. doi: 10.4049/jimmunol.169.7.3652. PubMed PMID: 12244157.

Efstathiou, J.A., Liu, D., Wheeler, J.M., Kim, H.C., Beck, N.E., Ilyas, M., Karayiannakis, A.J., Mortensen, N.J., Kmiot, W., Playford, R.J., Pignatelli, M., and Bodmer, W.F. (1999). Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells. *Proc Natl Acad Sci U S A* 96**,** 2316-2321.

Eksteen, B., Mora, J. R., Haughton, E. L., Henderson, N. C., Turner, L. L., Villablanca, E. J., Curbishley, S. M., Aspinall, A. I., von Andrian, U. H., Adams, D. H. (2009) Gut Homing Receptors on CD8 T Cells Are Retinoic Acid Dependent and Not Maintained by Liver Dendritic or Stellate Cells. Gastroenterology.

Erkelens MN, Mebius RE. Retinoic Acid and Immune Homeostasis: A Balancing Act. Trends Immunol. 2017;38(3):168-80. doi: 10.1016/j.it.2016.12.006. PubMed PMID: 28094101.

Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. J Cell Biol. 2009;187(5):733-47. doi: 10.1083/jcb.200904054. PubMed PMID: 19951918; PMCID: PMC2806590.

Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, Furtado GC, Lira SA, Shakhar G. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity. 2013;38(3):581-95. doi: 10.1016/j.immuni.2013.01.009. PubMed PMID: 23395676; PMCID: PMC4115273.

Figueiredo C, Van Doorn LJ, Nogueira C, Soares JM, Pinho C, Figueira P, Quint WG, Carneiro F. Helicobacter pylori genotypes are associated with clinical outcome in Portuguese patients and show a high prevalence of infections with multiple strains. Scand J Gastroenterol. 2001;36(2):128-35. doi: 10.1080/003655201750065861. PubMed PMID: 11252403.

Foitzik, K., Spexard, T., Nakamura, M., Halsner, U., Paus, R. (2005) Towards dissecting the pathogenesis of retinoid-induced hair loss: all-trans retinoic acid induces premature hair follicle regression (catagen) by upregulation of transforming growth factor-beta2 in the dermal papilla. J Invest Dermatol 124, 1119-26.

Gandhi, R., Anderson, D. E., Weiner, H. L. (2007) Cutting Edge: Immature human dendritic cells express latency-associated peptide and inhibit T cell activation in a TGF-beta-dependent manner. J Immunol 178, 4017-21.

Gout, S., Marie, C., Laine, M., Tavernier, G., Block, M.R., and Jacquier-Sarlin, M. (2004). Early enterocytic differentiation of HT-29 cells: biochemical changes and strength increases of adherens junctions. *Exp Cell Res* 299**,** 498-510.

Green HN, Mellanby E. Vitamin a as an Anti-Infective Agent. Br Med J. 1928;2(3537):691-6. doi: 10.1136/bmj.2.3537.691. PubMed PMID: 20774205; PMCID: PMC2456524.

Gu, Z., Noss, E.H., Hsu, V.W., and Brenner, M.B. (2011). Integrins traffic rapidly via circular dorsal ruffles and macropinocytosis during stimulated cell migration. *J Cell Biol* 193**,** 61-70.

Guerra, F., and Bucci, C. (2016). Multiple Roles of the Small GTPase Rab7. *Cells* 5**,** E34.

Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, Segura E, Tussiwand R, Yona S. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol. 2014;14(8):571-8. doi: 10.1038/nri3712. PubMed PMID: 25033907; PMCID: PMC4638219.

Gyongyosi A, Szatmari I, Pap A, Dezso B, Pos Z, Szeles L, Varga T, Nagy L. RDH10, RALDH2, and CRABP2 are required components of PPARgamma-directed ATRA synthesis and signaling in human dendritic cells. J Lipid Res. 2013;54(9):2458-74. doi: 10.1194/jlr.M038984. PubMed PMID: 23833249; PMCID: PMC3735943.

Habtezion, A., Nguyen, L.P., Hadeiba, H., and Butcher, E.C. (2016). Leukocyte Trafficking to the Small Intestine and Colon. *Gastroenterology* 150**,** 340-354.

Hadley, G. A. and Higgins, J. M. (2014) Integrin alphaEbeta7: molecular features and functional significance in the immune system. Advances in experimental medicine and biology 819, 97-110.

Haniffa, M., Collin, M., and Ginhoux, F. (2013). Ontogeny and functional specialization of dendritic cells in human and mouse. *Adv Immunol* 120**,** 1-49.

Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology. 1996;111(2):419-25. doi: 10.1053/gast.1996.v111.pm8690207. PubMed PMID: 8690207.

Harris, P.R., Wright, S.W., Serrano, C., Riera, F., Duarte, I., Torres, J., Pena, A., Rollan, A., Viviani, P., Guiraldes, E., Schmitz, J.M., Lorenz, R.G., Novak, L., Smythies, L.E., and Smith, P.D. (2008). Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology* 134**,** 491-499.

Higgins JM, Cernadas M, Tan K, Irie A, Wang J, Takada Y, Brenner MB. The role of alpha and beta chains in ligand recognition by beta 7 integrins. J Biol Chem. 2000;275(33):25652-64. doi: 10.1074/jbc.M001228200. PubMed PMID: 10837471.

Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, Belkaid Y, Mathis D, Benoist C. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition

from CD4+CD44hi Cells. Immunity. 2008;29(5):758-70. doi: 10.1016/j.immuni.2008.09.018. PubMed PMID: 19006694; PMCID: PMC3140207.

Hitzler, I., Oertli, M., Becher, B., Agger, E.M., and Muller, A. (2011). Dendritic cells prevent rather than promote immunity conferred by a helicobacter vaccine using a mycobacterial adjuvant. *Gastroenterology* 141**,** 186-196, 196 e181.

Huang Z, Liu Y, Qi G, Brand D, Zheng SG. Role of Vitamin A in the Immune System. J Clin Med. 2018;7(9). doi: 10.3390/jcm7090258. PubMed PMID: 30200565; PMCID: PMC6162863.

Hubert, P., Caberg, J.H., Gilles, C., Bousarghin, L., Franzen-Detrooz, E., Boniver, J., and Delvenne, P. (2005). E-cadherin-dependent adhesion of dendritic and Langerhans cells to keratinocytes is defective in cervical human papillomavirus-associated (pre)neoplastic lesions. *J Pathol* 206**,** 346-355.

Iliev ID, Spadoni I, Mileti E, Matteoli G, Sonzogni A, Sampietro GM, Foschi D, Caprioli F, Viale G, Rescigno M. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut. 2009;58(11):1481-9. doi: 10.1136/gut.2008.175166. PubMed PMID: 19570762.

Iliev, I.D., Mileti, E., Matteoli, G., Chieppa, M., and Rescigno, M. (2009a). Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol* 2**,** 340-350.

Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., Hill, C. S. (2002) SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 62, 65-74.

Ito, M., Maruyama, T., Saito, N., Koganei, S., Yamamoto, K., and Matsumoto, N. (2006). Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. *J Exp Med* 203**,** 289-295.

Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004;21(4):527-38. doi: 10.1016/j.immuni.2004.08.011. PubMed PMID: 15485630.

Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, Berg PL, Davidsson T, Powrie F, Johansson-Lindbom B, Agace WW. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. J Exp Med. 2008;205(9):2139-49. doi: 10.1084/jem.20080414. PubMed PMID: 18710932; PMCID: PMC2526207.

Jaensson-Gyllenback E, Kotarsky K, Zapata F, Persson EK, Gundersen TE, Blomhoff R, Agace WW. Bile retinoids imprint intestinal CD103+ dendritic cells with the ability

to generate gut-tropic T cells. Mucosal Immunol. 2011;4(4):438-47. doi: 10.1038/mi.2010.91. PubMed PMID: 21289617; PMCID: PMC3130189.

Jaensson-Gyllenback E, Kotarsky K, Zapata F, Persson EK, Gundersen TE, Blomhoff R, Agace WW. Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. Mucosal Immunol. 2011;4(4):438-47. doi: 10.1038/mi.2010.91. PubMed PMID: 21289617; PMCID: PMC3130189.

Jakob, T., Brown, M.J., and Udey, M.C. (1999). Characterization of E-cadherin-containing junctions involving skin-derived dendritic cells. *J Invest Dermatol* 112**,** 102-108.

Jarnicki AG, Conroy H, Brereton C, Donnelly G, Toomey D, Walsh K, Sweeney C, Leavy O, Fletcher J, Lavelle EC, Dunne P, Mills KH. Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. J Immunol. 2008;180(6):3797-806. doi: 10.4049/jimmunol.180.6.3797. PubMed PMID: 18322186.

Jenkinson, S. E., Whawell, S. A., Swales, B. M., Corps, E. M., Kilshaw, P. J., Farthing, P. M. (2011) The alphaE(CD103)beta7 integrin interacts with oral and skin keratinocytes in an E-cadherin-independent manner*. Immunology 132, 188-96.

Jin, C. J., Hong, C. Y., Takei, M., Chung, S. Y., Park, J. S., Pham, T. N., Choi, S. J., Nam, J. H., Chung, I. J., Kim, H. J., Lee, J. J. (2010) All-trans retinoic acid inhibits the differentiation, maturation, and function of human monocyte-derived dendritic cells. Leukemia research 34, 513-20.

Jones BG, Oshansky CM, Bajracharya R, Tang L, Sun Y, Wong SS, Webby R, Thomas PG, Hurwitz JL. Retinol binding protein and vitamin D associations with serum antibody isotypes, serum influenza virus-specific neutralizing activities and airway cytokine profiles. Clin Exp Immunol. 2016;183(2):239-47. doi: 10.1111/cei.12718. PubMed PMID: 26425827; PMCID: PMC4711169.

Kabisch R, Mejias-Luque R, Gerhard M, Prinz C. Involvement of Toll-like receptors on Helicobacter pylori-induced immunity. PLoS One. 2014;9(8):e104804. doi: 10.1371/journal.pone.0104804. PubMed PMID: 25153703; PMCID: PMC4143222.

Kam RK, Deng Y, Chen Y, Zhao H. Retinoic acid synthesis and functions in early embryonic development. Cell Biosci. 2012;2(1):11. doi: 10.1186/2045-3701-2-11. PubMed PMID: 22439772; PMCID: PMC3325842.

