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β8 integrin expression and activation of TGF-β by intestinal dendritic cells is determined by both tissue microenvironment and cell lineage

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Abstract

Activation of TGF-β by dendritic cells (DCs) expressing αvβ8 integrin is essential for the generation of intestinal regulatory T cells (Tregs) that in turn promote tolerance to intestinal antigens. We have recently shown that αvβ8 integrin is preferentially expressed by $CD103^+$ DCs, and confers their ability to activate TGF-β and generate Tregs. However, how these DCs become specialized for this vital function is unknown. Here we show that β8 expression is controlled by a combination of factors that include DC lineage, and signals derived from the tissue microenvironment and microbiota. Specifically, our data demonstrate that TGF-β itself, along with retinoic acid (RA) and Toll-like receptor (TLR) signaling, drive expression of αvβ8 in DCs. However, these signals only result in high levels of β8 expression in cells of the cDC1 lineage, $CD8α^+$ or $CD103^+CD11b^-$ DCs, and this is associated with epigenetic changes in the *Itgb8* locus. Together, these data provide a key illustrative example of how microenvironmental factors and cell

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DISCLOSURE

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lineage drive the generation of regulatory αvβ8-expressing DCs specialized for activation of TGF-β to facilitate Treg generation.

INTRODUCTION

Dendritic Cells (DCs) serve a unique sentinel role in the body, surveying tissues, integrating peripheral cues and instructing the adaptive immune system accordingly. DCs can both orchestrate powerful pathogen-directed immunity or regulate and suppress immune responses to self-associated or innocuous antigens, and the complexity of these roles is reflected in the diverse populations of DCs found in different tissues and under different conditions (1, 2). Determining how DCs differentiate to carry out specialized functions is critical for our understanding of immunity in health and disease.

The intestine provides a particular challenge for the immune system, containing a high local concentration of microbes, including commensals and potential pathogens, as well as diverse dietary and environmental antigens (3). To prevent inappropriate inflammatory responses to these mostly innocuous antigens, the mucosal immune system has robust immunoregulatory mechanisms, which include regulatory lymphocytes, which reside in the mucosa and associated lymphoid organs and actively suppress immune responses to intestinal antigens (4). The best characterized of these are $CD4^+$ $Foxp3^+$ regulatory T cells, which originate either in the thymus (‘thymic’ or ‘natural’ Tregs), or are generated in the periphery from naïve $CD4^+$ T cells (‘peripheral’ or ‘adaptive’ Tregs). In the intestine, peripheral Tregs are generated by DCs that constitutively acquire and present self and foreign antigens (5, 6), resulting in antigen-specific tolerance (7). The generation of peripheral Tregs requires TGF-β, and we and others have shown that an essential characteristic of DCs that generate Tregs is their ability to activate TGF-β from its inactive or ‘latent’ precursor, to an active form that can engage the TGF-β receptor (8, 9). This requires the action of a specific cell surface integrin, αvβ8, and underscoring the importance of this process, deletion of either the αv or β8 subset from DCs results in failure to generate intestinal Tregs and subsequent development of colitis (10, 11).

Recently we have shown that expression of αvβ8 is tightly regulated in DCs. While the αv subunit, the only known partner of β8 (12), is ubiquitously expressed, β8 expression is restricted to specific subsets of cells in the intestine (8). Under homeostatic conditions, αvβ8 is expressed predominantly on DCs from mesenteric lymph nodes (MLN) and intestinal lamina propria that express the mucosal integrin αEβ7 (CD103), conferring on these cells their preferential ability to activate TGF-β and generate Tregs (8, 9). $CD103^+$ DCs have previously been implicated in the generation of intestinal Tregs, which has also been attributed to their ability to synthesize all-trans retinoic acid (RA), which promotes Treg generation in the presence of TGF-β. These data therefore support the concept that subsets of intestinal DCs are specialized for generation of Tregs. However, the precise mechanisms by which this population of DCs acquires this specialized ability to activate TGF-β and how microenvironmental cues and cell lineage are integrated in this process remain to be fully determined.

In this study, we set out to identify the signals that regulate β8 expression in intestinal DCs. We report that αvβ8 is expressed preferentially on the $CD103^{+}CD11b^{-}$ subset of DCs in the MLNs, and that expression is acquired in the LP. We show that signals from the mucosal microenvironment, specifically TGF-β, retinoic acid (RA) and Toll-like receptor (TLR) agonists, together promote expression of β8 integrin in DCs and that inhibition of signaling through these pathways in mice leads to reduction in αvβ8 expression by DCs. Furthermore, we provide evidence that DC lineage is critical in establishing DC subset-specific expression of β8, demonstrating that DCs derived from the CD103/CD8α cDC1 lineage respond more robustly to these conditioning factors to upregulate β8. Together these data show that the combination of cell lineage, immune mediators and both dietary and microbe-derived environmental factors shape intestinal DCs into critical gatekeepers of TGF-β-dependent immune responses through regulation of β8 integrin expression.

MATERIALS AND METHODS

Mice

All animals were housed under specific pathogen free conditions at Plateau de Biologie Expérimentale de la Souris (PBES, ENS Lyon, France), at Benaroya Research Institute (BRI, Seattle, WA) or at Massachusetts General Hospital (MGH, Boston, MA). Female C57BL/6 mice from Charles River Laboratories (L'Arbesle, France) or The Jackson Laboratory (Bar Harbor, ME) were used between 6 and 15 weeks of age. Vitamin A deficient (VAD) mice were generated at MGH. Pregnant C57BL/6 mice were maintained on a vitamin A sufficient (VA+) diet (4 IU/g, TestDiet 58M1) through day 10 of gestation and then switched to a VAD diet (TestDiet 5T2P, both diets Pharmaserv Inc., Framingham, MA) through weaning. Pups were then maintained either on VA+ or VAD diets. Antibiotic-treated mice were generated at PBES. Mice were maintained on drinking water containing 1 g/L metronidazole, 1 g/L ampicillin, 1 g/L neomycin (all from Sigma-Aldrich, St. Quentin Fallavier, France) and 500 mg/L vancomycin (Mylan, St Priest, France) for 4 weeks. Transgenic CD11-Cre (B6.Cg-Tg(Itgax-cre)1-1Reiz/J) and *Ccr7*$^{-/-}$ (B6.129P2(C)-*Ccr7*^{tm1Rfor}/J) mice were obtained from The Jackson Laboratory. *Myd88*$^{-/-}$ and *Trif*$^{-/-}$ mice were provided by Rodrigo Mora (MGH, Boston, MA). T*gfbr2*$^{fl/fl}$ mice (B6.129S6-*Tgfbr2*^{tm1Hlm}) were obtained from Harold Moses (13) (Vanderbilt-Ingram Cancer Center, Nashville, TN). Foxp3$^{IRES\text{-}eGFP}$ reporter mice were obtained from Dominique Kaiserlian (CIRI, Lyon, France). All mice were on C57BL/6 background except for *Tgfbr2-flox* mice that were on a mixed B6.129S6 background. Animal experiments were performed under appropriate licenses within local and national guidelines for animal care.

Dendritic cell isolation

DCs from spleen, MLN and lamina propria were isolated as previously described (8). BMDCs generated in the presence of Flt3-L (FL-DCs) were cultured as previously described (14). Briefly, bone marrow cells were cultured in the presence of 100 ng/ml recombinant Flt3 ligand (Peprotech, Neuilly-sur-Seine, France). Fresh medium was added to the cultures on day 3 and 6, and cells were harvested at day 9. FL-DC subsets were then sorted by flow cytometry as indicated.

DC conditioning in culture

Cells were stimulated *in vitro* at 37°C in X-vivo 15 medium (Lonza, Levallois-Perret, France) supplemented with MEM non-essential amino acids, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-β-mercaptoethanol (all, Life Technologies, Saint Aubin, France). Unless stated otherwise, the stimuli were used at the following final concentrations: 0.2 ng/ml TGF-β (R&D biosystems, Lille, France), 0.2 μM all-trans retinoic acid (Sigma-Aldrich), 5 ng/ml TSLP, 5 ng/ml IL-10, 5 ng/ml IL-1β, 5 ng/ml IFN-γ (all, Peprotech), 2% culture supernatant containing GM-CSF (4 ng/ml final), 500 ng/ml Pam3CSK4, 10^8 cells/ml HKLM, 1 μg/ml poly(I:C), 100 ng/ml LPS-EK, 100 ng/ml ST-FLA, 100 ng/ml FSL1, 1 μg/ml ssRNA40/LyoVec, 2.5 μM ODN1826 (Mouse TLR1-9 agonist kit, InvivoGen, Toulouse, France).

