Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning

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Intestinal dendritic cells (DCs) have been shown to display specialized functions, including the ability to promote gut tropism to lymphocytes, to polarize noninflammatory responses, and to drive the differentiation of adaptive Foxp3$^+$ regulatory T (T_{reg}) cells. However, very little is known about what drives the mucosal phenotype of DCs. Here, we present evidence that the local microenvironment, and in particular intestinal epithelial cells (ECs), drive the differentiation of T_{reg}-cell-promoting DCs, which counteracts Th1 and Th17 development. EC-derived transforming growth factor-β (TGF-β) and retinoic acid (RA), but not thymic stromal lymphopoietin (TSLP), were found to be required for DC conversion. After EC contact, DCs upregulated CD103 and acquired a tolerogenic phenotype. EC-conditioned DCs were capable of inducing *de novo* T_{reg} cells with gut-homing properties that when adoptively transferred, protected mice from experimental colitis. Thus, we have uncovered an essential mechanism in which EC control of DC function is required for tolerance induction.

INTRODUCTION

The symbiosis between commensal bacteria and the gut has risks and benefits for the host organism because bacteria continuously challenge the fine balance of intestinal immune homeostasis with their cargo of microbial-associated molecular patterns (MAMPs). By binding to pattern recognition receptors, including Toll-like receptors, MAMPs can activate immune cells and lead to inflammation.[1,2] The polarized expression of pattern recognition receptors on the basolateral membrane (or intracellularly) in epithelial cells (ECs) can limit their activation.[3] However, dendritic cells (DCs) that can intercalate between ECs for direct bacterial uptake[4–6] could be continuously exposed to MAMP-dependent activation. If not held in check, the development of an exaggerated inflammatory response could lead to chronic inflammation and immune disorders such as inflammatory bowel disease and cancer. Unlike other tissues, lamina propria (LP) derived DCs (LP-DCs) have been shown to be less prone to the development of inflammatory responses.[7,8] Gut-derived CD103$^+$ mesenteric lymph node (MLN) DCs (MLN-DCs) and LP-DCs have been recently described to actively participate in the *de novo* generation of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T (T_{reg}) cells by a retinoic acid (RA)- and TGF-β-dependent mechanism.[9,10] This suggests that either a subset of DCs or the local gut microenvironment plays a major role in controlling the activity of DCs, the development of inflammatory reactions, and the induction of tolerance. Interestingly, only the CD103$^+$ population of LP-DCs or MLN-DCs is endowed with tolerogenic properties. As ECs express E-cadherin,[11] which is the ligand of CD103, we hypothesized that during the interaction of DCs with ECs, EC-derived factors could participate in driving the tolerogenic mucosal phenotype of gut DCs. Such a mechanism would guarantee that DCs that are in close contact with ECs could be less responsive to MAMP stimulation and might promote the development of adaptive T_{reg} cells. Indeed, we show here that ECs induced the expression of CD103 on DCs and conferred upon them a mucosal phenotype through the action of RA and TGF-β. EC-conditioned DCs (EC-DCs) drove the differentiation of gut-homing T_{reg} cells that were protective against experimental colitis. Thus, we have discovered that ECs are not only a barrier between the external and internal world but also play an active role in controlling DC function and intestinal tolerance.

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RESULTS

EC-DCs induce CD4$^+$CD25$^+$Foxp3$^+$ T_{reg}-cell differentiation in mice

It has been recently shown that DCs of gut origin have the ability to convert naive T cells into Foxp3$^+$ T_{reg} cells.[9,10] However, the mechanism of conferring tolerogenic properties upon gut DCs is still unknown. We reasoned that because of their close interaction with ECs, the ECs could participate in conferring the tolerogenic phenotype upon DCs. To evaluate whether DCs "educated" by ECs were able to promote T_{reg}-cell differentiation, bone marrow-derived DCs (BMDCs) were incubated for 24 h with supernatant derived from a murine epithelial cell line (MODE-K). Conditioned DCs were pulsed for 6 h with ovalbumin (OVA), washed, and co-incubated with naive CD4$^+$CD25$^-$ T cells from OVA-specific OT-II mice for 5 days. We then analyzed the development of CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells by flow cytometry. Conditioning with MODE-K supernatant in the presence of OVA favored T_{reg}-cell conversion (5.5 vs. 9.3%, **Figure 1a, c,** and **d),** and this effect was enhanced by the presence of TGF-β during the last 2 days of coculture (13.7 vs. 20.1%, **Figure 1a, c** and **d**). In addition, the capacity of EC-DCs to induce T_{reg}-cell differentiation was tested using enhanced green fluorescent proteins (eGFP)$^-$ T cells isolated from Foxp3-eGFP reporter mice (Foxp3-GFP). Again, EC-DCs induced a higher rate of conversion of eGFP-Foxp3$^-$ into eGFP-Foxp3$^+$ T cells than non-conditioned DCs (**Supplementary Figure 1a, c** and **d**).

To follow T-cell proliferation in culture, highly purified CD4$^+$CD25$^-$ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) before incubation with DCs. At 3 days later, T cells were stained for Foxp3 expression and analyzed for CFSE dilution. EC-DCs induced the development of a higher number of proliferating Foxp3$^+$ cells, whereas the number of proliferating Foxp3$^-$ cells was similar in the two conditions (**Figure 1b**). The increase of T_{reg} cells is likely because of their *de novo* differentiation rather than because of the expansion of already existing Foxp3$^+$ cells, as the CD4$^+$CD25$^-$ OTII cells used in the experiment expressed negligible levels of Foxp3 (from 0.3 to 0.5%, **Figure 1a**). The experiments were then repeated using T cells isolated from DO11.10 SCID mice that have only naive T cells that do not express Foxp3.[9] BMDCs were derived from BALB/c mice, conditioned with EC supernatants and loaded with OVA as described above. Again, MODE-K-conditioned DCs displayed a greater ability to induce T_{reg}-cell conversion than do control DCs (**Supplementary Figure 1b**).

We then compared the ability of EC-DCs with that of non-conditioned BMDCs to influence the development of inflammatory CD4$^+$ T cells. T cells incubated with EC-DCs produced lower amounts of interferon-γ (IFN-γ) and interleukin (IL)-17 in response to OVA than those incubated with non-conditioned DCs (**Figure 1e**). Confirming our previous observations that EC conditioning of human DCs also skewed toward a Th2 phenotype,[12] we observed an increase in IL-10, IL-13 and IL-5 levels in culture supernatants (**Supplementary Figure 2**). We were only able to detect low amounts of IL-5, IL-10, and IL-13 released by T cells, but we have to point out that this was a primary culture; whether these tiny amounts of ILs can exert a biological effect remains to be established, particularly in light of the still relevant Th1 and Th17 cytokines. These results indicate that EC-derived factors "educate" tolerogenic DCs to induce T_{reg}-cell differentiation and concomitantly limit Th1 and Th17 inflammatory responses.

Next, we compared the suppressor activity of CD25$^+$ T_{reg} cells generated by EC-DCs and by non-conditioned DCs with that of CD25$^+$ cells isolated from the mouse spleen. CD25$^+$ T cells were isolated from conditioned or non-conditioned DC/T-cell cultures, or from the spleen, and incubated in a second proliferating antigen presentation assay using lipopolysaccharide (LPS)-matured DCs and CFSE-labeled CD4$^+$ T cells in the presence of plate-bound α-CD3. We found that CD25$^+$ T_{reg} cells generated by EC-DCs were more suppressive than non-conditioned DC-generated or splenic CD25$^+$ T_{reg} cells (**Figure 1f**).

Collectively, our results indicate that ECs can induce tolerogenic DCs and suggest the *de novo* development of functionally active T_{reg} cells. This activity was observed when using conditioned BMDCs from both C57/BL6J and BALB/c mice, suggesting a general rather than a background-restricted phenomenon.