Kang, J. S., Liu, C., Derynck, R. (2009) New regulatory mechanisms of TGF-beta receptor function. Trends Cell Biol. 19, 385-394.

Kao JY, Zhang M, Miller MJ, Mills JC, Wang B, Liu M, Eaton KA, Zou W, Berndt BE, Cole TS, Takeuchi T, Owyang SY, Luther J. Helicobacter pylori immune escape is mediated by dendritic cell-induced Treg skewing and Th17 suppression in mice. Gastroenterology. 2010;138(3):1046-54. doi: 10.1053/j.gastro.2009.11.043. PubMed PMID: 19931266; PMCID: PMC2831148.

Karjalainen, M., Kakkonen, E., Upla, P., Paloranta, H., Kankaanpaa, P., Liberali, P., Renkema, G.H., Hyypia, T., Heino, J., and Marjomaki, V. (2008). A Raft-derived, Pak1-regulated entry participates in alpha2beta1 integrin-dependent sorting to caveosomes. *Mol Biol Cell* 19**,** 2857-2869.

Khandelwal, P., Ruiz, W.G., and Apodaca, G. (2010). Compensatory endocytosis in bladder umbrella cells occurs through an integrin-regulated and RhoA- and dynamin-dependent pathway. *EMBO J* 29**,** 1961-1975.

Kim JM, Kim JS, Yoo DY, Ko SH, Kim N, Kim H, Kim YJ. Stimulation of dendritic cells with Helicobacter pylori vacuolating cytotoxin negatively regulates their maturation via the restoration of E2F1. Clin Exp Immunol. 2011;166(1):34-45. doi: 10.1111/j.1365-2249.2011.04447.x. PubMed PMID: 21910723; PMCID: PMC3193917.

Kim, M. J., Jeong, E. K., Kwon, E. Y., Joo, J. Y., Lee, J. Y., Choi, J. (2014) Human CD103(+) dendritic cells promote the differentiation of Porphyromonas gingivalis heat shock protein peptide-specific regulatory T cells. J Periodontal Implant Sci 44, 235-41.

Klebanoff CA, Spencer SP, Torabi-Parizi P, Grainger JR, Roychoudhuri R, Ji Y, Sukumar M, Muranski P, Scott CD, Hall JA, Ferreyra GA, Leonardi AJ, Borman ZA, Wang J, Palmer DC, Wilhelm C, Cai R, Sun J, Napoli JL, Danner RL, Gattinoni L, Belkaid Y, Restifo NP. Retinoic acid controls the homeostasis of pre-cDC-derived splenic and intestinal dendritic cells. J Exp Med. 2013;210(10):1961-76. doi: 10.1084/jem.20122508. PubMed PMID: 23999499; PMCID: PMC3782040.

Kranzer, K., Eckhardt, A., Aigner, M., Knoll, G., Deml, L., Speth, C., Lehn, N., Rehli, M., Schneider-Brachert, W. (2004) Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. Infect.Immun. 72, 4416-4423.

Krishnan, M., Lapierre, L.A., Knowles, B.C., and Goldenring, J.R. (2013). Rab25 regulates integrin expression in polarized colonic epithelial cells. *Mol Biol Cell* 24**,** 818-831.

Lacaz-Vieira, F. (1997). Calcium site specificity. Early Ca2+-related tight junction events. *J Gen Physiol* 110**,** 727-740.

Larange A, Cheroutre H. Retinoic Acid and Retinoic Acid Receptors as Pleiotropic Modulators of the Immune System. Annu Rev Immunol. 2016;34:369-94. doi: 10.1146/annurev-immunol-041015-055427. PubMed PMID: 27168242.

Lay, M. K., Atmar, R. L., Guix, S., Bharadwaj, U., He, H., Neill, F. H., Sastry, K. J., Yao, Q., Estes, M. K. (2010) Norwalk virus does not replicate in human macrophages or dendritic cells derived from the peripheral blood of susceptible humans. Virology 406, 1-11.

Le Floc'h A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, Chouaib S, Mami-Chouaib F. Alpha E beta 7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. J Exp Med. 2007;204(3):559-70. doi: 10.1084/jem.20061524. PubMed PMID: 17325197; PMCID: PMC2137907.

Le Floc'h, A., Jalil, A., Franciszkiewicz, K., Validire, P., Vergnon, I., and Mami-Chouaib, F. (2011). Minimal engagement of CD103 on cytotoxic T lymphocytes with an E-cadherin-Fc molecule triggers lytic granule polarization via a phospholipase Cgamma-dependent pathway. *Cancer Res* 71**,** 328-338.

Lobert, V.H., Brech, A., Pedersen, N.M., Wesche, J., Oppelt, A., Malerod, L., and Stenmark, H. (2010). Ubiquitination of alpha 5 beta 1 integrin controls fibroblast migration through lysosomal degradation of fibronectin-integrin complexes. *Dev Cell* 19**,** 148-159.

Mane, V., and Muro, S. (2012). Biodistribution and endocytosis of ICAM-1-targeting antibodies versus nanocarriers in the gastrointestinal tract in mice. *Int J Nanomedicine* 7**,** 4223-4237.