β8 integrin expression analysis

Itgb8 gene expression by quantitative RT-PCR for MLN and spleen DCs was performed as previously described (8). For lamina propria DCs, *Itgb8* gene expression was quantified by nested RT-PCR using the following pre-amplification primers, Fw-AGTGAACAATAGATGTGGCTC, Rev-CCGTCATTCGGCACCACTAT, and qPCR primers, Fw-TGGCCCTTTATTCCCGTGAC, Rev-GGGTGGATACTAATGTATGGCGA. β8 integrin protein expression was measured by Western blot as previously described (8) using an anti-β8 antiserum generated in rabbit using the C-terminal tail of the human β8 cytoplasmic region, and provided by Joseph McCarty (15).

In vitro Treg generation assay

In vitro Treg generation assay was performed as previously described (8). For RGD blockade experiments, cRGD or control cRAD peptides (both Enzo Life Sciences, Villeurbanne, France) were added at 2 μg/ml, as previously described (16).

Antibodies

The following antibodies were used: anti-CD11c-PE-Cy7 (HL3), anti-I-A/I-E-FITC (2G9), anti-CD8α-APC (53-6.7), anti-CD45RB/B220-PerCP-Cy5.5 (RA3-6B2), anti-CD103-PE (M290), anti-CD172/Sirpα-PE (P84), anti-CD24-FITC (M1/69), anti-CD11b-PE (M1/70; all from BD Biosciences, Pont de Claix, France), anti-I-A/I-E-APC (M5/114.15.2), anti-CD103-PerCP-Cy5.5 (2E7; Biolegend, Ozyme, Sant-Quentin-Yvelines, France), and anti-CD86-FITC (B7-2), anti-CD11b-APC-eFluor780 (M1/70; eBioscience, Paris, France).

Computational analysis

ChIP-seq datasets of the $CD24^+$ and $CD172^+$ FL-DCs were retrieved from Gene Expression Omnibus (GEO, GSE66899) (17). Raw reads were cleaned using Trimmomatic-0.33 (18) with the following parameters: ILLUMINACLIP:adapters.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:30. The remaining sequences were aligned to the mouse genome (NCBI37/mm9) by bowtie2 version 2.2.5 with default parameters (19). Density tracks were generated by the *makeUCSCfile* program from the HOMER software suite v4.7 (20). To allow comparison between the two subpopulations density profiles were normalized based on the library size.

Statistical analysis

Data were analyzed using GraphPad Prism Software 6.0a. Statistics were calculated using either unpaired *t* test when comparing two groups or one-way ANOVA with Dunnett's post-hoc test when comparing more than two groups. For grouped analysis, statistics were calculated using two-way ANOVA with Tukey's or Sidak's post-hoc test, where appropriate.

RESULTS

αvβ8 is preferentially expressed by CD103$^+$CD11b$^-$ DCs

Our previous studies revealed that under homeostatic conditions integrin αvβ8 is expressed preferentially in the CD103$^+$ subpopulation of MLN DCs, and confers on these DCs the ability to generate peripheral Tregs through activation of TGF-β (8, 9). Intestinal CD103$^+$ DCs comprise two distinct populations, distinguished by expression of CD11b, and in further analysis of MLN DCs we found that *Itgb8* (the gene encoding β8) was expressed at much higher levels in CD103$^+$CD11b$^-$ DCs than in the CD103$^+$CD11b$^+$ subset (Figure 1A–B), while αv integrin gene (*Itgav*) did not show any significant difference (supplementary figure 1A). Preferential expression of β8 integrin protein by CD103$^+$CD11b$^-$ MLN DCs was confirmed by Western Blot analysis (Figure 1C). The expression of high levels of β8 by these DCs was associated with their preferential ability to activate latent TGF-β (l-TGF-β) and generate Tregs (Figure 1D). Furthermore, this difference in TGF-β activation activity was dependent on αvβ8 ligand binding integrins, as treatment with an αv ligand mimetic peptide, cRGD specifically reduced the effects of l-TGF-β on induction of Tregs by CD103$^+$CD11b$^-$ DCs. The partial reduction of the effects of l-TGF-β by RGD on Treg generation is likely due to contaminating active TGF-β in the l-TGF-β preparation or to an additional αv-independent TGF-β activation pathway. β8 only pairs with the αv integrin subunit, and deletion of either integrin results in identical effects on TGF-β activation and Treg generation (8, 16) (9, 11), and we therefore feel confident attributing this activity to αvβ8. As these results closely resemble those we had previously reported with pooled CD103$^+$ DCs from MLN or from intestinal LP (8), we concluded that CD103$^+$CD11b$^-$ DCs were the cells responsible for the high levels of expression of αvβ8 and activation of TGF-β in the CD103$^+$ fraction.

CD103$^+$ DCs that acquire β8 expression are derived from the intestine

CD103$^+$CD11b$^-$ DCs in the MLN are composed of cells that develop in the intestinal LP and then migrate (21), and others that reside in the MLN, and these populations can be distinguished by relative levels of CD11c and MHC-II expression (22). Previous findings by ourselves and others that CD103$^+$ DCs derived from the LP can preferentially activate TGF-β in an αvβ8-dependent mechanism (8, 9) suggested that DCs may acquire expression of αvβ8 in the LP. Supporting this, we found that *Itgb8* expression is restricted to LP-derived migratory CD11c$^+$MHC-II^{hi}CD103$^+$CD11b$^-$ MLN DCs (mR1), while neither of the resident DC subsets expressed significant levels of β8 integrin (figure 2A). To investigate this further, we analyzed expression of *Itgb8* in DCs from *Ccr7*$^{-/-}$ mice. CCR7 is required for migration of DCs from the LP to MLN (6, 23, 24) and MLN of *Ccr7*$^{-/-}$ mice contain predominantly blood-derived DCs with reduced numbers of DCs that have migrated from the intestine ((22) and figure 2B). Notably, the expression of *Itgb8* was much lower in CD103$^+$CD11b$^-$ DCs

from *Ccr7*$^{-/-}$ cells than in cells from control mice, and was not significantly different from expression on CD103^{+}CD11b^{+} DCs (Figure 2C). These data therefore confirm that *Itgb8* is preferentially expressed on MLN CD103^{+}CD11b^{-} DCs that have migrated from the LP. We also measured expression of *Itgb8* in CD11c^{hi}MHC-II^{hi} cells purified directly from the LP, but in contrast to our findings with MLN DCs, we found that none of the major populations of CD11c^{hi}MHC-II^{hi} cells isolated from the LP (CD103^{+}CD11b^{-} DCs, CD103^{+}CD11b^{+} DCs or CD103^{-}CD11b^{+} DCs/macrophages) expressed *Itgb8* at levels equivalent to those seen in MLN (Figure 2D) when freshly isolated from tissue. This was in agreement with data reported by Atarashi *et al.*, who showed that αvβ8 was not expressed at significant levels by LP CD11c^{+} cells in the steady state (25).

TGF-β and RA contribute to DC β8 expression *in vivo*

The ability of MLN DCs to generate Tregs and regulate local and systemic immune responses is thought to be due to conditioning events that occur in the intestinal LP, and has been postulated to depend on a number of conditioning factors produced by epithelial cells, including TGF-β and retinoic acid (RA) (26–30). To determine whether these factors regulate expression of β8 integrin in intestinal DCs, we used genetic and dietary manipulation to inhibit potential conditioning factors in mice *in vivo*. We first investigated TGF-β signaling in DCs, making use of conditional knockout mice in which the TGF-β type II receptor is specifically deleted in DCs (*Cd11c-cre; Tgfbr2*$^{fl/fl}$, referred to as *Cd11c-Tgfbr2* KO mice). *Cd11c-Tgfbr2* KO mice develop normally until 4 weeks of age but then gradually develop severe multi-organ autoimmune inflammation associated with spontaneous colitis (31). In order to exclude the potential contribution of intestinal inflammation on β8 integrin, we used mice less than 5 week old with no clinical signs of colitis or wasting disease (figure 3A–B). CD103^{+}CD11b^{-} and CD103^{+}CD11b^{+} DCs were both present in MLNs of *Cd11c-Tgfbr2*-KO mice, at frequencies similar to those seen in control mice, suggesting that development of these cells was largely normal in the absence of TGF-β signaling (Figure 3C). However, CD103^{+}CD11b^{-} DCs from MLNs of *Cd11c-Tgfbr2* KO mice expressed much lower levels of *Itgb8* than equivalent DCs from control mice; indeed, expression was not significantly different from that seen in CD103^{+}CD11b^{+} DCs from either control or *Cd11c-Tgfbr2* KO mice (Figure 3D). These findings therefore established that TGF-β signaling in DCs is required for preferential expression of β8 in CD103^{+}CD11b^{-} DCs *in vivo*. We next assessed the role of RA in β8 expression. Mice were depleted of RA by maintenance on a vitamin A-deficient diet (VAD), as previously described (32, 33). Confirming that we had depleted RA signaling in DCs, expression of *Raldh1a2*, which is induced by RA in immune cells (30, 34), was completely lost in both CD103^{+}CD11b^{-} and CD103^{+}CD11b^{+} DCs from VAD mice (Figure 3E–F). *Itgb8* expression was also significantly reduced in CD103^{+}CD11b^{-} MLN DCs from VAD mice (Figure 3F, right panel), indicating that RA signaling also contributed to the preferential expression of αvβ8 in this DC subset *in vivo*.