EC-DCs are noninflammatory

As EC-DCs showed a reduced ability to drive Th1 and Th17 differentiation, we investigated the pattern of cytokines released by EC-DCs. We incubated BMDCs with supernatant from MODE-K cells and stimulated them with LPS (1 μg ml^{-1}) for 6 h. We observed that, consistent with their ability to induce T_{reg}-cell differentiation, EC-DCs produced significantly more IL-10 and IL-2, and less IL-12p70 in comparison with non-conditioned DCs (**Figure 2a**). In addition, together with the augmented production of cytokines involved in T_{reg}-cell homeostasis, EC-DCs expressed lower amounts of mRNA coding for inflammatory cytokines involved in the polarization of Th1 (IL-12p35 coded by *Il12a*) and Th17 (IL-23p19 coded by *Il23a*) cells (**Figure 2b**). Hence, EC-DCs display limited production of inflammatory cytokines and drive T_{reg}-cell differentiation.

Spleen DCs acquire mucosal DC properties and induce T_{reg}-cell differentiation after "education" by ECs

LP and MLNs have been reported to be the sites of peripheral T_{reg}-cell generation, and DCs isolated from these tissues play a major role in this process.[9,10] Accordingly, we found that MLN-DCs were able to induce the development of T_{reg} cells (8.9%) with considerably higher efficiency compared with spleen DCs (3.2%) (**Figure 3a**). However, if splenic DCs were first incubated with MODE-K supernatant, the frequency of T_{reg} cells in the cocultures was dramatically increased (9.1%) (**Figure 3a** and **b**). These data indicate that MLN-DCs display a superior ability to drive T_{reg}-cell differentiation when compared with spleen DCs, a feature that is likely acquired in the periphery after EC conditioning, suggesting that "mucosal" DCs become so only after environmental education.

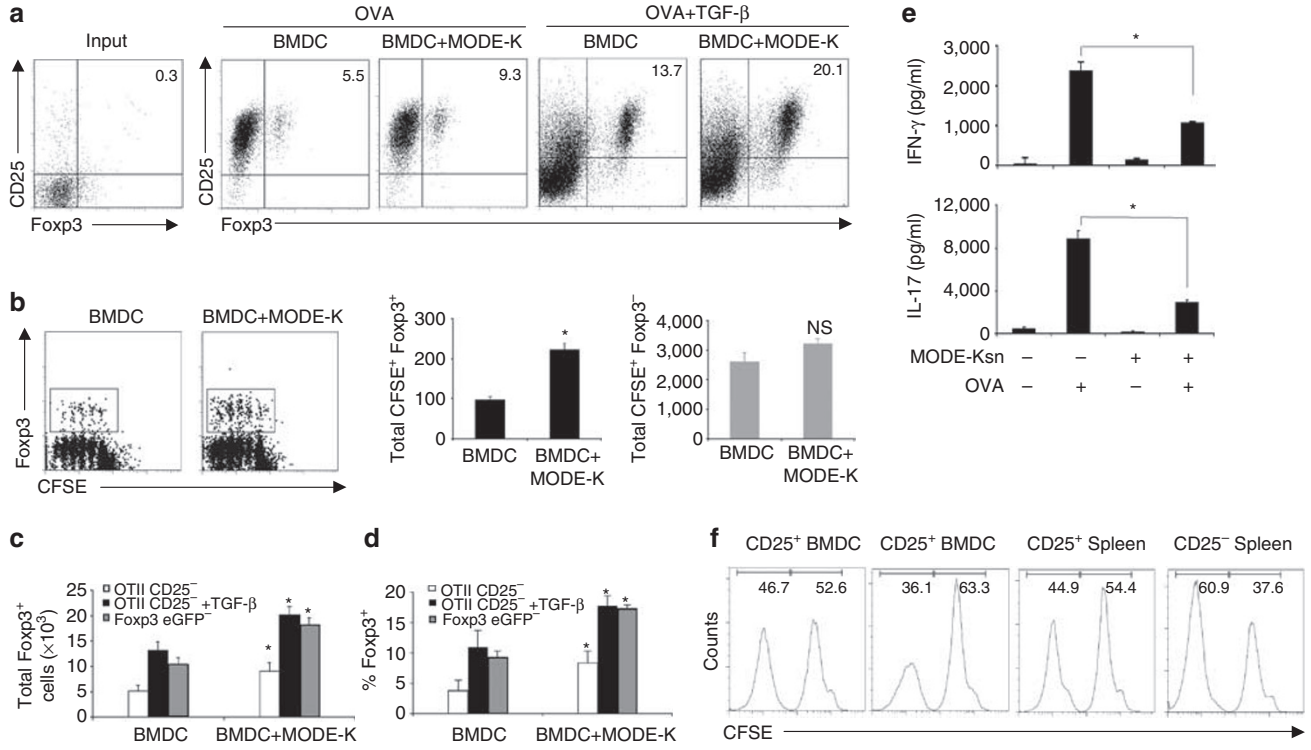

Figure 1 ECs promote tolerogenic DCs able to induce T_{reg}-cell differentiation. BMDCs (1×10^4) were pretreated with or without MODE-K cell supernatant for 24 h and pulsed with OVA (250 μg ml^{-1}). After washing, cells were incubated together with naive CD4$^+$CD25$^-$ OT II cells (**a, c–e**) for 5 days (**a**). Dot plots show CD25$^+$ and Foxp3$^+$ T cells in the CD4$^+$ gate. TGF-β (3 ng ml^{-1}) was added during the last 2 days of culture. Left panel: input cells. Numbers indicate the percentage of positive cells in the quadrant. Data are representative of five independent experiments. (**b**) CFSE-labeled CD4$^+$CD25$^-$ OTII cells were cultured with OVA-loaded BMDCs as above. After 3 days, the cells were harvested and stained for CD4 and intracellular Foxp3. Dot plots represent Foxp3 vs. CFSE staining. Data are representative of three independent experiments. Graphs show total numbers of CFSE$^+$Foxp3$^+$ cells in the gate (left graph) or CFSE$^+$Foxp3$^-$ cells outside the gate (right graph). (**c**, **d**) Pooled data showing absolute numbers (**c**) or percentage (**d**) of Foxp3$^+$ T cells in the assay in panel a or **Supplementary Figure 1a** using DCs pulsed with OVA without (open bars) or with TGF-β (black bars); or eGFP-Foxp3$^+$ T cells (grey bars). (**e**) IFN-γ and IL-17 were measured in culture supernatants by ELISA. Data are representative of three independent experiments. Treatments are reported in the legends below the graphs. (**f**) CFSE-based suppression assays using CD4$^+$CD25$^+$ T cells derived from DC/T-cell cocultures with non-conditioned or MODE-K-conditioned BMDCs. The proliferation of CFSE-labeled responder T cells was analyzed by flow cytometry. One out of two representative experiments is shown. The numbers show percentage of proliferating (left) and non-proliferating (right) cells. (**b–e**) Error bars, s.d. *$P<0.05$. BMDC, bone marrow-derived DC; CFSC, carboxyfluorescein succinimidyl ester; DC, dendritic cells; IFN-γ, interferon-γ; IL-17, interleukin-17; OVA, ovalbumin; T_{reg}, regulatory T cells.

The supernatant from intestinal ECs drives the development of T_{reg} cells

To exclude the possibility that the observed ability of ECs to drive tolerogenic DCs could be due to the use of spent culture medium, which could nonspecifically inhibit DC maturation, we compared the supernatant of MODE-K cells with that of either a mouse mammary tumor epithelial cell line (4T1) or a highly proliferating fibroblast cell line (NIH-3T3) for their ability to drive tolerogenic DCs. As shown in **Figure 4a** and **b**, only DCs incubated with MODE-K cell supernatant were able to significantly increase the frequency of T_{reg} cells in culture. It has been shown that gut DCs drive the development of T_{reg} cells by a mechanism that is dependent on TGF-β and RA.[9,10] Thus, we evaluated whether TGF-β and *aldh1a2*, an enzyme involved in the conversion of retinal into RA, were expressed by ECs. We also wanted to assess whether differences in the expression of these molecules could explain the diverse abilities of the tested cell lines to induce tolerogenic DCs. We found that only MODE-K cells expressed both *aldh1a2* and TGF-β, whereas 4T1 and NIH-3T3 cells expressed only TGF-β (**Figure 4c**). We then purified primary ECs from the small intestine of mice and collected their supernatant after 24 h of culture to rule out the possibility that the ability of MODE-K cells to induce tolerogenic DCs was an artifact of this cell line (**Supplementary Figure 3a** and **b**). BMDCs incubated with RA were used as a positive control for the experiment. Similar to MODE-K cells, the supernatant from primary ECs of the small intestine strongly favored the development of T_{reg}-polarizing DCs (**Supplementary Figure 3a** and **b**).