Manicassamy S, Pulendran B. Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin Immunol. 2009;21(1):22-7. doi: 10.1016/j.smim.2008.07.007. PubMed PMID: 18778953; PMCID: PMC2642591.

Mann, E. R., Bernardo, D., English, N. R., Landy, J., Al-Hassi, H. O., Peake, S. T., Man, R., Elliott, T. R., Spranger, H., Lee, G. H., Parian, A., Brant, S. R., Lazarev, M., Hart, A. L., Li, X., Knight, S. C. (2015) Compartment-specific immunity in the human gut: properties and functions of dendritic cells in the colon versus the ileum. Gut.

Maritzen, T., Schachtner, H., and Legler, D.F. (2015). On the move: endocytic trafficking in cell migration. *Cell Mol Life Sci*.

Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S, Hisatake K, Nogi Y. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. J

Biol Chem. 2004;279(44):45969-79. doi: 10.1074/jbc.M408795200. PubMed PMID: 15304486.

McDole, J. R., Wheeler, L. W., McDonald, K. G., Wang, B., Konjufca, V., Knoop, K. A., Newberry, R. D., Miller, M. J. (2012) Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature 483, 345-9.

Megraud F, Neman-Simha V, Brugmann D. Further evidence of the toxic effect of ammonia produced by Helicobacter pylori urease on human epithelial cells. Infect Immun. 1992;60(5):1858-63. PubMed PMID: 1563774; PMCID: PMC257085.

Miehlke S, Yu J, Schuppler M, Frings C, Kirsch C, Negraszus N, Morgner A, Stolte M, Ehninger G, Bayerdorffer E. Helicobacter pylori vacA, iceA, and cagA status and pattern of gastritis in patients with malignant and benign gastroduodenal disease. Am J Gastroenterol. 2001;96(4):1008-13. doi: 10.1111/j.1572-0241.2001.03685.x. PubMed PMID: 11316139.

Mokrani M, Klibi J, Bluteau D, Bismuth G, Mami-Chouaib F. Smad and NFAT pathways cooperate to induce CD103 expression in human CD8 T lymphocytes. J Immunol. 2014;192(5):2471-9. doi: 10.4049/jimmunol.1302192. PubMed PMID: 24477908.

Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, Mebius RE. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. J Immunol. 2011;186(4):1934-42. doi: 10.4049/jimmunol.1001672. PubMed PMID: 21220692.

Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science. 2006;314(5802):1157-60. doi: 10.1126/science.1132742. PubMed PMID: 17110582.

Moss, S.F., and Malfertheiner, P. (2007). Helicobacter and gastric malignancies. *Helicobacter*. 12 Suppl 1**,** 23-30.

Moustakas, A. and Heldin, C. H. (2009) The regulation of TGFbeta signal transduction. Development 136, 3699-3714.

Murphy TL, Grajales-Reyes GE, Wu X, Tussiwand R, Briseno CG, Iwata A, Kretzer NM, Durai V, Murphy KM. Transcriptional Control of Dendritic Cell Development. Annu Rev Immunol. 2016;34:93-119. doi: 10.1146/annurev-immunol-032713-120204. PubMed PMID: 26735697; PMCID: PMC5135011.

Muzaki, A. R., Tetlak, P., Sheng, J., Loh, S. C., Setiagani, Y. A., Poidinger, M., Zolezzi, F., Karjalainen, K., Ruedl, C. (2015) Intestinal CD103CD11b dendritic cells restrain colitis via IFN-gamma-induced anti-inflammatory response in epithelial cells. Mucosal Immunol.

Nagafuchi, A., and Takeichi, M. (1988). Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO J* 7**,** 3679-3684.

Nakahara T, Uchi H, Urabe K, Chen Q, Furue M, Moroi Y. Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. Int Immunol. 2004;16(12):1701-9. doi: 10.1093/intimm/dxh171. PubMed PMID: 15477228.

Napoli, J. L. (1986) Quantification of physiological levels of retinoic acid. Methods in enzymology 123, 112-24.

Necchi, V., Manca, R., Ricci, V., and Solcia, E. (2009). Evidence for transepithelial dendritic cells in human H. pylori active gastritis. *Helicobacter* 14**,** 208-222.

Ni, H., Li, A., Simonsen, N., and Wilkins, J.A. (1998). Integrin activation by dithiothreitol or Mn2+ induces a ligand-occupied conformation and exposure of a novel NH2-terminal regulatory site on the beta1 integrin chain. *J Biol Chem* 273**,** 7981-7987.

Niles RM. Signaling pathways in retinoid chemoprevention and treatment of cancer. Mutat Res. 2004;555(1-2):81-96. doi: 10.1016/j.mrfmmm.2004.05.020. PubMed PMID: 15476854.

Nizzoli G, Krietsch J, Weick A, Steinfelder S, Facciotti F, Gruarin P, Bianco A, Steckel B, Moro M, Crosti M, Romagnani C, Stolzel K, Torretta S, Pignataro L, Scheibenbogen C, Neddermann P, De Francesco R, Abrignani S, Geginat J. Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. Blood. 2013;122(6):932-42. doi: 10.1182/blood-2013-04-495424. PubMed PMID: 23794066.