Intestinal microbes and TLR signaling contribute to expression of *Itgb8* in DCs

The presence of intestinal microbes has been linked to effective development of LP DCs and establishment of normal mucosal immunoregulatory responses (30, 35, 36). To determine whether the intestinal microbiota contributed to induction of αvβ8 expression in DCs, we

treated mice with antibiotics, which reduced the presence of microbes in the intestine by 16-fold, assessed by measurement of 16S rDNA in stool (Figure 4A). As expected based on previous studies (22), intestinal $CD103^+$ $CD11b^-$ DCs were present and migrated normally to the MLN of antibiotic-treated animals; however, they expressed significantly lower levels of *Itgb8* than MLN DCs from untreated mice (Figure 4B–C), indicating a role for the intestinal microbiota in conditioning of these regulatory DCs. We next analyzed *Itgb8* expression in intestinal DCs from mice deficient in *Myd88* and *Trif*, components of the Toll-like receptor (TLR) signaling pathway by which DCs sense microbes. $CD103^+CD11b^-$ DCs from $Myd88^{-/-}$ mice had significantly reduced expression of *Itgb8* compared to DCs from control mice (Figure 4D). In contrast, deletion of TRIF did not significantly affect β8 expression, and showed no additive effect with MyD88 deficiency (Figure 4E–F). Importantly, in both $Myd88^{-/-}$ and in $Myd88^{-/-}$; $Trif^{-/-}$ double knockout mice (DKO), $CD103^+CD11b^-$ and $CD103^+CD11b^+$ MLN DCs were present in similar proportion than in control mice (figure 4G–H). Notably, the effects of *Myd88* deficiency on *Itgb8* expression were more pronounced than those of antibiotic treatment, which may be due to the continued presence of microbes in antibiotic treated mice, endogenous TLR ligands, or additional MyD88-dependent signaling pathways such as IL-1β.

Signals encountered in the mucosal environment induce β8 expression by direct effects on DCs

Together, these data indicated that TGF-β, RA and TLR signaling contributed to the expression of αvβ8 in intestinal DCs. To determine whether these stimuli could promote αvβ8 expression in DCs directly, we treated spleen DCs, which do not express β8 when freshly isolated (8), with TGF-β, RA and TLR ligands. Culture of spleen DCs with TGF-β induced a small increase in *Itgb8* (Figure 5A), whereas other cytokines associated with the intestinal microenvironment either had no effect on *Itgb8* expression (TSLP, IL-1β) or reduced expression (IL-10, IFN-γ, GM-CSF) (Figure 5A). Culture of DCs with RA alone had no effect on *Itgb8* expression, but when combined with TGF-β further promoted expression of *Itgb8* (Figure 5B–C). Hence, RA synergized with TGF-β signaling to increase gene expression, as has been previously shown for induction of $FoxP3^+$ Tregs in the intestine (28, 29). This synergistic effect did not appear to extend to other cytokines however, as combined treatment with TGF-β/RA with other cytokines or factors did not significantly increase *Itgb8* expression (Figure 5D).

We next tested the role of TLR signaling in DC induction of *Itgb8*. Stimulation of spleen DCs with the TLR9 ligand CpG oligodeoxynucleotide (ODN), and the TLR5 ligand Flagellin, both stimulated expression of *Itgb8* (Figure 6A). This induction of *Itgb8* was significantly enhanced by addition of RA/TGF-β which in combination with CpG ODN increased expression by greater than 10-fold over unstimulated DCs (Figure 6B), in line with the preferential expression of *Itgb8* that we have seen in MLN DCs. Together these data therefore show that TGF-β, RA and TLR ligands were sufficient to induce expression of *Itgb8* in DCs, and in combination induced levels similar to those seen *in vivo*.

β8 expression is differentially regulated in distinct DC subsets

These data therefore support the hypothesis that factors encountered in the intestinal LP induce β8 integrin expression in DCs. However, it is unclear why αvβ8 is expressed preferentially by the $CD103^+CD11b^-$ subset of intestinal DCs, and not by other DCs exposed to the intestinal microenvironment. One possible explanation is that distinct lineages of DCs show differential responses to *Itgb8*-inducing signals and we therefore tested the ability of individual subsets of spleen DCs to up-regulate *Itgb8* in response to conditioning signals in culture. Spleen DCs consist of two main subsets, $CD11b^-CD8\alpha^+$ DCs and $CD11b^+CD8\alpha^-$ DCs (Figure 7A: referred to here as $CD8\alpha^+$ and $CD11b^+$ DCs respectively). Sorted spleen $CD8\alpha^+$ DCs did not express significant levels of *Itgb8* when freshly isolated (Figure 7B), but gene expression could be detected after 8 hours in culture without additional stimulation (Figure 7C). Furthermore, treatment of $CD8\alpha^+$ DCs with the combination of TGF-β, RA and CpG ODN induced significant further increase in *Itgb8* expression (Figure 7C), as we had seen for cultures of total spleen DCs. Notably, western blot analysis showed that combined treatment with TGF-β, RA and CpG ODN increased both levels and duration of β8 protein expression (Figure 7D). In contrast, spleen $CD11b^+$ DCs did not express *Itgb8* spontaneously in culture (Figure 7C–D). Furthermore, although these cells did express *Itgb8* mRNA and β8 protein after stimulation with TGF-β, RA and CpG ODN, expression was significantly lower than seen in $CD8\alpha^+$ DCs, and was not sustained, diminishing to near background levels by 24–36 hours (Figure 7D). This differential induction of *Itgb8* in DC subsets was not due to a generalized inability of $CD11b^+$ DCs to respond to the conditioning factors, as these cells expressed similar or higher levels of TLRs and receptors for TGF-β and RA than MLN $CD103^+CD11b^-$ and spleen $CD8\alpha^+$ DCs (Supplementary Figure 1A and 2) and both spleen subsets showed equivalent levels of TGF-β and TLR signaling when stimulated (Supplementary Figure 3). Furthermore, consistent with their differential ability to express αvβ8 in culture, spleen $CD8\alpha^+$ DCs were able to generate Tregs in the presence of l-TGF-β through an αv-dependent mechanism whereas $CD11b^+$ DCs could not (Figure 7E). To determine whether similar differences in response to inducing stimuli may underlie differential expression of αvβ8 in intestinal DCs, we cultured MLN DCs with TGF-β, RA and CpG ODN and followed *Itgb8* expression. As we have shown in previous figures, in freshly isolated cells, *Itgb8* was expressed preferentially in $CD103^+CD11b^-$ DCs (R1) compared with $CD103^+CD11b^+$ DCs (R2) from MLN, or either population of spleen DCs (Figure 7B). Culture under *Itgb8*-inducing conditions for 8 hours increased expression of *Itgb8* in both $CD103^+CD11b^-$ and $CD103^+CD11b^+$ MLN DCs. However, the absolute level of *Itgb8* remained significantly lower in $CD103^+CD11b^+$ cells than $CD103^+CD11b^-$ (Supplementary figure 1B). Based on these results, we therefore concluded that the difference in αvβ8 expression between $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs could not be explained solely by differential exposure to conditioning factors, but was also determined by the ability of those factors to induce *Itgb8* expression.

Furthermore, recent studies establishing that splenic $CD8\alpha^+$ DCs (R4) and intestinal $CD103^+CD11b^-$ DCs (R1) share a common lineage (cDC1) (37–39), whereas spleen $CD11b^+$ DCs (R5) appear to be more closely related to intestinal $CD103^+CD11b^+$ DCs (R2)

of the cDC2 lineage (40–42) indicate that the CD8α$^+$/CD103$^+$CD11b$^-$ DC lineage may be uniquely specialized for high expression of αvβ8 and activation of TGF-β.