EC-derived TGF-β and RA induce tolerogenic DCs

As we have shown that MODE-K cells expressed TGF-β and an enzyme involved in RA synthesis, we analyzed whether TGF-β and RA played any role in driving tolerogenic DCs, which would be able to induce T_{reg}-cell development. Thus, BMDCs were incubated with MODE-K supernatant, with or without antibody neutralization of TGF-β, before T-cell activation. Indeed, neutralization of TGF-β in the MODE-K supernatant

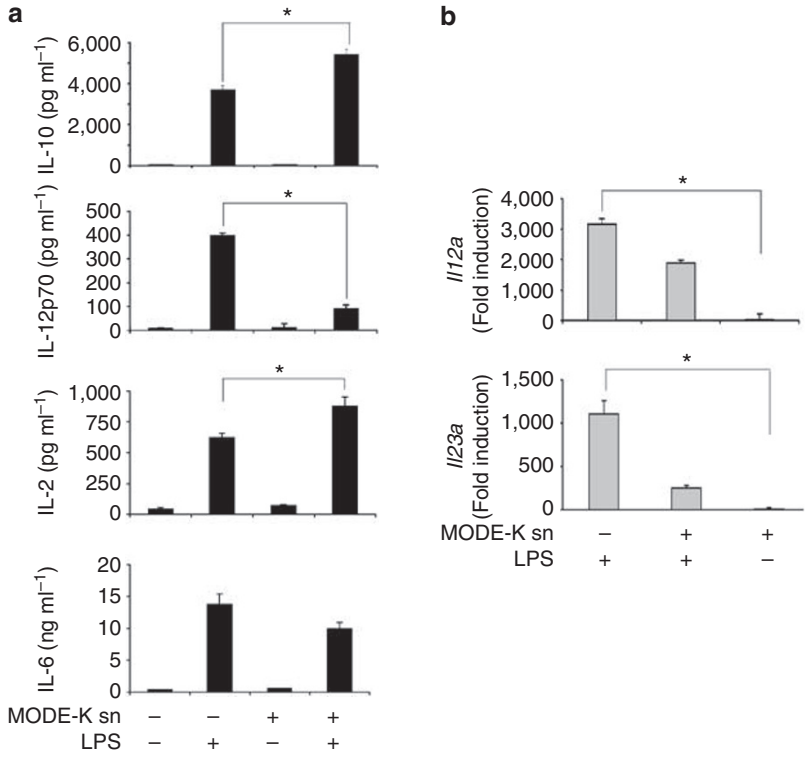

Figure 2 Epithelial cell-conditioned BMDCs are noninflammatory. BMDCs were pretreated with or without MODE-K cell supernatant for 24 h and stimulated with or without LPS (1 μg ml^{-1}) for 6 h. (**a**) IL-10, IL-12p70, IL-2, and IL-6 released in the culture supernatants were measured by ELISA after 24 h. (**b**) *Il23a* (IL-23p19) and *Il12a* (IL-12p35) expressions were analyzed by quantitative RT-PCR and normalized to *Tbp* mRNA. Data represent fold induction over immature BMDCs. Treatments are reported in the legends below the graphs. The difference in cytokine production or RNA expression between MODE-K conditioned and non-conditioned BMDCs was statistically significant (*P<0.05). Data are representative of three independent experiments. BMDC, bone marrow-derived DC; IL-17, interleukin-17; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-PCR.

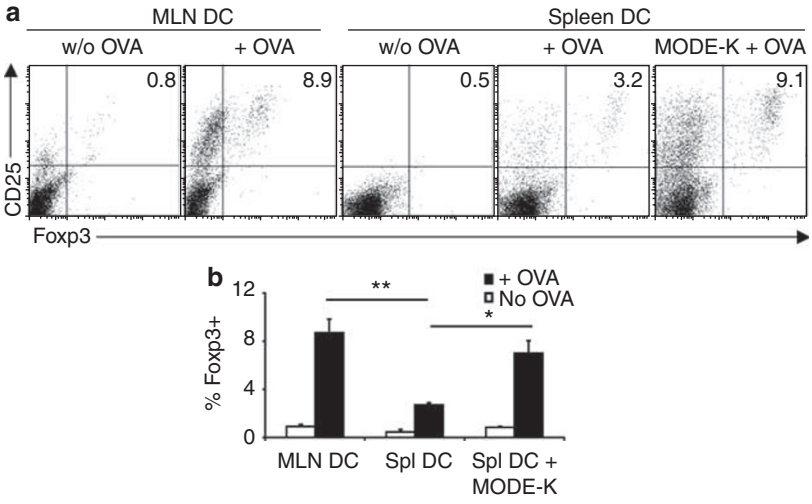

Figure 3 ECs imprint spleen DCs with a mucosal phenotype. (**a**) CD11c$^+$ DCs were isolated from MLNs and the spleens of C57/BL6J mice. MLN-DCs and spleen DCs (with or without MODE-K conditioning) were pulsed with OVA (250 μg ml^{-1}) for 6 h, washed, and incubated with naive CD4$^+$CD25$^-$ OTII cells for 5 days. Development of CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells was analyzed by FACS. Dot plots representative of two independent experiments are shown. (**b**) Graph represents pooled data from the experiments described in (**a**). DC, dendritic cells; EC, epithelial cells; MLN, mesenteric lymph node; OVA, ovalbumin *P<0.05; **P<0.01.

lead to a strong reduction in the frequency of T_{reg} cells in EC-DC/T-cell cultures (23.0% vs. 11.6%) (**Figure 5a** and **b**) and correlated with increased IFN-γ production (**Figure 5c**). This was not due to unspecific effects of the neutralizing antibody because an isotype control had no effect on T_{reg}-cell development (**Figure 5a** and **b**). We then tested whether EC-derived RA was also required for the differentiation of tolerogenic DCs. DCs were incubated with an RA receptor inhibitor (LE540) for 1 h

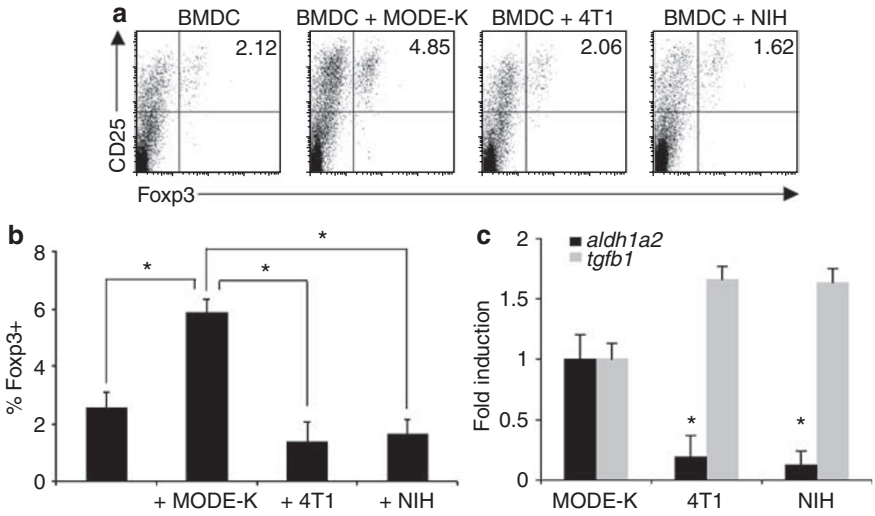

Figure 4 IEC, but not supernatants from fibroblast or mammary ECs, promote tolerogenic DCs. BMDCs were either incubated with supernatants derived from MODE-K cells, mammary tumor ECs (4T1), or a fibroblast cell line (NIH-3T3). At 24 h later, cells were collected, pulsed with OVA, and cocultured with naïve OTII cells, as described in **Figure 1**. (**a**) Dot plots show CD25$^+$ Foxp3$^+$ T cells. Numbers indicate the percentage of positive cells in the quadrant. (**b**) The graph shows pooled data from the experiments described in panel a. (**c**). Fold induction of *aldh1a2* and *tgfb1* in cell lines normalized to *Tbp* mRNA. Error bars, s.d. **P*<0.05. Data are representative of two independent experiments. BMDC, bone marrow-derived DC; DC, dendritic cells; EC, epithelial cells; OVA, ovalbumin.

