Classical dendritic cells are required for dietary antigen–mediated induction of peripheral T\textsubscript{reg} cells and tolerance

Daria Esterházy\textsuperscript{1}, Jakob Loschko\textsuperscript{2}, Mariya London\textsuperscript{1}, Veronica Jove\textsuperscript{1}, Thiago Y Oliveira\textsuperscript{2} & Daniel Mucida\textsuperscript{1}

Oral tolerance prevents pathological inflammatory responses to innocuous foreign antigens by peripheral regulatory T cells (pT\textsubscript{reg} cells). However, whether a particular subset of antigen-presenting cells (APCs) is required during dietary antigen exposure for the ‘instruction’ of naive CD4\textsuperscript{+} T cells to differentiate into pT\textsubscript{reg} cells has not been defined. Using myeloid lineage–specific APC depletion in mice, we found that monocyte-derived APCs were dispensable, while classical dendritic cells (cDCs) were critical, for pT\textsubscript{reg} cell induction and oral tolerance. CD11b\textsuperscript{−} cDCs from the gut-draining lymph nodes efficiently induced pT\textsubscript{reg} cells and, conversely, loss of transcription factor IRF8–dependent CD11b\textsuperscript{−} cDCs impaired their polarization, although oral tolerance remained intact. These data reveal the hierarchy of cDC subsets in the induction of pT\textsubscript{reg} cells and their redundancy during the development of oral tolerance.

RESULTS

Break in oral tolerance in the systemic absence of cDCs

We first set out to determine whether one of the two main myeloid lineages, the macrophage-monocyte lineage or the common DC precursor lineage, was required for the induction of oral tolerance (Supplementary Fig. 1a). Throughout our study, we focused on the populations present in the mLNs, the main inductive sites of oral tolerance\textsuperscript{14}. The macrophage-monocyte lineage in mLNs is dominated by macrophages, identified as Lin\textsuperscript{−}MHCII\textsuperscript{+}CD11c\textsuperscript{+}CD64\textsuperscript{+} cells, and these DCS are believed to induce the conversion of naive CD4\textsuperscript{+} T cells into pT\textsubscript{reg} cells after they migrate to the mLNs\textsuperscript{14,15}. Indeed, both lamina propria–derived DCS and mLN-derived DCS, particularly integrin \alpha\textsubscript{E} (CD103)--positive or DEC205\textsuperscript{+} DCS, produce large amounts of RA and TGF-\beta and efficiently induce pT\textsubscript{reg} cells\textsuperscript{1,6–8,16–19}. However, whether these pT\textsubscript{reg} cell–inducing APCs are also required for the induction of oral tolerance has not been investigated. Furthermore, because the strategies used so far that rely on cell surface markers have targeted multiple APC lineages, the exact nature and origin of APCs responsible for the induction of pT\textsubscript{reg} cells \textit{in vivo} are still unclear.

Here we identified an essential role for pre-DC–derived classical dendritic cells (cDCs) for the induction of both pT\textsubscript{reg} cells and oral tolerance, while macrophages and monocyte-derived cells seemed to be dispensable for this. Furthermore, we identified a hierarchical pattern for the pT\textsubscript{reg} cell–inducing capacity of mLN-derived cDC subsets, whereby dietary antigen–mediated polarization of pT\textsubscript{reg} cells was the most dependent on migratory CD11b\textsuperscript{−} cDCs dependent on the transcription factor IRF8. Oral tolerance was intact, however, in absence of this cDC subset, which highlights the robustness of this process and the functional redundancy of cDCs.

1Laboratory of Mucosal Immunology, The Rockefeller University, New York, New York, USA. 2Laboratory of Molecular Immunology, The Rockefeller University, New York, New York, USA. Correspondence should be addressed to D.M. (mucida@rockefeller.edu).