DC lineage determines the pattern of β8 gene expression

To further investigate whether lineage determines the ability of DCs to express αvβ8 in the intestinal microenvironment, DCs were generated from bone marrow precursors in culture with Flt3 ligand, and their ability to express β8 integrin measured. This method generates 2 main populations of DCs, CD24$^+$CD172$^-$CD11b^{low} DCs (CD24$^+$ FL-DCs), which resemble CD8α$^+$ or CD103$^+$CD11b$^+$ DCs, and CD24$^-$CD172$^+$CD11b$^+$ DCs, (CD172$^+$ FL-DCs), which are more related to CD11b$^+$ DCs (14, 38, 39) (Figure 8A). CD24$^+$ FL-DCs showed consistently higher levels of *Itgb8* expression than CD172$^+$ DCs from the same culture. Furthermore, this difference was significantly enhanced after stimulation with TGF-β, RA and CpG ODN (Figure 8B). These findings are consistent with our data from sorted spleen DCs subsets (Figure 7C), and suggest that the preferential ability of CD8α$^+$/CD103$^+$CD11b$^-$ DCs to express *Itgb8* expression is acquired during development. To determine whether this might be due to epigenetic programming, we compared the epigenetic landscape of the *Itgb8* locus in CD24$^+$ and CD172$^+$ FL-DCs, using existing chromatin immunoprecipitation followed by sequencing (ChIP-seq) datasets (17). In CD24$^+$ FL-DCS, we identified an active enhancer marked by both monomethylation of histone 3 lysine 4 (H3K4me1) and acetylation of histone 3 lysine 27 (H3K27ac) 74 kb upstream of the *Itgb8* coding region (Figure 8C; upper panel). This active enhancer was also associated with binding to the lineage specific transcription factors Batf3 and IRF8. The same site in CD172$^+$ FL-DCs showed reduced H3K4me1 marks and no H3K27ac (Figure 8C, lower panel), and no other H3K27ac peak could be detected in the vicinity of *Itgb8*, suggesting that this locus is poised but not active in these cells. The presence of an active enhancer 74 kb upstream of *Itgb8* specifically in CD24$^+$ FL-DCs is consistent with the preferential ability of these cells to express β8 integrin. Altogether, these data therefore demonstrate that cell lineage plays a critical role in permitting expression of β8 in response to conditioning factors from the microenvironment, and support the concept that the ability of DCs to express high levels of β8 integrin is determined during development.

DISCUSSION

Despite major advances in determining the origin of DCs subpopulations, our understanding of the factors that determine their functional diversity and tissue specialization remains limited. In this study, we have identified factors that contribute to differentiation of DCs specialized for generation of Tregs in the intestine. We show that expression of integrin αvβ8, which is required for activation of TGF-β by intestinal DCs, is controlled by factors that include DC lineage, tissue microenvironment and the microbiota. Specifically, we show that the combination of TGF-β and RA, factors found at high levels in the intestinal microenvironment, together with TLR signaling, promote expression of β8. However, these signals only result in high levels of αvβ8 in the CD8α$^+$/CD103$^+$CD11b$^-$ lineage of DCs. Together, these data provide a key illustrative example of how microenvironmental factors and cell lineage drive the generation of DCs that perform critical specialized functions, in this case the local activation of TGF-β to facilitate Treg generation.

Our data emphasize the critical role of signals derived from the mucosal environment, namely TGF-β, RA and TLR signaling, in promoting expression of αvβ8 both *in vitro* and *in vivo*. Furthermore, we find that MLN DCs expressing high levels of αvβ8 have emigrated from the intestine, further supporting the importance of intestinal conditioning in generation of $\alpha v\beta 8^{+}$ DCs. However, we find that expression of αvβ8 is consistently lower in $CD11c^{hi}$ cells freshly isolated from the LP than those from MLNs, suggesting to us that the process of migration and 'maturation' of DCs is also required for high expression of αvβ8. Supporting this, we have previously reported that $CD103^{+}$ LP DCs develop the ability to activate TGF-β through an αv-dependent mechanism when cultured with T cells (8), conditions that promote activation or maturation of DCs (43, 44). Whether $CD103^{+}$ DCs in the LP can acquire high levels of αvβ8, such as during inflammation, remains to be determined. Although RA and TGF-β can potentially be derived from various stromal cells in the intestine (27, 45), work from the Rescigno laboratory strongly supports epithelial cells as a likely source of RA and TGF-β for DC conditioning. Their laboratory have previously shown that co-culture of spleen DCs with intestinal epithelial cells or epithelial cell-derived culture supernatant promotes the ability of spleen DCs to generate gut-trophic Tregs (26), and that this is dependent on epithelial cell-derived TGF-β and RA. Our data indicate that up-regulation of expression of αvβ8 on DCs is the likely mechanism for this observation. Furthermore, the close association of $CD103^{+}$ DCs with the intestinal epithelium (46, 47) suggests this DC subset is ideally placed to receive these signals. Furthermore, there is increasing evidence that DCs associated with the intestinal epithelium interact with intestinal microbes or their products, providing a potential source of TLR signals to promote αvβ8 expression (47–49). It is likely, therefore, that intestinal DCs differ in their exposure to some or all of these stimuli due to their localization, and this may contribute to differential expression of αvβ8 in DC subsets. However, even when cultured under conditions that strongly promote αvβ8 upregulation, expression remained lower in $CD103^{+}CD11b^{+}$ mucosal DCs than $CD103^{+}CD11b^{-}$ DC. Our observation that $CD8\alpha^{+}$ DCs from spleen and $CD24^{+}$ FL-DCs also show high and sustained expression of αvβ8 when cultured in TGF-β, RA and TLR ligands led us to conclude that DC lineage also contributes to αvβ8 expression. Recent studies indicate that these DCs share a common cDC1 lineage with $CD103^{+}CD11b^{-}$ DCs, distinct from $CD11b^{+}$ DCs found in the intestine and spleen (38, 39, 50). We therefore propose a model in which the cDC1 lineage is programmed to respond to stimulation through TGF-β, RA and TLRs to express high levels of *Itgb8*, and that this leads to preferential expression of αvβ8 in $CD103^{+}CD11b^{-}$ DCs *in vivo*. Similar lineage-specific regulation of the *Itgb8* promoter has been reported by the Nishimura laboratory in mesenchymal cells, where the pro-inflammatory cytokine IL-1β promotes expression of *Itgb8* in lung fibroblasts and astrocytes, but not dermal fibroblasts. In further parallels with our studies, they show that this requires p38 and NF-κB signaling, which are activated downstream of both IL-1β and TLR signaling. IL-1β activates *Itgb8* expression by chromatin remodeling and exposure of binding sites for additional transcription factors, and the expression of *Itgb8* in different cell types corresponds with the chromatin state of the *Itgb8* core promoter (51, 52). p38α is reported to be required for *Itgb8* expression by $CD103^{+}$ MLN DCs, as well as for Treg generation and induction of oral tolerance in mice (53). Hence, we speculate that TLR stimulation likewise promotes expression of *Itgb8* through regulating accessibility of the core promoter, possibly governing access of TGF-β

and RA-induced transcription factors, and this is further controlled by lineage-specific factors acting at more distal sites. Our model is supported by the presence of an enhancer 74 kb upstream of *Itgb8,* which is preferentially active in the developmentally related $CD24^+$ BMDCs. It is currently unclear what transcription factors dictate this lineage specificity, and this is an active ongoing area of investigation. However, likely candidates include Batf3 and IRF8, which both bind this enhancer and are required for effective generation and maintenance of $CD8\alpha^+$ and $CD103^+CD11b^-$ DCs *in vivo* (Figure 8) (17, 38, 50). Batf3 and IRF8 might thus contribute to licensing the $CD8\alpha^+/CD103^+CD11b^-$ DCs lineage to express β8.

DC expression of αvβ8 is essential for generation of intestinal Tregs (8–11), and our data therefore implicate $CD103^+CD11b^-$ DCs as the major DC subset responsible for Treg generation in the intestine. $CD103^+$ DCs have also been implicated in intestinal Treg generation in previous studies (28, 29, 54), although this function has also been attributed to mucosal $CD103^+CD11b^+$ DCs, $CD103^-CX3CR1^{int}F4/80^-$ DCs or $CD103^-CX3CR1^+F4/80^+$ macrophages (55, 56). Deletion of all $CD103^+$ DC subsets also leads to a significant loss of intestinal Tregs in mice (57), further supporting the key role for this subset in Treg generation. However, although other *in vitro* studies support the specific involvement of the $CD103^+$ $CD11b^-$ subset in this process (53), deletion of either the $CD103^+CD11b^-$ population, through knockout of the transcription factors IRF8 or Batf3 (38, 50) or the $CD103^+CD11b^+$ population (41) alone does not lead to a detectable loss of intestinal Tregs or intestinal inflammation. Hence it is likely that both populations make some contribution to Treg generation *in vivo*, or can compensate for each other in this process. Although we have focused on $CD103^+CD11b^-$ DCs, low levels of *Itgb8* transcript can be detected in $CD103^+CD11b^+$ DCs at homeostasis, and these and other DC subsets can upregulate expression during infection or inflammation. Further studies will therefore be needed to determine the exact contribution of different DC subsets to Treg generation and regulation of intestinal inflammation.