before conditioning. At 24 h later, DCs were washed and incubated with naive OTII T cells, as above. We found that inhibition of RA signaling to DCs affected the induction of T_{reg} cells (**Figure 5d** and **e**) and restored the development of Th17 responses (**Figure 5f**). However, we could not detect any synergistic effect in the inhibition of T_{reg}-cell differentiation by blocking both TGF-β and RA (data not shown). RA is likely derived from ECs in our experimental setting, as we found no increase in *aldh1a2* expression in EC-DCs as compared with DCs alone, whereas intestinal ECs displayed a nearly three times higher expression of the enzyme than DCs (**Figure 5g**). On the other hand, *aldh1a2* was highly expressed in colon tissue and MLN than in the spleen (**Figure 5g**). In particular, the CD103$^+$ DC subset isolated from MLN showed the highest expression of *aldh1a2* (**Figure 5g**), suggesting that DC-derived RA may also participate in T_{reg} conversion at mucosal sites *in vivo*.

Similar to what we already described in humans,[12] we found that MODE-K cells and primary murine ECs constitutively expressed TSLP (**Supplementary Figure 4**). Thus, we compared the ability of DCs isolated from MLN- or BM-DCs from TSLPR$^{-/-}$ mice to induce T_{reg}-cell differentiation. The functional receptor for TSLP comprises two chains: IL7Rα chain, which is shared with IL-7 receptor, and a TSLP receptor chain (TSLPR).[13] Our results show that both TSLPR$^{-/-}$ and WT DCs (**Figure 6a** and **b**) had a similar ability to convert naive T cells into T_{reg} cells, both before and after EC conditioning. All together, these results suggest that EC-derived TGF-β and RA are needed for the promotion of the tolerogenic phenotype of DCs in mice, but TSLP could be dispensable.

EC contact drives CD103 expression on DCs that become tolerogenic

As DCs interact closely with ECs in the gut, we evaluated whether the proximity to ECs had differentially effected DC conditioning. Hence, we opted for the mouse CMT93 epithelial cell line that, unlike MODE-K cells, is able to form EC monolayers. CMT93 cells were grown on a transwell and murine BMDCs were added to the basolateral side of the EC monolayer. We measured the ability of DCs conditioned by CMT93 supernatant or direct CMT93 contact to drive the development of T_{reg} cells. We found that in agreement with MODE-K cells, CMT93 supernatants were capable of driving the differentiation of tolerogenic DCs, but EC contact did not confer further differentiation (**Figure 7b** and **e**). As it has been shown that murine intestinal CD103$^+$ DCs are able to convert naive T cells into Foxp3$^+$ T_{reg} cells [9] and are required for T-cell-mediated prevention of experimental colitis,[14] we asked whether EC contact could induce CD103 expression on BMDCs. Interestingly, CD103 was strongly upregulated on BMDCs after 24 h of direct contact with ECs (**Figure 7a** and **d**). DCs incubated with EC supernatants were also able to upregulate CD103, but to a lower extent (**Figure 7a** and **d**). When α-TGF-β (10 or 50 μg ml^{-1}) or LE540 (1 or 10 nM) were added to the EC-DC cocultures, we could not inhibit CD103 upregulation (data not shown). We then analyzed whether CD103$^+$ and CD103$^-$ cells obtained after contact with ECs had different abilities to drive T_{reg} cells. As shown in **Figure 7c–e**, FACS (fluorescence-activated cell sorting)-sorted CD103$^+$ cells showed a more than fourfold greater ability to drive T_{reg}-cell development than did CD103$^-$ DCs. This correlated with the ability of CD103$^+$ but not CD103$^-$ DCs to induce the expression of α4β7, an integrin involved in the homing of lymphocytes to the gut, on Foxp3$^+$ cells and with a reduced production of IFN-γ and IL-17 (**Figure 7f** and **g**). Interestingly, the contact with ECs induced the expression of *aldh1a2* in DCs to a similar level as RA (**Figure 7h**). Together, these results suggest that EC contact by DCs promotes a full CD103$^+$ DC mucosal tolerogenic phenotype.