Nizzoli G, Larghi P, Paroni M, Crosti MC, Moro M, Neddermann P, Caprioli F, Pagani M, De Francesco R, Abrignani S, Geginat J. IL-10 promotes homeostatic proliferation of human CD8(+) memory T cells and, when produced by CD1c(+) DCs, shapes naive CD8(+) T-cell priming. Eur J Immunol. 2016;46(7):1622-32. doi: 10.1002/eji.201546136. PubMed PMID: 27129615.

Oertli M, Sundquist M, Hitzler I, Engler DB, Arnold IC, Reuter S, Maxeiner J, Hansson M, Taube C, Quiding-Jarbrink M, Muller A. DC-derived IL-18 drives Treg differentiation, murine Helicobacter pylori-specific immune tolerance, and asthma protection. J Clin Invest. 2012;122(3):1082-96. doi: 10.1172/JCI61029. PubMed PMID: 22307326; PMCID: PMC3287234.

Ohoka, Y., Yokota-Nakatsuma, A., Maeda, N., Takeuchi, H., Iwata, M. (2014) Retinoic acid and GM-CSF coordinately induce retinal dehydrogenase 2 (RALDH2) expression through cooperation between the RAR/RXR complex and Sp1 in dendritic cells. PLoS One 9, e96512.

Osz J, Brelivet Y, Peluso-Iltis C, Cura V, Eiler S, Ruff M, Bourguet W, Rochel N, Moras D. Structural basis for a molecular allosteric control mechanism of cofactor binding to nuclear receptors. Proc Natl Acad Sci U S A. 2012;109(10):E588-94. doi: 10.1073/pnas.1118192109. PubMed PMID: 22355136; PMCID: PMC3309777.

Parsonnet J, Friedman GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. Gut. 1997;40(3):297-301. doi: 10.1136/gut.40.3.297. PubMed PMID: 9135515; PMCID: PMC1027076.

Paul, N.R., Jacquemet, G., and Caswell, P.T. (2015). Endocytic Trafficking of Integrins in Cell Migration. *Curr Biol* 25**,** R1092-1105.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45. doi: 10.1093/nar/29.9.e45. PubMed PMID: 11328886; PMCID: PMC55695.

Piskunov A, Rochette-Egly C. A retinoic acid receptor RARalpha pool present in membrane lipid rafts forms complexes with G protein alphaQ to activate p38MAPK. Oncogene. 2012;31(28):3333-45. doi: 10.1038/onc.2011.499. PubMed PMID: 22056876.

Prahalad P, Dakshanamurthy S, Ressom H, Byers SW. Retinoic acid mediates regulation of network formation by COUP-TFII and VE-cadherin expression by TGFbeta receptor kinase in breast cancer cells. PLoS One. 2010;5(4):e10023. doi: 10.1371/journal.pone.0010023. PubMed PMID: 20386594; PMCID: PMC2850308.

Qian F, Deng J, Cheng N, Welch EJ, Zhang Y, Malik AB, Flavell RA, Dong C, Ye RD. A non-redundant role for MKP5 in limiting ROS production and preventing LPS-induced vascular injury. EMBO J. 2009;28(19):2896-907. doi: 10.1038/emboj.2009.234. PubMed PMID: 19696743; PMCID: PMC2760111.

Racke MK, Burnett D, Pak SH, Albert PS, Cannella B, Raine CS, McFarlin DE, Scott DE. Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. J Immunol. 1995;154(1):450-8. PubMed PMID: 7527821.

Rad, R., Ballhorn, W., Voland, P., Eisenacher, K., Mages, J., Rad, L., Ferstl, R., Lang, R., Wagner, H., Schmid, R. M., Bauer, S., Prinz, C., Kirschning, C. J., Krug, A. (2009) Extra- and Intracellular Pattern Recognition Receptors Cooperate in the Recognition of Helicobacter pylori. Gastroenterology.

Rainero, E., and Norman, J.C. (2013). Late endosomal and lysosomal trafficking during integrin-mediated cell migration and invasion: cell matrix receptors are trafficked through the late endosomal pathway in a way that dictates how cells migrate. *Bioessays* 35**,** 523-532.

Rampal, R., Awasthi, A., Ahuja, V. (2016) Retinoic acid-primed human dendritic cells inhibit Th9 cells and induce Th1/Th17 cell differentiation. J Leukoc Biol.

Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine. 2015;74(1):5-17. doi: 10.1016/j.cyto.2014.09.011. PubMed PMID: 25458968; PMCID: PMC4416069.

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol. 2001;2(4):361-7. doi: 10.1038/86373. PubMed PMID: 11276208.

Riedl, E., Stockl, J., Majdic, O., Scheinecker, C., Rappersberger, K., Knapp, W., Strobl, H. (2000) Functional involvement of E-cadherin in TGF-beta 1-induced cell cluster formation of in vitro developing human Langerhans-type dendritic cells. J Immunol 165, 1381-6.

Roberts MS, Woods AJ, Dale TC, Van Der Sluijs P, Norman JC. Protein kinase B/Akt acts via glycogen synthase kinase 3 to regulate recycling of alpha v beta 3 and alpha 5 beta 1 integrins. Mol Cell Biol. 2004;24(4):1505-15. doi: 10.1128/mcb.24.4.1505-1515.2004. PubMed PMID: 14749368; PMCID: PMC344170.