In this study, we have focused on the factors that regulate αvβ8 expression by DCs during immune homeostasis, where the principal immunological role is likely to be generation of Tregs. In inflammatory settings, where DC αvβ8 is required for Th17 cell generation (16, 58), different mechanisms may be involved. Atarashi and colleagues have reported that microbe-derived ATP triggers Th17 responses *in vivo,* and that exogenous ATP promoted αvβ8 expression in DCs (25). However, in our experiments, ATP did not induce significant αvβ8 expression in spleen DCs when given alone or in combination with RA and TGF-β (data not shown). This discrepancy most likely reflects responses of different DC subsets, and whereas we focused on $CD103^+$ DCs, during intestinal infection ATP promotes expression of αvβ8 in $CD103^-$ $CD11b^+$ $CX3CR1^+$ $CD70^{hi}$ DCs. Hence Treg and Th17 responses appear to differ in both the conditioning signals that induce αvβ8 expression and the subsets of DCs involved. In this context, it is interesting that TGF-β, RA and TLR stimulation (our studies) or ATP stimulation (25) induce only transient expression of αvβ8 in inflammatory DCs, which is likely to favor Th17 over Treg differentiation (59). Conversely, we find that $CD103^+CD11b^-$ DCs exhibit high and sustained αvβ8 expression, which would be expected to induce regulatory responses. Despite these differences, our data

indicate that regulation of αvβ8 expression is a major factor determining the ability of DCs to generate Tregs and Th17 cells.

TGF-β and RA have been previously implicated in promoting Treg generation through direct effects on T cells, inducing expression of Foxp3 and intestinal homing receptors (28, 29, 32, 60–62). Our findings, and those of Rescigno and colleagues, that these same factors promote differentiation of Treg-generating DCs indicate that these environmental factors produce coordinated effects on the immune system, and highlight a 'feed-forward' effect, in which TGF-β signaling in DCs promotes expression of αvβ8, which then promotes TGF-β signaling in T cells. Likewise, RA signaling induces the expression of RA-synthesizing enzymes in DCs (30, 34). In this way, the intestinal microenvironment promotes DCs to 'recapitulate' the same environment in the MLN during T cell presentation, which we propose allows fine spatio-temporal regulation of TGF-β signaling and thus induction of appropriate mucosal responses and homing in T cells. In addition, it has recently been shown that αvβ8 is expressed by Tregs, and contributes to ongoing immune regulation (63); whether β8 expression is also activated by RA and TGF-β in T cells remains to be determined.

In conclusion, here we show that cell lineage and gut-derived factors together shape DCs into critical gatekeepers of TGF-β dependent intestinal immune responses via regulation of β8 expression. These results also provide a detailed demonstration of how acquisition of a specialized function comes from the unique ability of DC to integrate signals from the periphery and communicate this contextual information forward for induction of appropriate adaptive immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

αvβ8	alpha-v beta8 integrin
BMDC	bone marrow-derived dendritic cell

ChIP-seq	chromatin immunoprecipitation sequencing
CpG ODN	CpG oligodeoxynucleotides
DC	dendritic cell
Flt3-L	FMS-like tyrosine kinase 3 ligand
FL-DCs	BMDCs generated in the presence of Flt3-L
Itgb8	integrin beta8 gene
l-TGF-β	latent TGF-β
LP	lamina propria
MLN	mesenteric lymph node
RA	retinoic acid
SI	small intestine
Treg	regulatory T cell
TGFBR2	TGF-β receptor 2
TRIF	Toll/IL-1R-domain-containing adapter-inducing IFN-β
VAD	vitamin A deficient
VA+	vitamin A sufficient
WT	wild-type

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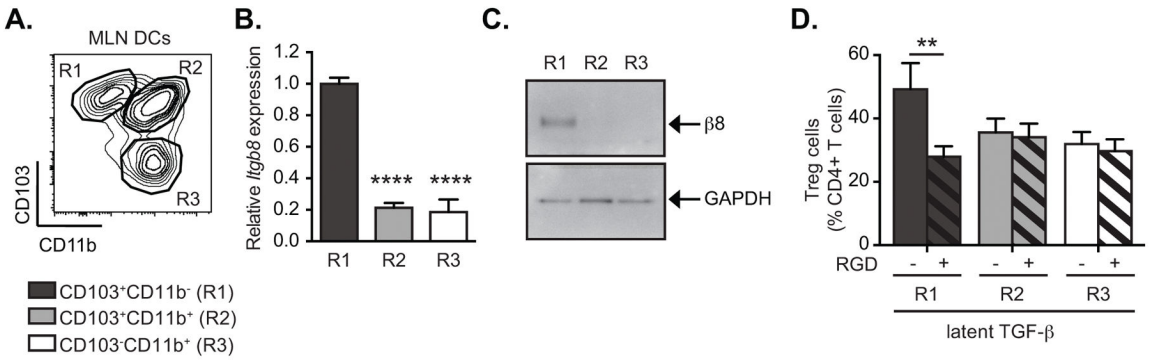

Figure 1. CD103^{+}CD11b^{-} MLN DCs express high levels of αvβ8 expression and activate TGF-β to generate Tregs

CD11c^{+} MLN DC subsets were sorted by FACS into three populations based on expression of CD103 and CD11b. (A) Representative FACS plot gated on CD11c^{+}MHC-II^{+} DCs from MLN shows gating strategy for isolating indicated populations. (B) Quantitative RT-PCR analysis of β8 integrin gene (*Itgb8*) expression relative to β-Actin (*Actb*) and presented relative to levels in CD103^{+}CD11b^{-} DCs (R1) in indicated MLN DC subpopulations. Histogram shows mean ± SEM from 3 individual experiments and at least 9 individual mice. (C) Western blot analysis of β8 integrin and GAPDH expression in indicated MLN DC subsets. (D) FACS-sorted MLN DC subsets were cultured with naïve CD4^{+}Foxp3^{GFP-} T cells in serum-free medium with or without addition of latent TGF-β, RGD (+, hatched bars) and/or RAD (−, solid bars) peptides as indicated. After 4 days in culture, Treg generation was assessed by Foxp3^{GFP} expression. Data show mean ± SEM of individual DC:T cell cultures with six independant pools of MLN DCs from two separate experiments. **, $p<0.005$; ****, $p<0.0001$; ANOVA with Dunnet's (B) or Sidak's (D) post-hoc test.

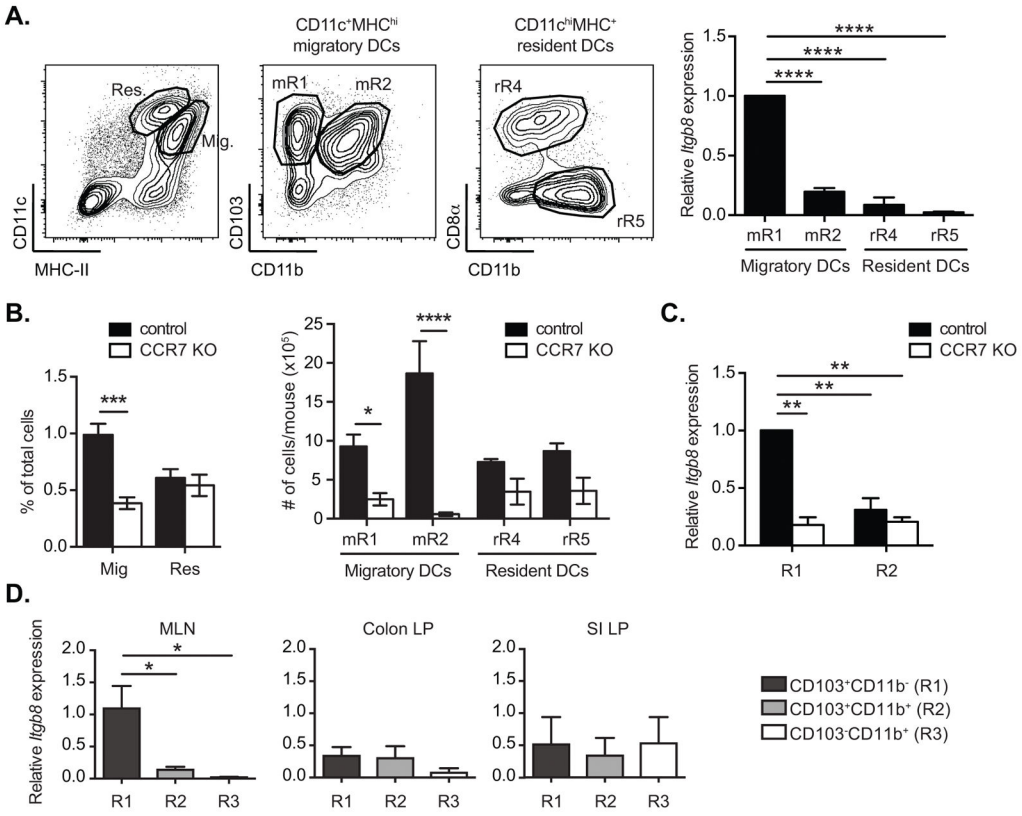

Figure 2. Migratory CD103^{+}CD11b^{-} DCs acquire β8 integrin expression in the lamina propria