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(CD8<sup>+</sup>CD11b<sup>−</sup>CD11c<sup>+</sup> versus CD8<sup>+</sup>CD11b<sup>−</sup>CD11c<sup>+</sup>) and two migratory MHCII<sup>hi</sup> populations (CD103<sup>+</sup>CD11b<sup>−</sup> versus CD103<sup>+</sup>CD11b<sup>+</sup>) (Fig. 1a).

We first used a mouse model of delayed-type hypersensitivity of the Th<sub>1</sub> subset of helper T cells<sup>13</sup> to address whether a specific APC lineage was required for the induction phase of oral tolerance. Tolerance was assessed by measurement of the cellular and humoral inflammatory immune response to ovalbumin (OVA) in mice pre-exposed to oral OVA or oral PBS (as a control) and immunized with OVA in complete Freund’s adjuvant (CFA) (Fig. 1b). We targeted the macrophage-monocyte lineage through the use of ‘MM DTR mice’ (which express the gene encoding Cre recombinase under control of the myeloid cell–specific Lyz2 promoter (Lyz2<sup>Cre</sup>) and sequence encoding the diphtheria toxin receptor (DTR) gene preceded by a loxp site–flanked stop cassette under control of the macrophage-specific Csf1r promoter (Csf1r<sup>LoxP-LoxP-DTR</sup>).<sup>20,21</sup> We targeted the pre-DC–derived lineage through the use of ‘zDC DTR mice’ (which have sequence encoding DTR knocked in 3′ of Zbtb46, which encodes a transcription factor (Zbtb46) of the pre-DC lineage (Zbtb46<sup>DTR</sup>)<sup>21</sup>). Because diphtheria toxin (DT) is lethal to zDC DTR mice, due to the expression of Zbtb46 in vascular endothelium<sup>21</sup>, we used chimeras generated by the transfer of total bone marrow (BM) cells from zDC<sup>DTR</sup> or MM DTR mice or DTR-negative control mice (called ‘wild-type’ mice here) into lethally irradiated C57BL/6 mice. The administration of DT to the resultant MM<sup>DTR</sup> chimeras led to broad depletion of monocytes, monocyte- and yolk sac–derived tissue macrophages and inflammatory DCs, which lasted for at least 36 h (ref. 20) (Supplementary Fig. 1c–f), while the administration of DT to the resultant zDC<sup>DTR</sup> chimeras resulted in a compensatory surge in the cytokine Ifnγ in the serum, as well as a complete loss of cDCs, including migratory cDC subsets in the mLNs<sup>21</sup> (Supplementary Fig. 1c,d). To induce oral tolerance, we fed OVA to mice by gavage (‘OVA gavage’) at 12 and 30 h after the first of three DT injections given 36 h apart, while mice fed PBS served as non-tolerant controls, and we timed the administration of DT and OVA or PBS such that the clearance of OVA (completed after 24 h) preceded the reappearance of APCs<sup>20</sup> (Supplementary Fig. 1c–h). We ruled out the possibility that the administration of DT to the chimeras resulted in altered absorption of oral antigen, as we observed similar uptake of OVA into various tissues in DT-treated zDC<sup>DTR</sup>, MM<sup>DTR</sup> and wild-type mice at 4 h after OVA gavage (Supplementary Fig. 1i–m).

Because APCs are required for immune responses, we immunized mice with OVA plus CFA 8 d after the final DT dose, when the APC niches were replenished (Fig. 1b and Supplementary Fig. 1n,o), followed by subcutaneous injection of OVA in the ear 14 and 24 d after immunization.