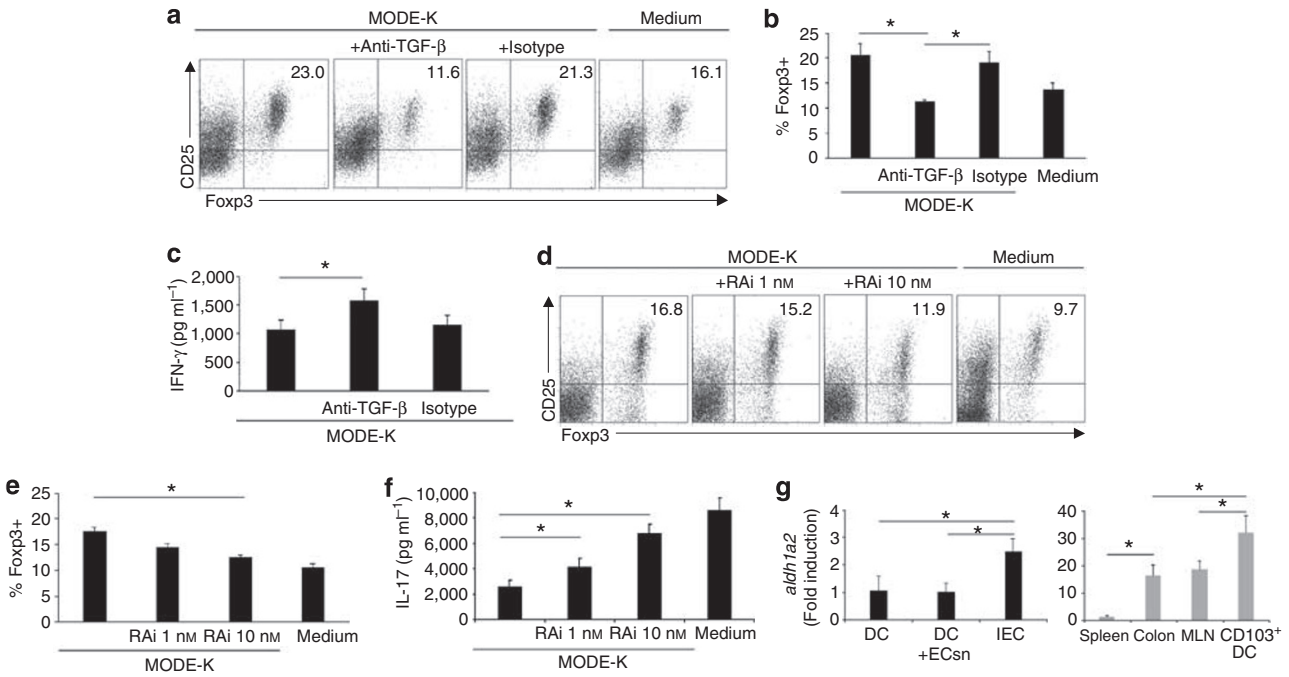

Figure 5 ECs induce tolerogenic DCs by a TGF-β and RA-dependent mechanism. MODE-K supernatants were pre-incubated for 2 h at 4 °C with (**a–c**) 50 μg ml^{-1} anti-TGF-β or an isotype control. BMDCs were pre-incubated (**d–f**) either with or without RAi (LE540) for 1 h at 37 °C were then conditioned for 24 h with neutralized or control supernatants, pulsed with OVA, and cocultured with naive OTII cells as described in **Figure 1**. (**a, d**) Dot plots show CD25$^+$ and Foxp3$^+$ T cells in the CD4$^+$ gate. Numbers show the percentage of cells in the gate. Graphs (**b**) and (**e**) show pooled data from the experiments described in panel **a** and **d**, respectively. (**c**) IFN-γ production in the cocultures from panel **a** was assessed by ELISA. (**f**) IL-17 production in cocultures from panel d was measured by ELISA. (**g**) *aldh1a2* expression level in BMDCs (DC), BMDC + MODE-K supernatant (DC + ECsn), primary IEC, spleen, colon, MLN, and FACS-sorted CD103$^+$ DC isolated from MLNs (CD103$^+$ DC). *aldh1a2* mRNA expression was analyzed by quantitative RT-PCR, normalized to *rpl32* mRNA and displayed as fold induction vs. untreated DCs (left) or spleen (right). (**b, c, e–g**) Error bars, s.d. *$P<0.05$. Data are representative of three independent experiments (**a–c**) or two independent experiments (**d–g**). BMDC, bone marrow-derived DC; DC, dendritic cells; EC, epithelial cells; IFN-γ, interferon-γ; IL-17, interleukin-17; LPS, lipopolysaccharide; MLN, mesenteric lymph node; OVA, ovalbumin; RT-PCR, reverse transcriptase-PCR; TGF-β, transforming growth factor-β.

EC-DC-induced T_{reg} cells are protective *in vivo* against experimental colitis

We showed that EC-DCs acquired the ability to drive T_{reg}-cell differentiation. Thus, we asked whether these newly generated T_{reg} cells might have a protective function *in vivo* in the gut. T_{reg} cell-enriched populations were generated using either BMDCs conditioned or unconditioned for 24 h with MODE-K supernatants after culture with CD4$^+$CD25$^-$ T cells from the same mice. After 5 days of coculture, the frequency of CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells in the two conditions was analyzed by FACS (**Figure 8a**). As we found that EC-DCs induce twice as many T_{reg} cells, we wondered whether their frequency could be functionally significant and have an impact on the development of colitis. Thus we injected intravenous 1×10^6 total T cells (**Figure 8**) or purified CD25$^+$ T cells (**Supplementary Figure 5**)—or PBS (phosphate buffered saline) as a control—into C57/BL6J mice 8 h before colitis induction with 2% (w/v) dextran sulfate sodium (DSS). The onset of DSS colitis was characterized by weight loss in all groups. Interestingly, mice adoptively transferred with total or purified CD25$^+$ T cells generated using EC-DCs experienced reduced weight loss and a faster recovery as compared with the other two groups (**Figure 8b** and **Supplementary Figure 5**). Twelve days from the beginning of DSS administration, mice receiving PBS or T cells generated with non-conditioned DCs still displayed signs of robust colitis, including swollen colons, contracted ceca, and watery stools (**Figure 8c**). Histopathological analysis confirmed that mice with gross alterations in intestinal appearance also displayed altered histology, including focal crypt epithelial destruction and extensive areas of epithelial hyperplasia associated with mononuclear infiltrates (**Figure 8d**). Consistent with the histology, mice receiving PBS or T cells generated with non-conditioned DCs displayed significantly increased amounts of IFN-γ and TNF-α in the colons, compared with those receiving EC-DC-derived T cells (**Figure 8e**). In all groups of mice, we could not detect any significant difference in IL-6 production (data not shown). These data suggest that EC-DCs are able to effectively induce the generation of colitis-protective T_{reg} cells.

DISCUSSION

It is becoming increasingly clear that immune cells isolated from mucosal tissues exhibit very peculiar phenotypes.[8] Macrophages, for instance, display bactericidal activity without inducing inflammation,[15] drive the development of T_{reg} cells, and control DC function.[16] DCs isolated from either Peyer's patches or the LP are also noninflammatory, [8,17,12,18,19,35] and a subtype of MLN-DCs expressing CD103 is involved in T-cell-mediated regulation of experimental colitis.[14] CD103$^+$ DCs impart gut-homing properties to T cells [20] and drive the development of T_{reg} cells by a RA- and TGF-β-dependent mechanism.[9,10]

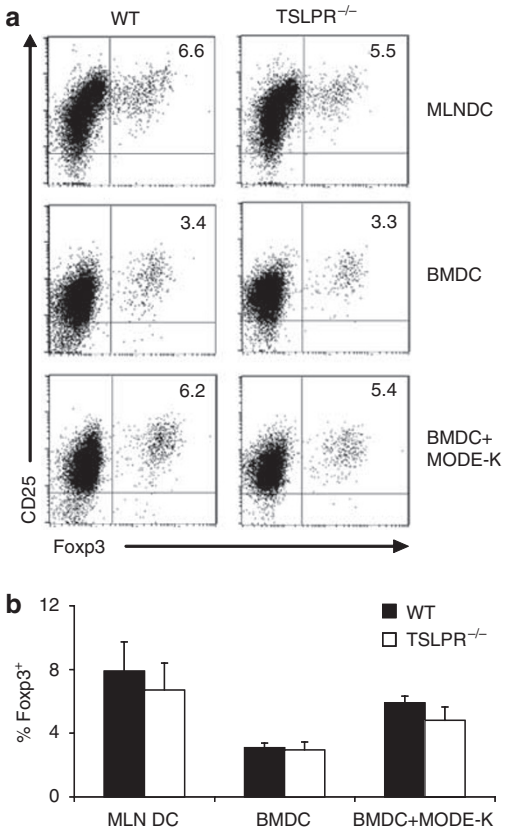

Figure 6 TSLP is not involved in the induction of tolerogenic DCs in mice. (**a**) CD11c$^+$ DCs isolated from MLNs or BMDCs (conditioned or unconditioned with MODE-K supernatants) from wild-type C57/BL6J and TSLPR$^{-/-}$ mice were pulsed with OVA (250 μg ml^{-1}), washed, and incubated together with naive CD4$^+$CD25$^-$ OT II cells for 5 days. The number of T_{reg} cells in the cultures was evaluated by FACS. Dot plots represent CD25$^+$Foxp3$^+$ T cells inside the CD4$^+$ gate. Numbers indicate the percentage of positive cells in the quadrant. Data are representative of three experiments. (**b**) Graph shows pooled data from the experiments described in panel **a**. BMDC, bone marrow-derived DC; DC, dendritic cells; MLN, mesenteric lymph node; OVA, ovalbumin; T_{reg}, regulatory T cells; TSLP, thymic stromal lymphopoietin.