(A) Quantitative RT-PCR analysis of β8 integrin gene (*Itgb8*) expression in migratory (Mig) CD11c^{+}MHC-II^{hi} and resident (Res) CD11c^{hi}MHC-II^{+} MLN DC subsets sorted by FACS based on expression of CD103 and CD11b (mR1, mR2) or CD8α and CD11b (rR4, rR5), respectively, using the gating strategy indicated in the representative FACS plots. Histogram shows mean ± SEM of 6 independent pools of mice from 2 separate experiments, presented relative to levels in CD103^{+}CD11b^{-} DCs (mR1). (B) Frequencies and numbers of indicated migratory and resident DC subsets gated as in (A) from MLN of CCR7$^{-/-}$ and control mice. (C–D) Quantitative (C) and nested (D) RT-PCR analysis of *Itgb8* expression relative to β-Actin (*Actb*) in indicated cell subsets sorted as in figure 1 from total CD11c^{+}MHC-II^{+} DCs from MLN of CCR7$^{-/-}$ and control mice (C) or CD11c^{hi}MHC-II^{hi} cells from MLN, colon and small intestinal (SI) lamina propria (LP) of WT mice (D). (B–D) Histogram shows mean ± SEM from at least 3 individual experiments and in (C) are presented relative to levels in CD103^{+}CD11b^{-} DCs. *, p< 0.05; ***, p<0.0005; two-way ANOVA with Tukey's (A, D) or Sidak's (B–C) post-hoc test.

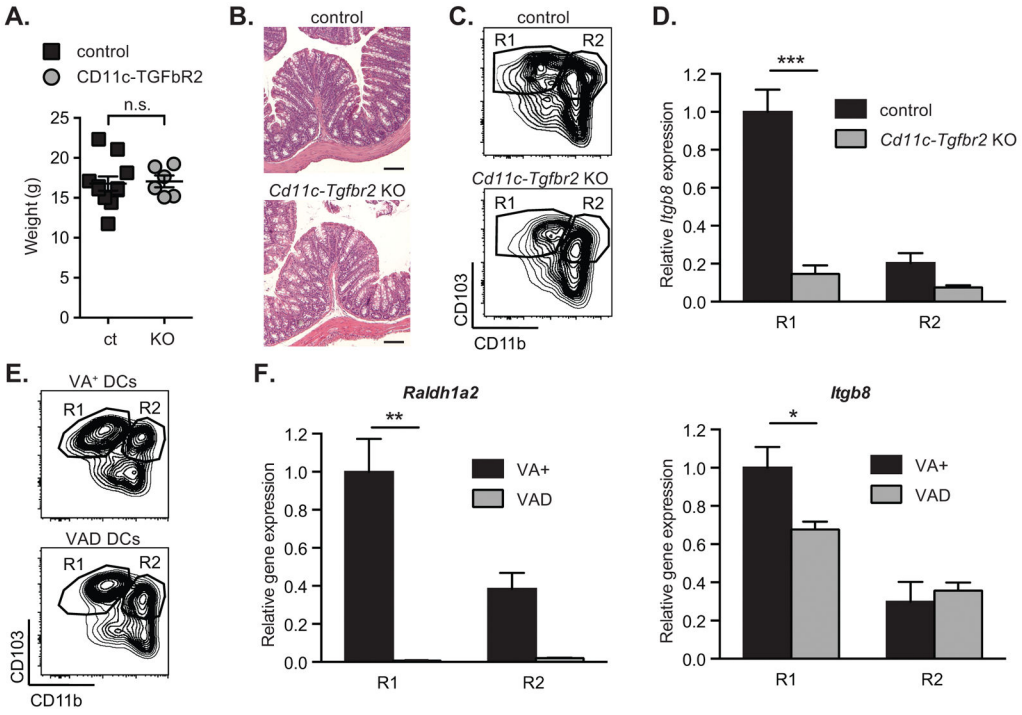

Figure 3. Reduced expression of β8 integrin expression in retinoic acid- and TGF-β signaling-deficient mice

β8 integrin (*Itgb8*) expression was assessed in MLN DC subpopulations FACS-sorted from control, *CD11c-Tgfbr2* KO mice (A–D) and animals fed with a vitamin A-deficient (VAD) or -sufficient (VA+) diet (E–F). (A) Weight and (B) representative haematoxylin- and eosin-stained sections of colons from *CD11c-Tgfbr2* KO and control mice. Scale bar, 100 μm. (C, E) Representative FACS plot of $CD11c^{+}MHC\text{-}II^{+}$ DCs from MLN showing gating strategy for isolating indicated populations. (D, F) Quantitative RT-PCR analysis of *Itgb8* (D, F right panel) and *Raldh1a2* (F, left panel) expression relative to *Actb* and presented relative to levels in control $CD103^{+}CD11b^{-}$ DCs (R1) in indicated MLN DC subpopulations. All histograms show mean ± SEM of at least 6 individual mice from 3 separate experiments. *, $p< 0.05$; **, $p<0.005$; ***, $p<0.0005$; two-way ANOVA with Tukey's post-hoc test.

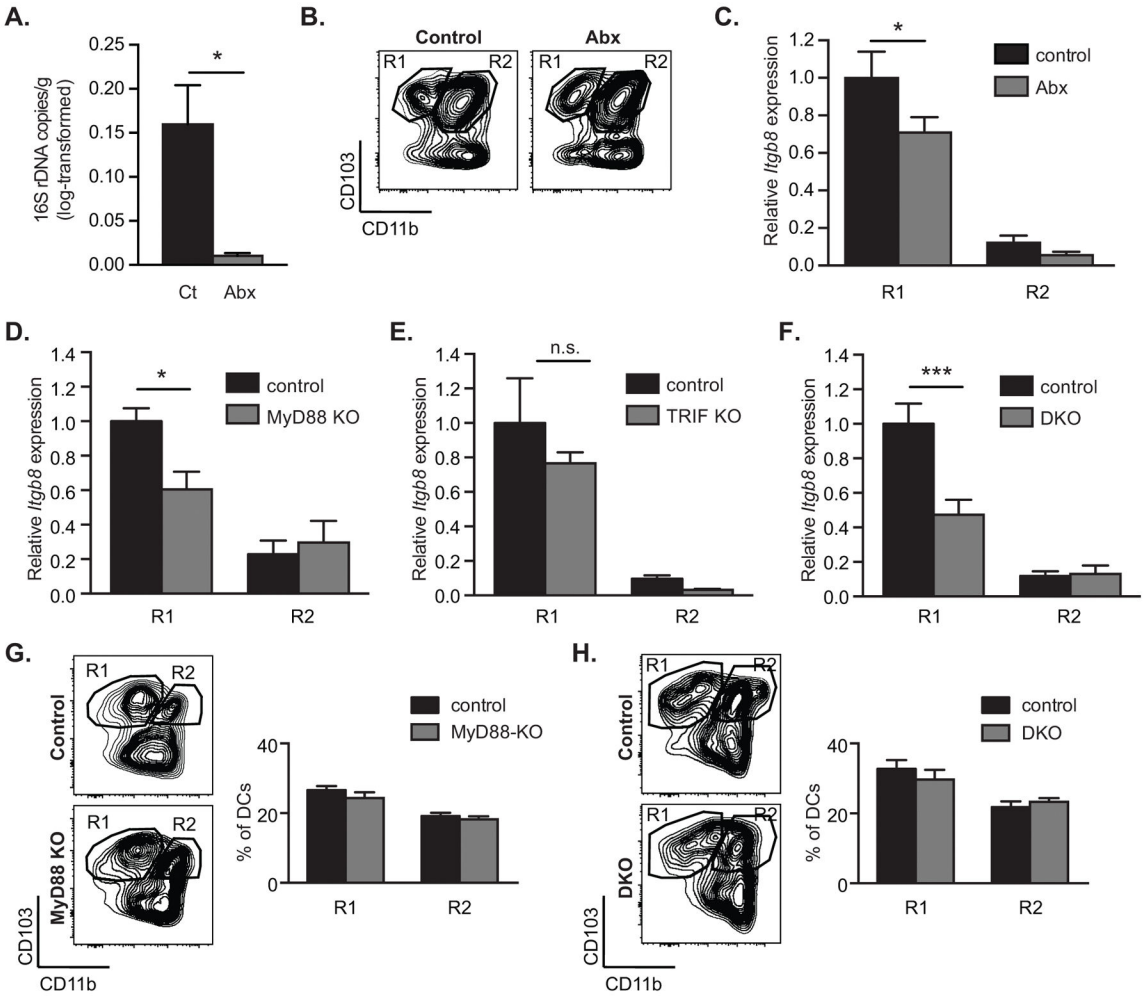

Figure 4. Intestinal microbes and MyD88 signaling promote β8 integrin expression in CD103$^+$CD11b$^-$ MLN DCs