Robinson, K., Kenefeck, R., Pidgeon, E.L., Shakib, S., Patel, S., Polson, R.J., Zaitoun, A.M., and Atherton, J.C. (2008). Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T cell responses. *Gut* 57**,** 1375-1385.

Robinson, P. W., Green, S. J., Carter, C., Coadwell, J., Kilshaw, P. J. (2001) Studies on transcriptional regulation of the mucosal T-cell integrin alphaEbeta7 (CD103). Immunology 103, 146-54.

Roe MM, Swain S, Sebrell TA, Sewell MA, Collins MM, Perrino BA, Smith PD, Smythies LE, Bimczok D. Differential regulation of CD103 (alphaE integrin) expression in human dendritic cells by retinoic acid and Toll-like receptor ligands. J Leukoc Biol. 2017;101(5):1169-80. doi: 10.1189/jlb.1MA0316-131R. PubMed PMID: 28087652; PMCID: PMC5380378.

Roesler BM, Rabelo-Goncalves EM, Zeitune JM. Virulence Factors of Helicobacter pylori: A Review. Clin Med Insights Gastroenterol. 2014;7:9-17. doi: 10.4137/CGast.S13760. PubMed PMID: 24833944; PMCID: PMC4019226.

Rubino M, Miaczynska M, Lippe R, Zerial M. Selective membrane recruitment of EEA1 suggests a role in directional transport of clathrin-coated vesicles to early endosomes. J Biol Chem. 2000;275(6):3745-8. doi: 10.1074/jbc.275.6.3745. PubMed PMID: 10660521.

Russell GJ, Parker CM, Cepek KL, Mandelbrot DA, Sood A, Mizoguchi E, Ebert EC, Brenner MB, Bhan AK. Distinct structural and functional epitopes of the alpha E beta 7 integrin. Eur J Immunol. 1994;24(11):2832-41. doi: 10.1002/eji.1830241138. PubMed PMID: 7525307.

Satpathy AT, Kc W, Albring JC, Edelson BT, Kretzer NM, Bhattacharya D, Murphy TL, Murphy KM. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. J Exp Med. 2012;209(6):1135-52. doi: 10.1084/jem.20120030. PubMed PMID: 22615127; PMCID: PMC3371733.

Saurer, L., McCullough, K. C., Summerfield, A. (2007) In vitro induction of mucosa-type dendritic cells by all-trans retinoic acid. J Immunol. 179, 3504-3514.

Schittenhelm, L., Hilkens, C.M., and Morrison, V.L. (2017). beta2 Integrins As Regulators of Dendritic Cell, Monocyte, and Macrophage Function. *Front Immunol* 8**,** 1866.

Schlickum, S., Sennefelder, H., Friedrich, M., Harms, G., Lohse, M. J., Kilshaw, P., Schon, M. P. (2008) Integrin alpha E(CD103)beta 7 influences cellular shape and motility in a ligand-dependent fashion. Blood 112, 619-25.

Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, Ho AW, See P, Shin A, Wasan PS, Hoeffel G, Malleret B, Heiseke A, Chew S, Jardine L, Purvis HA, Hilkens CM, Tam J, Poidinger M, Stanley ER, Krug AB, Renia L, Sivasankar B, Ng LG, Collin M, Ricciardi-Castagnoli P, Honda K, Haniffa M, Ginhoux F. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. Immunity. 2013;38(5):970-83. doi: 10.1016/j.immuni.2013.04.011. PubMed PMID: 23706669; PMCID: PMC3666057.

Schon, M.P., Arya, A., Murphy, E.A., Adams, C.M., Strauch, U.G., Agace, W.W., Marsal, J., Donohue, J.P., Her, H., Beier, D.R., Olson, S., Lefrancois, L., Brenner, M.B., Grusby, M.J., and Parker, C.M. (1999). Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J Immunol* 162**,** 6641-6649.

Schulz, O., Jaensson, E., Persson, E. K., Liu, X., Worbs, T., Agace, W. W., Pabst, O. (Dec 21) Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med 206, 3101-14.

Scott, C.L., Aumeunier, A.M., and Mowat, A.M. (2011). Intestinal CD103+ dendritic cells: master regulators of tolerance? *Trends Immunol* 32**,** 412-419.

Segura E, Durand M, Amigorena S. Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells. J Exp Med. 2013;210(5):1035-47. doi: 10.1084/jem.20121103. PubMed PMID: 23569327; PMCID: PMC3646495.

Segura, E. (2016). Review of Mouse and Human Dendritic Cell Subsets. *Methods Mol Biol* 1423**,** 3-15.

Serrano, C., Wright, S.W., Bimczok, D., Shaffer, C.L., Cover, T.L., Venegas, A., Salazar, M.G., Smythies, L.E., Harris, P.R., and Smith, P.D. (2013). Downregulated Th17 responses are associated with reduced gastritis in Helicobacter pylori-infected children. *Mucosal Immunol*.

Shen B, Delaney MK, Du X. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. Curr Opin Cell Biol. 2012;24(5):600-6. doi: 10.1016/j.ceb.2012.08.011. PubMed PMID: 22980731; PMCID: PMC3479359.