All this evidence strongly supports the notion that gut DCs confer "mucosal" properties to effector immune cells, but still very little is known on what drives the "mucosal" phenotype of the DCs. Our results emphasize the role of the microenvironment, and in particular of intestinal ECs, in the control of DC function by driving the development of "tolerogenic mucosal" DCs. EC-DCs released higher amounts of IL-2 and IL-10 in response to LPS when compared with non-conditioned DCs, and drove the *de novo* generation of CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells, which were suppressive. In agreement with an involvement of the mucosal environment, spleen DCs incubated with EC supernatant displayed increased ability to generate *de novo* T_{reg} cells, which was similar to that of MLN-DCs. The ability of DCs to drive T_{reg} cells correlated with a decreased production of IL-12 and IL-23, and a reduced ability to drive Th1 and Th17 development. Furthermore, DCs in close proximity with ECs upregulated the expression of CD103, a marker that characterizes MLN tolerogenic DCs coming from mucosal sites.[20] When FACS-sorted, primarily the CD103$^+$ EC-DCs displayed tolerogenic properties and upregulated α4β7, confirming that CD103 is a marker of gut-derived tolerogenic DCs and that the interaction with ECs confers full mucosal properties to DCs. Despite an increase in CD103$^+$ cells and the induction of *aldh1a2* after EC contact, the number of converted Foxp3$^+$ T cells was similar to that obtained after simple supernatant conditioning, suggesting that the contact-dependent interaction might also confer different properties. It is possible that CD103$^+$ DCs might play a role in IgA class switching, as RA has been shown to play a fundamental role in driving IgA development [21] and that mouse LP-DCs express higher levels of *aldh1a2* and are involved in IgA class switching.[22–24] Our data would support the notion that MLN CD103$^+$ DCs could migrate from the LP, where they had received signals from the local environment, resulting in an inhibited inflammatory potential, the upregulation of CD103 after contact with Ecs, and the ability to induce T_{reg} cells. The finding that *aldh1a2* is upregulated after EC contact and is expressed by MLN CD103$^+$ cells supports this hypothesis. Thus, we describe that ECs are conductors of DC function and disrupting this balance could result in inflammatory disorders.

What are the factors involved in generating tolerogenic DCs? We found that EC-derived TGF-β and RA are necessary to drive the tolerogenic phenotype of DCs, because neutralization of either one was sufficient to affect the differentiation of T_{reg} cells. However, TGF-β alone is not sufficient to drive tolerogenic DCs as cell lines such as NIH-3T3 or 4T1 that are producing it even at higher levels than MODE-K cells still were unable to drive tolerogenic DCs at the same frequency. This is likely because of the reduced expression of the RA-converting enzyme 5 by 4T1 and NIH-3T3 cells, as RA signaling synergizes with TGF-β to favor T_{reg}-cell conversion.[9,10] We could detect an upregulation of *aldh1a2* in DCs after EC contact, but not after incubation with EC supernatant, suggesting that EC-derived RA is sufficient for the polarization of T_{reg}-promoting DCs. In agreement with recently published reports,[25,26] EC-derived RA was also involved in inhibiting Th17 responses as suppression of RA signaling restored IL-17 production in EC-DC/T-cell cocultures. It is likely that in proximity to ECs, RA is present at higher concentrations and hence inhibits Th17 induction. By contrast at a greater distance from ECs, the RA concentration might decrease and it has been shown that low levels of RA favor Th17 induction.[22] In addition, a subset of LP-DCs that expresses CD70 (CD11c^{low}CD70^{high}) and a unique series of ATP receptors was shown to respond to bacteria-derived ATP and to favor the development of Th17 cells.[27,28] This suggests that CD103$^+$ and CD70$^+$ DC subsets may respond to the local microenvironmet differently according to their surface receptors and exert diverse activities. Finally, TSLP, which is produced by Intestinal epithelial cells, was not involved in the induction of tolerogenic DCs in mice, as DCs isolated from MLNs of TSLPR$^{-/-}$ mice displayed a phenotype similar to WT MLN-DCs. It is likely that EC-derived TSLP primarily plays a role in Th1/Th2 control in the mouse system, as described previously by Zaph *et al.*[29]

T_{reg} cells play a major role in the immune homeostasis of the gut: adoptive transfer of antigen-experienced T cells, includ-

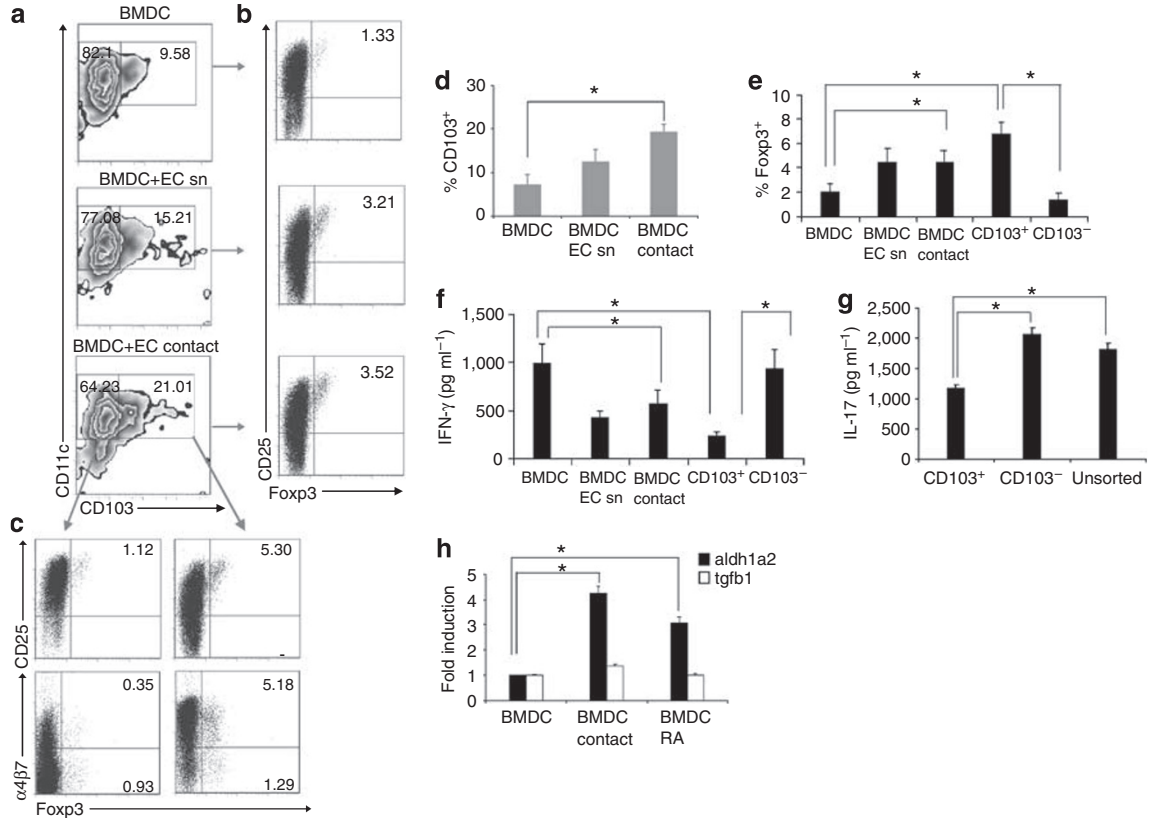

Figure 7 Epithelial cells drive the development of tolerogenic CD103$^+$ DCs. (**a**) Density plots show CD11c$^+$CD103$^+$ cells. Epithelial cells (ECs, CMT93) were grown on a transwell. BMDCs were incubated for 24 h on the basolateral side of the EC monolayer (BMDC EC contact; lower panel). For comparison, BMDCs were either pretreated (BMDC EC sn; middle panel) or untreated (BMDC, upper panel) with CMT93 supernatants. DCs were stained for CD11c and CD103, and analyzed by FACS. Numbers represent the percentage of cells in the gate. (**b**) Dot plots display CD25$^+$ and Foxp3$^+$ cells arisen from cocultures with BMDCs treated as above. (**c**) CD103$^+$ and CD103$^-$ cells were FACS-sorted from EC contact-conditioned DCs (arrows) and used in T-cell cocultures. Dot plots of CD25$^+$Foxp3$^+$ (upper dot plots) and á4β7$^+$Foxp3$^+$ (lower dot plots) cells are shown. (**d**) Pooled data from two experiments as in panel a showing the percentage of generated CD103$^+$ DCs. (**e**) Pooled data from two experiments as in panels b and c showing percentage of Foxp3$^+$ T cells. (**f**) IFN-γ and (**g**) IL-17 production in coculture supernatants was measured by ELISA. (**h**) Fold induction of *aldh1a2* and *tgfb1* in DCs after EC contact or RA treatment normalized to *Tbp* mRNA. (**d–h**) *$P<0.05$. Error bars, s.d. BMDC, bone marrow-derived DC; DC, dendritic cells; EC, epithelial cells; IFN-γ, interferon-γ; IL, interleukin; RA, retinoic acid.