At 72 h after the injection of OVA into the ear, ear swelling and serum titers of anti-OVA immunoglobulin G1 (IgG1) and anti-OVA IgG2c were lower in OVA-fed wild-type and MM<sup>DTR</sup>, but not in OVA-fed zDC<sup>DTR</sup> chimeras, than in their PBS-fed counterparts (Fig. 1c–e). We noticed that PBS-fed zDC<sup>DTR</sup> chimeras immunized with OVA in CFA produced less serum IgG than did their wild-type or MM<sup>DTR</sup> counterparts, probably a consequence of the overall reduced frequency of CD4<sup>+</sup> T cells after recent depletion of cDCs (Supplementary Fig. 1o). As a positive control for the APC dependence of oral tolerance induction, we also studied chimeras lacking all CD11c-expressing APCs<sup>20,22</sup>, generated by the transfer of BM from mice expressing the DTR under control of the promoter of Ilgax, which encodes the integrin and common APC marker CD11c (‘CD11c<sup>DTR</sup> mice’), into lethally irradiated C57BL/6 mice (to generate ‘CD11c<sup>DTR</sup> chimeras’)<sup>20,22</sup>. PBS-fed and OVA-fed CD11c<sup>DTR</sup> chimeras showed similar ear swelling and serum anti-OVA immunoglobulin responses (Fig. 1c–e), which suggested a lack of tolerance to OVA. These observations indicated that monocyte-macrophage–derived APCs were dispensable for oral tolerance induction, while pre-DC–derived cells were critical for this.

Next we assessed the requirement for cDCs in preventing allergic responses of the Th<sub>2</sub> subset of helper T cells<sup>2,5,6</sup>. Following the same regimen used for the administration of DT and oral OVA described above, we immunized mice with OVA in the adjuvant alum at 8 and 15 d after the final injection of DT and challenged the mice intranasally with OVA three times at 3-day intervals (Fig. 2a). Eosinophilia in the bronchoalveolar lavage fluid and lungs, as well as total lung-tissue cellular infiltrates and serum IgE, were similarly lower in OVA-fed wild-type and MM<sup>DTR</sup> chimeras, but not in OVA-fed zDC<sup>DTR</sup> chimeras, than in the corresponding PBS-fed chimeras (Fig. 2b–g). These data showed that the absence of cDCs during antigen feeding resulted in a failure to establish tolerance to both Th<sub>1</sub> and Th<sub>2</sub> immunity.

**Requirement for cDCs in pTreg cell induction**

Oral tolerance is mediated by pTreg cells<sup>2,3</sup>. We thus addressed whether the pre-DC lineage was required for the induction of pTreg cells after antigen feeding by adoptively transferring sorted naïve CD45.1<sup>+</sup>
OVA-specific OT-II cells into CD45.2 BM chimeras (generated as described above) depleted of APCs, then assessing the appearance of CD45.1+ (donor) Foxp3+ T cells in various tissues after the administration of OVA by gavage (Fig. 3a). The administration of DT alone did not induce the activation, proliferation or differentiation of OT-II cells in the chimeric host mice (data not shown). At 48 h after the initial administration of OVA, MM(DTR) chimeric host mice displayed compromised (but perceptible) induction of pTreg cells in mLNs compared with that of wild-type chimeric host mice (Fig. 3b,c), an effect that was accompanied by an unaltered degree of OT-II cell engraftment but reduced overall activation and division of OT-II cells (Fig. 3b–f and Supplementary Fig. 2a). In contrast, pTreg cells were almost completely undetectable in OVA-fed zDC(DTR) chimeric host mice (Fig. 3b,c), and the activation of OT-II cells was decreased compared with that of OVA-fed wild-type chimeric host mice (Fig. 3d). The engraftment of OT-II cells was lower in zDC(DTR) chimeric host mice than in wild-type chimeric host mice (Fig. 3d), an observation we also made after the administration of DT alone (Supplementary Fig. 2b). At this time point we did not find substantial activation of OT-II cells or induction of pTreg cells in the spleen or distal lymph nodes of any of the groups studied (data not shown). The effect of the depletion of monocytes-macrophages on the induction of pTreg cells was compensated for over time, as by 7.5 d after OVA feeding, the frequency of pTreg cells in MM(DTR) chimeric host mice was similar to that of their wild-type counterparts (Fig. 3g), in line with the intact tolerance found in these MM(DTR) chimeric mice (Figs. 1c–e and 2b–g). In zDC(DTR) chimeric host mice, the frequency of OT-II cells 7.5 d after OVA gavage were proportional to the CD4+ T cell compartment (Supplementary Fig. 2c); however, the frequency of pTreg cells among the transferred cells was still substantially reduced in the mLNs (Fig. 3g and Supplementary Fig. 2d,e), spleen and distal lymph nodes (data not shown) compared with the induction of pTreg cells in wild-type chimeric hosts. These data indicated that the activation and proliferation of CD4+ T cells and the induction of pTreg cells depended on cDCs and excluded the possibility of an essential contribution of monocyte-derived APCs to the generation of pTreg cells after oral exposure to antigen.