Shen, B., Delaney, M.K., and Du, X. (2012). Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Curr Opin Cell Biol* 24**,** 600-606.

Shiu, J., and Blanchard, T.G. (2013). Dendritic cell function in the host response to Helicobacter pylori infection of the gastric mucosa. *Pathog Dis* 67**,** 46-53.

Sittig SP, Bakdash G, Weiden J, Skold AE, Tel J, Figdor CG, de Vries IJ, Schreibelt G. A Comparative Study of the T Cell Stimulatory and Polarizing Capacity of Human Primary Blood Dendritic Cell Subsets. Mediators Inflamm. 2016;2016:3605643. doi: 10.1155/2016/3605643. PubMed PMID: 27057096; PMCID: PMC4761397.

Sommer A. Vitamin A deficiency, child health, and survival. Nutrition. 1997;13(5):484-5. doi: 10.1016/s0899-9007(97)00013-0. PubMed PMID: 9225350.

Sonnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol. 2000;149(4):901-14. doi: 10.1083/jcb.149.4.901. PubMed PMID: 10811830; PMCID: PMC2174575.
Sonnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol. 2000;149(4):901-14. doi: 10.1083/jcb.149.4.901. PubMed PMID: 10811830; PMCID: PMC2174575.

Stevens GA, Bennett JE, Hennocq Q, Lu Y, De-Regil LM, Rogers L, Danaei G, Li G, White RA, Flaxman SR, Oehrle SP, Finucane MM, Guerrero R, Bhutta ZA, Then-Paulino A, Fawzi W, Black RE, Ezzati M. Trends and mortality effects of vitamin A deficiency in children in 138 low-income and middle-income countries between 1991 and 2013: a pooled analysis of population-based surveys. Lancet Glob Health. 2015;3(9):e528-36. doi: 10.1016/S2214-109X(15)00039-X. PubMed PMID: 26275329.

Strauch UG, Grunwald N, Obermeier F, Gurster S, Rath HC. Loss of CD103+ intestinal dendritic cells during colonic inflammation. World J Gastroenterol. 2010;16(1):21-9. doi: 10.3748/wjg.v16.i1.21. PubMed PMID: 20039445; PMCID: PMC2799913.

Suzuki, R., Nakao, A., Kanamaru, Y., Okumura, K., Ogawa, H., Ra, C. (2002) Localization of intestinal intraepithelial T lymphocytes involves regulation of alphaEbeta7 expression by transforming growth factor-beta. Int Immunol 14, 339-45.

Swain S, Roe MM, Sebrell TA, Sidar B, Dankoff J, VanAusdol R, Smythies LE, Smith PD, Bimczok D. CD103 (alphaE Integrin) Undergoes Endosomal Trafficking in Human Dendritic Cells, but Does Not Mediate Epithelial Adhesion. Front Immunol. 2018;9:2989. doi: 10.3389/fimmu.2018.02989. PubMed PMID: 30622531; PMCID: PMC6308147.

Tang, A., Amagai, M., Granger, L.G., Stanley, J.R., and Udey, M.C. (1993). Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* 361**,** 82-85.

Taraszka, K.S., Higgins, J.M., Tan, K., Mandelbrot, D.A., Wang, J.H., and Brenner, M.B. (2000). Molecular basis for leukocyte integrin alpha(E)beta(7) adhesion to epithelial (E)-cadherin. *J Exp Med* 191**,** 1555-1567.

Tobita T, Takeshita A, Kitamura K, Ohnishi K, Yanagi M, Hiraoka A, Karasuno T, Takeuchi M, Miyawaki S, Ueda R, Naoe T, Ohno R. Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid. Blood. 1997;90(3):967-73. PubMed PMID: 9242525.

Tokuda H, Hatakeyama D, Akamatsu S, Tanabe K, Yoshida M, Shibata T, Kozawa O. Involvement of MAP kinases in TGF-beta-stimulated vascular endothelial growth factor synthesis in osteoblasts. Arch Biochem Biophys. 2003;415(1):117-25. doi: 10.1016/s0003-9861(03)00225-x. PubMed PMID: 12801520.

Triantafilou, M., Gamper, F. G., Lepper, P. M., Mouratis, M. A., Schumann, C., Harokopakis, E., Schifferle, R. E., Hajishengallis, G., Triantafilou, K. (2007) Lipopolysaccharides from atherosclerosis-associated bacteria antagonize TLR4, induce formation of TLR2/1/CD36 complexes in lipid rafts and trigger TLR2-induced inflammatory responses in human vascular endothelial cells. Cell Microbiol 9, 2030-9.

Ullrich O, Reinsch S, Urbe S, Zerial M, Parton RG. Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol. 1996;135(4):913-24. doi: 10.1083/jcb.135.4.913. PubMed PMID: 8922376; PMCID: PMC2133374.

Uno, K., Kato, K., Atsumi, T., Suzuki, T., Yoshitake, J., Morita, H., Ohara, S., Kotake, Y., Shimosegawa, T., Yoshimura, T. (2007) Toll-like receptor (TLR) 2 induced

through TLR4 signaling initiated by Helicobacter pylori cooperatively amplifies iNOS induction in gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 293, G1004-12.