ing T_{reg} cells, can protect against experimental colitis.[30,31] MLN CD103$^+$ DCs are involved in T-cell-mediated regulation of experimental colitis [14] presumably by the generation of T_{reg} cells. However, the functionality of mucosally derived adaptive T_{reg} cells has not been tested previously. As EC-DCs display properties very similar to mucosal tolerogenic DCs, we used them to generate T_{reg} cells *ex vivo* to study their function during experimental colitis. We show that *ex vivo* EC-DCs generated a greater number of T_{reg} cells, which were effective suppressors of T-cell proliferation *in vitro* and extremely potent in protecting against colitis *in vivo*. T_{reg} cells were found to be generated by EC-DCs twofold above the rate observed using non-conditioned DCs, and although the physiological consequence of this difference cannot be predicted, still the observed effect was clear and consistently striking. This could be because of an increased suppressor activity (as shown in **Figure 1f**), a higher frequency, or a greater gut-homing ability of EC-DC-generated T_{reg} cells. Indeed, we describe that EC-CD103$^+$ DCs upregulated the expression of the gut-homing marker, α4β7, on T_{reg} cells, thus suggesting that not only can EC-DCs drive T_{reg}-cell development but they can also imprint them with gut-homing properties, similar to gut DCs.[26]

In conclusion, we describe that ECs play a major role in controlling DC function by conferring upon them a tolerogenic "mucosal" phenotype. EC-DCs can induce the development of gut-homing T_{reg} cells with potent suppressor activity that protect against experimental colitis. This could open new avenues for possible therapeutic intervention. For instance, in a hypothetical treatment of inflammatory bowel disease, the use of gut-derived DCs for the generation of T_{reg} cells is not feasible because of their paucity and difficulty of isolation. Thus, we propose a new therapeutic tool of imprinting *ex vivo* a "mucosal" tolerogenic phenotype to DCs for the development of gut-homing T_{reg} cells. Our approach could offer a valid alternative to standard anti-inflammatory therapies that could revolutionize the treatment of inflammatory bowel disease.

METHODS

Mice

Female C57BL6J mice or BALB/c mice (6–7 weeks old) were purchased from Charles River, Milan, Italy. DO11.10 BALB/c and DO11.10 SCID

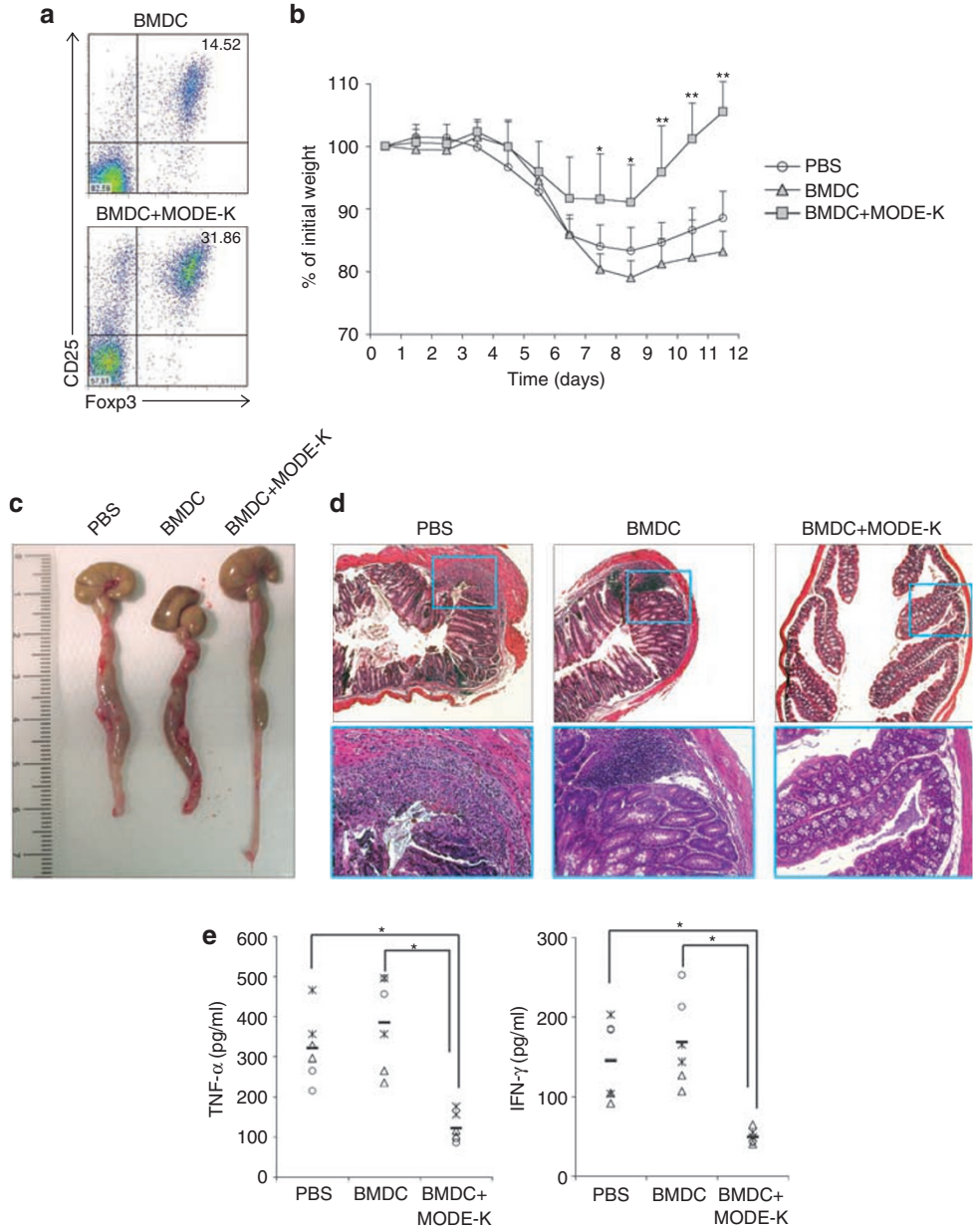

Figure 8 EC-DC generated T_{reg} cells are strongly protective and ameliorate inflammation in DSS colitis. BMDCs were incubated with or without MODE-K supernatants for 24 h, cocultured with naive $CD4^+CD25^-$ T cells for 5 days and TGF-β (3 ng ml^{-1}) was added during the last 3 days of culture. (**a**) The number of T_{reg} cells in the cocultures was evaluated by FACS. Dot plots show $CD25^+Foxp3^+$ T cells in the $CD4^+$ gate. Numbers show the percentage of cells in the quadrant. (**b**) Total T cells (1×10^6) from the cocultures of naive T cells with non-conditioned BMDC (BMDC) or with MODE-K-conditioned BMDC (BMDC + MODE-K) were injected intravenously 8 h before induction of DSS colitis. The control group was injected only with PBS. Data represent mean body weight of the mice as a percentage of the initial weight on day 0 ($n=4$). *$P<0.05$; **$P<0.02$, for the BMDC group against the BMDC + MODE-K group. (**c**) Gross appearance of cecum and colon 12 days after colitis induction. (**d**) Hematoxylin and eosin staining of mouse colonic sections 12 days after colitis induction. Original magnification, ×10 (upper panel), ×20 (lower panel). (**e**) Colon specimens (1 cm^2) were collected on day 12 and were incubated in triplicate for 24 h in RPMI. Cytokine production in the supernatants was measured by ELISA. Data are representative of two experiments. Error bars, s.d. *$P<0.05$. BMDC, bone marrow-derived DC; DSS, dextran sulfate sodium; EC-DC, EC-conditioned DCs; FACS, fluorescence-activated cell sorting; PBS, phosphate buffered saline; T_{reg}, regulatory T cells.