β8 integrin (*Itgb8*) expression was assessed in MLN DC subpopulations FACS-sorted from control, antibiotics-treated (A–C), MyD88- (D, G), TRIF- (E) and MyD88-TRIF-deficient (F, H) mice. (A) Log-transformed 16S rDNA copy numbers per gram of stool in control and antibiotics-treated (Abx) animals, as determined using real-time PCR. (B) Representative FACS plot of CD11c$^+$MHC-II$^+$ DCs from MLN showing gating strategy for isolating indicated populations. (C–F) Quantitative RT-PCR analysis of *Itgb8* expression relative to *Actb* and presented relative to levels in control CD103$^+$CD11b$^-$ DCs (R1) in indicated MLN DC subpopulations in control and antibiotics-treated (C), MyD88-deficient (MyD88 KO) (D), TRIF-deficient (TRIF KO) (E) and MyD88-TRIF-deficient mice (DKO) (F). (G–H) Representative FACS plot and frequencies of CD11c$^+$MHC-II$^+$ DCs from MLN of MyD88-KO (G) and Myd88-TRIK DKO mice (H). Histograms show mean ± SEM of n individual mice from 1 (E, n=3), 2 (D, n≥8, F, n≥6) or 3 (C, n≥5) separate experiments. *, $p<0.05$; ***, $p<0.0005$; unpaired student *t* test (A) or two-way ANOVA with Tukey's post-hoc test (C–H).

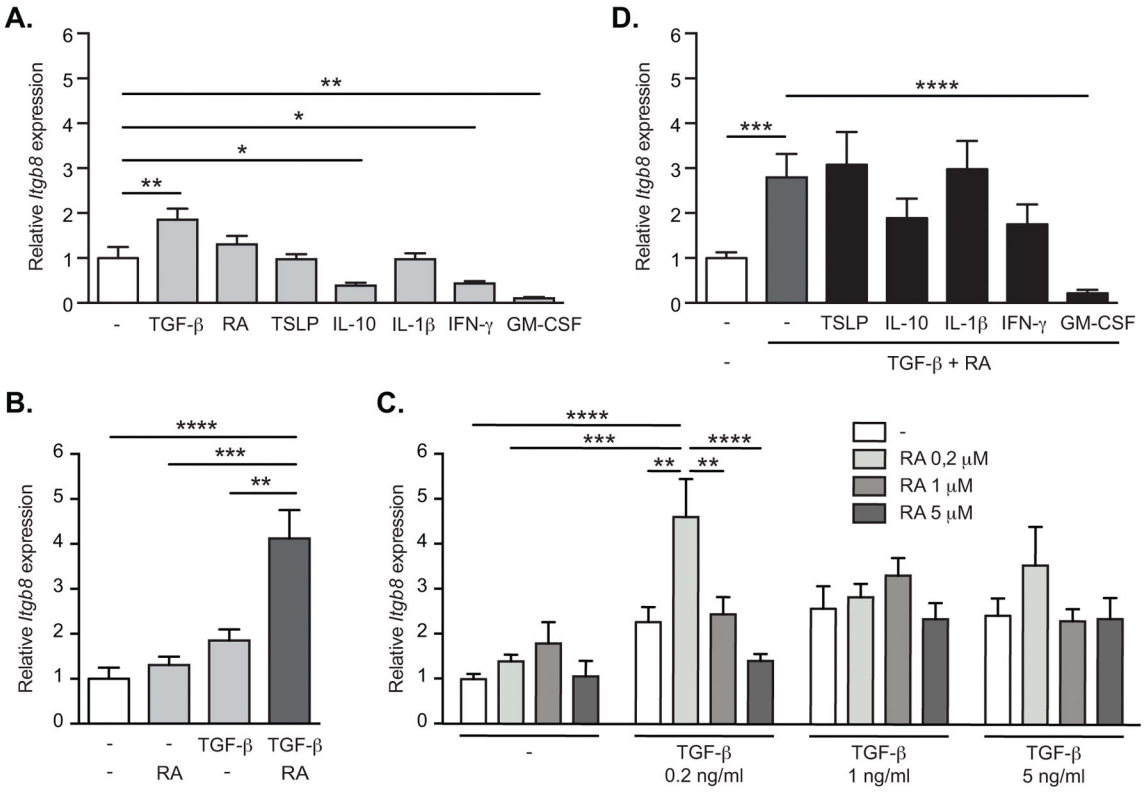

Figure 5. TGF-β and RA directly induce *Itgb8* expression in spleen DCs

$CD11c^+$ spleen DCs were sorted with magnetic beads and then cultured in serum free medium for 16h. Cells were either left untreated (−) or stimulated with TGF-β, retinoic acid (RA), or both (A–C). In addition cells were treated with TSLP, IL-10, IL-1β, IFN-γ or GM-CSF, without (B) or with (D) addition of TGF-β and RA. 0.2 ng/ml TGF-β and 0.2 μM RA were used unless indicated otherwise. β8 integrin (*Itgb8*) gene expression was assessed by quantitative RT-PCR analysis relative to *Actb* and presented relative to levels in unstimulated spleen DCs. Histograms show mean ± SEM of cultures from 15 (A), 6 (B; D) or 9 (C) independent pools of mice from at least 3 separate experiments. *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$; ****, $p<0.0001$; one-way ANOVA with Dunnet's post-hoc test (A–B; D) or two-way ANOVA with Tukey's post-hoc test (C).

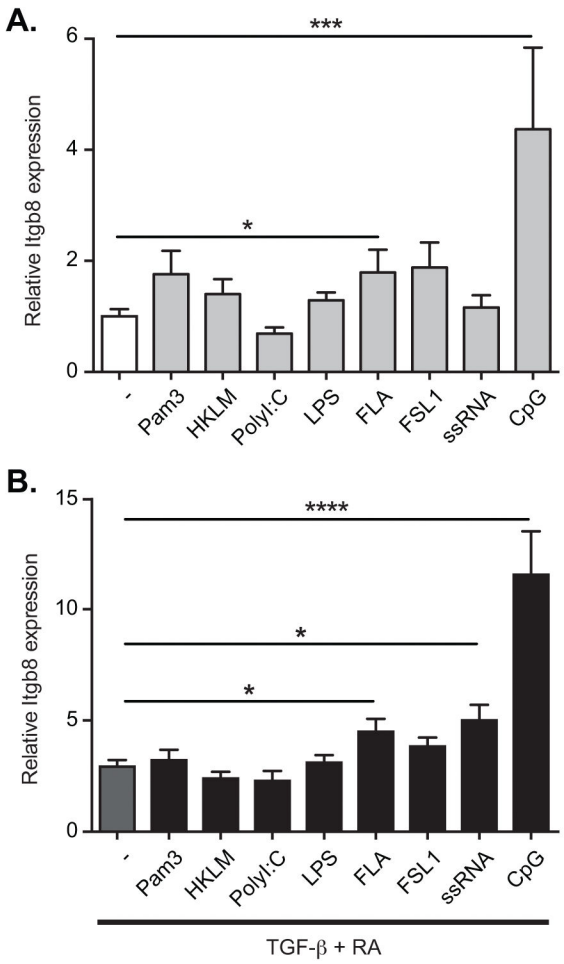

Figure 6. TLR stimulation induces *Itgb8* expression in spleen DCs

$CD11c^+$ spleen DCs were sorted with magnetic beads and then cultured in serum free medium for 16h. Cells were either left untreated (−) or stimulated with agonists for TLR-1 (Pam3), TLR-2 (HKLM), TLR-3 (PolyI:C), TLR-4 (LPS), TLR-5 (FLA), TLR-6 (FSL1), TLR-7 (ssRNA) or TLR-9 (CpG), with (B) or without (A) addition of TGF-β and RA. β8 integrin (*Itgb8*) gene expression was assessed by quantitative RT-PCR analysis relative to *Actb* and presented relative to levels in unstimulated spleen DCs. Histograms show mean ± SEM of cultures from 9 independent pools of mice from 3 separate experiments. *, $p<0.05$; ***, $p<0.0005$; ****, $p<0.0001$; one-way ANOVA with Dunnet's post-hoc test.