Van Den Bossche, J., Malissen, B., Mantovani, A., De Baetselier, P., and Van Ginderachter, J.A. (2012). Regulation and function of the E-cadherin/catenin complex in cells of the monocyte-macrophage lineage and DCs. *Blood* 119**,** 1623-1633.

Van Roy, F., and Berx, G. (2008). The cell-cell adhesion molecule E-cadherin. *Cell Mol Life Sci* 65**,** 3756-3788.

Venerito M, Vasapolli R, Rokkas T, Malfertheiner P. Gastric cancer: epidemiology, prevention, and therapy. Helicobacter. 2018;23 Suppl 1:e12518. doi: 10.1111/hel.12518. PubMed PMID: 30203589.

Vermot J, Gallego Llamas J, Fraulob V, Niederreither K, Chambon P, Dolle P. Retinoic acid controls the bilateral symmetry of somite formation in the mouse embryo. Science. 2005;308(5721):563-6. doi: 10.1126/science.1108363. PubMed PMID: 15731404.

Vernerey, F.J., and Farsad, M. (2014). A mathematical model of the coupled mechanisms of cell adhesion, contraction and spreading. *J Math Biol* 68**,** 989-1022.

Viladomiu, M., Bassaganya-Riera, J., Tubau-Juni, N., Kronsteiner, B., Leber, A., Philipson, C.W., Zoccoli-Rodriguez, V., and Hontecillas, R. (2017). Cooperation of Gastric Mononuclear Phagocytes with Helicobacter pylori during Colonization. *J Immunol* 198**,** 3195-3204.

Vlasova AN, Chattha KS, Kandasamy S, Siegismund CS, Saif LJ. Prenatally acquired vitamin A deficiency alters innate immune responses to human rotavirus in a gnotobiotic pig model. J Immunol. 2013;190(9):4742-53. doi: 10.4049/jimmunol.1203575. PubMed PMID: 23536630; PMCID: PMC3633669.

Wagner, M., Han, B., Jessell, T. M. (1992) Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. Development 116, 55-66.

Wang G, Ge Z, Rasko DA, Taylor DE. Lewis antigens in Helicobacter pylori: biosynthesis and phase variation. Mol Microbiol. 2000;36(6):1187-96. doi: 10.1046/j.1365-2958.2000.01934.x. PubMed PMID: 10931272.

Watanabe K, Sugai M, Nambu Y, Osato M, Hayashi T, Kawaguchi M, Komori T, Ito Y, Shimizu A. Requirement for Runx proteins in IgA class switching acting downstream of TGF-beta 1 and retinoic acid signaling. J Immunol. 2010;184(6):2785-92. doi:

Watchmaker PB, Lahl K, Lee M, Baumjohann D, Morton J, Kim SJ, Zeng R, Dent A, Ansel KM, Diamond B, Hadeiba H, Butcher EC. Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. Nat Immunol. 2014;15(1):98-108. doi: 10.1038/ni.2768. PubMed PMID: 24292363; PMCID: PMC3942165.

Watchmaker, P. B., Lahl, K., Lee, M., Baumjohann, D., Morton, J., Kim, S. J., Zeng, R., Dent, A., Ansel, K. M., Diamond, B., Hadeiba, H., Butcher, E. C. (2014) Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. Nat Immunol 15, 98-108.

Wu CC, Hsu SC, Shih HM, Lai MZ. Nuclear factor of activated T cells c is a target of p38 mitogen-activated protein kinase in T cells. Mol Cell Biol. 2003;23(18):6442-54. doi: 10.1128/mcb.23.18.6442-6454.2003. PubMed PMID: 12944472; PMCID: PMC193716.

Ye, F., Kim, C., and Ginsberg, M.H. (2012). Reconstruction of integrin activation. *Blood* 119**,** 26-33.

Yoshizawa, M., Miyazaki, H., Kojima, S. (1998) Retinoids potentiate transforming growth factor-beta activity in bovine endothelial cells through up-regulating the expression of transforming growth factor-beta receptors. J Cell Physiol 176, 565-73.

Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J. 2002;21(14):3749-59. doi: 10.1093/emboj/cdf366. PubMed PMID: 12110587; PMCID: PMC126112.

Zamani M, Ebrahimtabar F, Zamani V, Miller WH, Alizadeh-Navaei R, Shokri-Shirvani J, Derakhshan MH. Systematic review with meta-analysis: the worldwide prevalence of Helicobacter pylori infection. Aliment Pharmacol Ther. 2018;47(7):868-76. doi: 10.1111/apt.14561. PubMed PMID: 29430669.

Zeng R, Bscheider M, Lahl K, Lee M, Butcher EC. Generation and transcriptional programming of intestinal dendritic cells: essential role of retinoic acid. Mucosal Immunol. 2016;9(1):183-93. doi: 10.1038/mi.2015.50. PubMed PMID: 26129652; PMCID: PMC4698111.

Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, Butcher EC. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. Mucosal Immunol. 2013;6(4):847-56. doi: 10.1038/mi.2012.123. PubMed PMID: 23235743; PMCID: PMC3612556.

Zhang, K., and Chen, J. (2012). The regulation of integrin function by divalent cations. *Cell Adh Migr* 6**,** 20-29.