TCR transgenic mice were maintained in microisolator cages in our specific pathogen-free animal facility. $TSLPR^{-/-}$ mice on C57/BL6J background were kindly provided by Dr Warren J Leonard[32] and OT-II mice were purchased from Charles River. Foxp3-eGFP reporter mice were provided by M Oukka (Brigham and Women's Hospital, Cambridge, MA)[33] and were kindly provided by Dr Yasmine Belkaid (NIH). Animals were bred and maintained in our conventional animal facility. All experiments were carried out in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC).

DC cultures and EC isolation

BMDCs were generated from the bone marrows of C57/BL6J, BALB/c, or $TSLPR^{-/-}$ mice, cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mmol l^{-1} glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μmol l^{-1}

2-mercaptoethanol, and 30% supernatant from granulocyte-macrophage colony-stimulating factor-producing NIH-3T3 cells. Spleen and MLN-DCs were isolated by gentle collagenase treatment (1 mg ml^{-1}) and purification of CD11c$^+$ cells was carried out by FACS (MLN-DC purity>97%) or MACS (spleen DC, Miltenyi, Caldenara di Reno, BO, Italy). CD103$^+$ and CD103$^-$ DCs were sorted by flow cytometry on FACSAria (Becton Dickinson, San Jose, CA) (purity >94%) after 24 h cocultures of BMDCs with polarized EC monolayers. Murine intestinal ECs were isolated from the small intestine or colon of C57/BL6J mice, using the protocol described above. A murine small intestinal EC line MODE-K (kindly provided by Dr D Kaiserlian[34]); CMT93, a mouse rectal carcinoma cell line (kindly provided by Dr D Artis Department of Pathobiology, University of Pennsylvania, Philadelphia); 4T1 invasive mouse mammary tumor cells (CRL-2539); and the NIH-3T3 cells (CRL-1658) (obtained from The American Type Culture Collection, Manassas, VA) were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mmol l^{-1} glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and 50 µmol l^{-1} 2-mercaptoethanol.

DC conditioning and activation

Supernatants of confluent MODE-K, CMT93, 4T1, or NIH-3T3 fibroblast cell lines, or supernatants collected after 24 h culture of primary IECs were used for DC conditioning. Alternatively, CMT93 cells were cultured for 78 hours in the upper chambers of Transwell filters (3 µm in pore diameter; Costar Europe, Badhoevedorp, The Netherlands) until a transepithelial resistance of 300 Ω times per cm^2 was achieved (polarized monolayer).

Murine BMDCs or splenic DCs were incubated for 24 h with medium alone or with cell line supernatants. In EC-DC contact-dependent interaction experiments, BMDCs were seeded for 24 h on the basolateral side of polarized monolayers of CMT93. In some of the experiments, MODE-K supernatants were pre-incubated with anti-TGF-β antibody (clone 1D11, R&D Systems, Minneapolis, MN) for 2 h at 37 °C. Alternatively, DCs were pre-incubated with LE540 (Wako Chemicals, Osaka, Japan) for 1 h at 37 °C and then used for conditioning as described above. When cytokine secretion by BMDCs (EC-conditioned or control DCs) was examined, cells were activated for 6 h with 1 µg ml^{-1} LPS (Alexis Biochemicals, San Diego, CA), were washed, and cultured for additional 24 h in the complete medium. Culture supernatants were analyzed using ELISA (IL-2, IL-6, IL-10, and IL-12p70; R&D Systems) according to the manufacturers' instructions.

T-cell differentiation assays

BMDCs were conditioned with MODE-K supernatants for 24 h or were FACS-separated into CD103$^+$ and CD103$^-$ after incubation with polarized EC monolayers, as described above. Then DCs were incubated with or without OVA (250 µg ml^{-1}, Grade VI, Sigma-Aldrich) for 6 h and were placed in 96-well plates at a ratio of 1:10 (DC/T cell) with CD4$^+$CD25$^-$ T cells purified according to the manufacturer's protocol (Miltenyi) from the spleens of OVA-specific OTII transgenic mice. For each condition, the experiment was carried out in a triplicate wells. For the experiments described in **Supplementary Figure 1b**, T cells were isolated from the spleens of DO11.10 SCID mice. CFSE-labeled CD4$^+$CD25$^-$ T cells were used for proliferation experiments. After 5 days of culture, supernatants were collected and were analyzed by ELISA for IFN-γ and IL-17 (from R&D Systems). IL-10, IL-5, and IL-13 production in the supernatants was analyzed using the cytokine bead array Flex Set System (BD Bioscience). In some cases, TGF-β (2 ng ml^{-1}) was added at day 3 of the coculture. Cells from the cocultures were stained for CD4 (Pharmigen, San Jose, CA), CD25 (Pharmigen), α4β7 (BD Biosciences), and Foxp3 (e-Bioscience, San Diego, CA) after permeabilization. In some experiments, control or conditioned BMDCs were cocultured for 5 days with CD4$^+$ Foxp3-eGFP$^-$ T cells FACS sorted from the spleens of Foxp3-eGFP reporter mice. TGF-β (2 ng ml^{-1}) was added at day 3 of the coculture.

Suppression assay

We isolated CD25$^+$ T cells from the BMDC (±MODE-K sn) T-cell cocultures and seeded them in a 1:1 ratio in a secondary coculture composed of responder CFSE-labeled CD4$^+$ T cells (1×10^5), LPS-matured DCs (2×10^4), and plate-bound anti-CD3 (0.5 µg ml^{-1}). As a positive control of the assay, CD4$^+$CD25$^+$ purified from mouse spleens were used, whereas for maximal proliferation CD4$^+$CD25$^-$ T cells from the mouse spleen were used. The proliferation of CFSE-labeled T cells from the second MLR was monitored by FACS.

RT-PCR and quantitative real-time RT-PCR

RNA was isolated from treated or untreated DCs or MODE-K cells, from FACS-sorted CD103$^+$ MLN-DCs, primary IECs, colon, MLNs or spleen, and retro-transcribed. Real-time RT-PCR (reverse transcriptase-PCR) analyses were carried out in triplicate on the Applied Biosystems 7500 Fast Real-Time PCR System with the SYBR Green PCR kit as instructed by the manufacturer (Applied Biosystems, Carlsbad, CA). The amount of mRNA was normalized to the amount of *tbp* or *rpl32* mRNA. Samples were analyzed for the expression of *Il23a*, *Il12a*, *aldh1a2*, *tgfb1* or *tslp*, using the primers listed in **Supplementary Figure 6**.

Induction of DSS colitis, T_{reg}-cell-adoptive transfer and histopathology

To induce colitis, mice were housed in cages with drinking water supplemented with 2% (w/v) DSS (TdB Consultancy AB, Uppsala, Sweden) for 4 days. BMDCs were conditioned with MODE-K supernatants as described above. After washing, DCs were placed in 48-well plates at a ratio of 1:10 (DC/T cell) with CD4$^+$CD25$^-$ T cells purified from the spleens of C57/BL6J mice and cocultured for 5 days. TGF-β was added during the last 3 days of the coculture. Total or purified CD25$^+$ cells (1×10^6) were injected intravenously 8 h before colitis induction. Body weight was measured daily as well as stool consistency and occult blood. At day 12 after colitis induction, mice were killed and intestines removed for gross examination, histopathology, and cytokine evaluation. Paraffin-embedded colon tissues were sectioned and stained with hematoxylin and eosin for pathology assessment. Colon specimens (1 cm^2) were collected (three per mice) divided into four groups and cultured in complete RPMI for 24 h. Cytokines (TNF-α, IFN-γ, and IL-6) were tested by ELISA in the supernatants.

Statistical analysis

Student's paired *t*-test was used to determine the statistical significance of the data. Significance was defined at $P<0.05$ (two-tailed test and two-sample equal variance parameters). Statistic calculations were carried out by JMP 5.1 software (SAS Cary, NC).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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