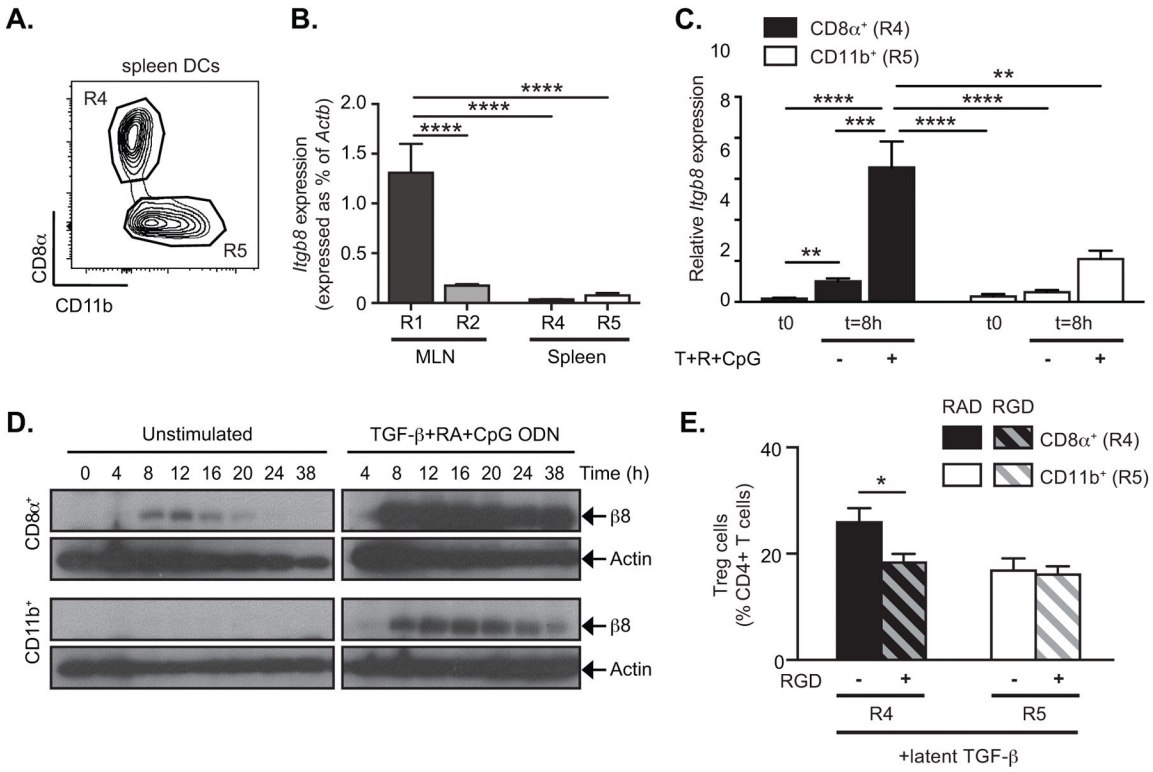

Figure 7. CD8α$^+$ spleen DCs preferentially express αvβ8 and generate Treg *in vitro*

DC subsets were sorted by FACS. (A) Representative FACS plot gated on CD11c$^+$MHC-II$^+$ DCs from spleen shows gating strategy for isolating indicated populations. MLN DC subsets were sorted as is Figure 1. (B) β8 integrin gene (*Itgb8*) expression was analyzed by quantitative RT-PCR in freshly isolated DC subsets from MLN and spleen and expressed as % of *Actb*. (C–D) Sorted DC subsets were cultured in serum free medium for 8h (C) or indicated times (D). Cells were either left untreated (−) or stimulated with TGF-β, RA and CpG ODN (+) and harvested either in TRIzol (C) or RIPA buffer (D) for analysis of β8 integrin expression. Alternatively for each freshly isolated DC subsets, their ability to induce Treg generation was assessed *in vitro* (E). (C) Quantitative RT-PCR analysis of *Itgb8* expression relative to *Actb* and presented relative to levels in unstimulated (−) CD8α$^+$ DCs (R4). (D) Western blot analysis of β8 integrin and β-actin. (E) FACS-sorted spleen DC subsets were cultured with naïve CD4$^+$Foxp3^{GFP-} T cells in serum-free medium with or without addition of latent TGF-β, RGD (+, hatched bars) and/or RAD (−, solid bars) peptides as indicated. After 4 days in culture, Treg generation was assessed by Foxp3^{GFP} expression. Data show mean ± SEM of individual DC:T cell cultures with 8 independent pools of spleen DCs from two separate experiments. Histograms show mean ± SEM of n independent pools of mice from 2 (B, n=5) or 4 (C, n=10) separate experiments. *, p<0.05; **, p<0.005; ***, p<0.0005; ****, p<0.0001; one-way ANOVA with Dunnet's post-hoc test (B) or two-way ANOVA with Tukey's post-hoc test (C, E).

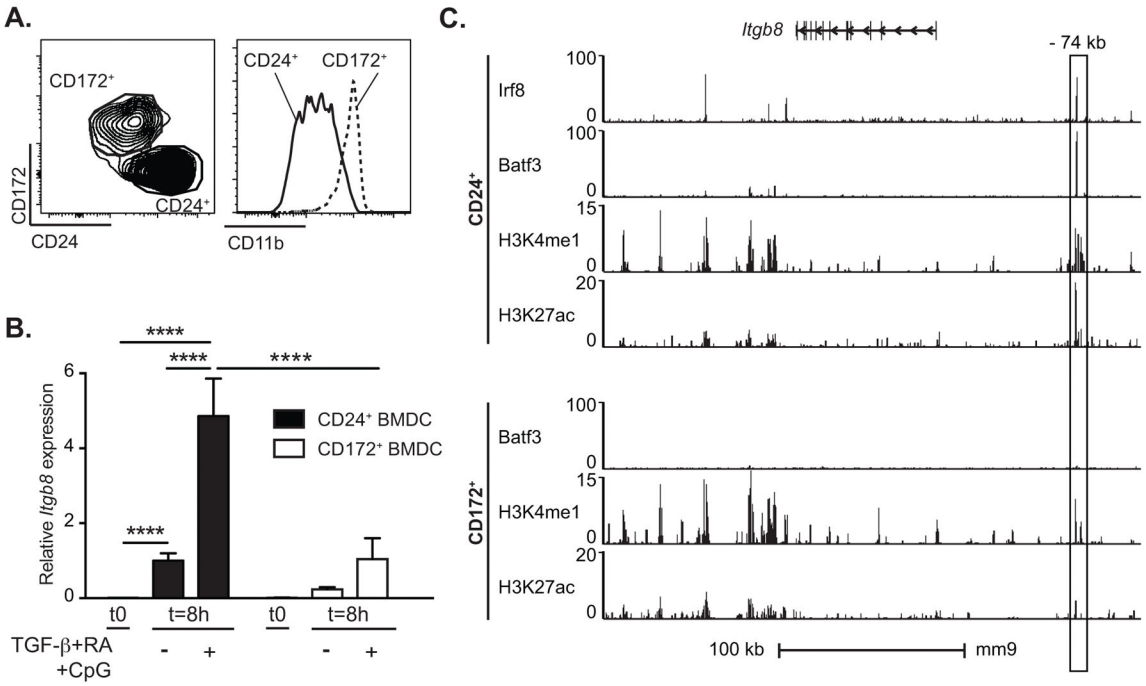

Figure 8. CD24$^+$ FL-DCs preferentially express αvβ8 and present an active enhancer in *Itgb8* locus

(A–B) Bone marrow-derived DCs were generated in culture in presence of Flt-3 ligand (FL-DCs). After 9 days in culture, FL-DC subsets were sorted by FACS and cultured in serum free medium for 8h. Cells were either left untreated (−) or stimulated with TGF-β, RA and CpG ODN (+).β8 integrin gene (*Itgb8*) expression was measured by quantitative RT-PCR relative to *Actb* and presented relative to levels in unstimulated (−) CD24$^+$ DCs at 8h. (A) Representative FACS plot of CD11c$^+$MHC-II$^+$ FL-DCs showing gating strategy for isolating indicated populations. (B) Histogram shows mean ± SEM of individual cultures from 4 independent experiments. ****, p<0.0001; two-way ANOVA with Tukey's post-hoc test. (C) ChIP-seq analysis of Batf3, IRF8, H3K4me1 and H3K27ac in CD24$^+$ and CD172$^+$ FL-DCs purified by sorting (ChIP-seq data from (17), GEO accession code GSE66899). Box represents outlined area at −74 kb relative to the *Itgb8* TSS. mm9, NCBI37/mm9 assembly of the mouse genome.