**Contribution of CX3CR1+ APCs to pTreg cell polarization in mLNs**

Here we distinguished APC subsets by ontogeny, but other studies have ascribed roles to gut-associated APCs on the basis of CX3CR1- targeting approaches. To define the lineage of origin of CX3CR1-expressing APC populations in steady-state mLNs, we generated chimeras by transferring BM from the progeny of Cx3cr1+/GFP mice (with heterozygous expression of green fluorescent protein (GFP) from Cx3cr1) crossed to zDC(DTR), MM(DTR) or wild-type control mice into lethally irradiated C57BL/6 mice, then injected DT into the resultant chimeras and characterized their loss of GFP+ cells. GFP+ cells represented roughly 25% of all CD11c+ APCs in the mLNs (Fig. 4a,b). The results obtained by the administration of DT to MM(DTR)Cx3cr1+/GFP chimeras indicated that most monocyte-macrophage-derived cells were among the GFP+ cells (Fig. 4b,c). In addition to CD64+ macrophages, about half of CD86+ and CD11b+ cells, and roughly 20% of CD103+CD11b+ populations, among the GFP+ cells underwent depletion via DT in MM(DTR)Cx3cr1+/GFP chimeras (Fig. 4b,c), which indicated that a portion of the GFP+CD64+ cells were of monocyte-macrophage origin. Notwithstanding those results, targeting of DT in zDC(DTR)Cx3cr1+/GFP chimeras revealed that cDC-derived cells represented the majority of APCs among CX3CR1+.
cells (Fig. 4b,c). These analyses highlighted the heterogeneity of CX3CR1 expression by APC subsets.

Microbiota-derived signals have been linked to regulation of the migration of CX3CR1+ mononuclear phagocytes \(^1\). To better characterize these cells, we transplanted BM from zDCDTR Cx3cr1+/GFP or wild-type Cx3cr1+/GFP mice into lethally irradiated germ-free (GF) or specific pathogen–free (SPF) wild-type host mice. We found a selective expansion of the CD103+/CD11b+ population in the mLN of all GF chimeric host mice relative to its size in the SPF host mice, regardless of the genotype of the BM transferred (Fig. 4d and Supplementary Fig. 3a). However, we observed the same fraction of CX3CR1+ cells among CD103+/CD11b+ cells in GF and SPF chimeric host mice (data not shown), which indicated that the enrichment could not be explained by specific mobilization of CX3CR1+ cells. Depleting the mice of cDCs indicated that CD11c+CD64− cells, including migratory subtypes, remained pre-DIC depleted in the zDCDTR Cx3cr1+/GFP GF chimeras (Fig. 4d and Supplementary Fig. 3b).

Finally, we sought to determine whether the altered cDC composition in the GF chimeras had an effect on the induction of pTreg cells by analyzing the fate of adaptively transferred OT-II cells upon OVA gavage in these mice relative to the fate of such cells in their SPF counterparts. The GF chimeric hosts displayed reduced induction and cell division of pTreg cells compared with that of SPF chimeric hosts, and the administration of DT completely prevented the appearance of Foxp3+ OT-II cells in both zDCDTR Cx3cr1+/GFP GF hosts and zDCDTR Cx3cr1+/GFP SPF hosts (Fig. 4e). The number of CD4+ T cells and engraftment of OT-II cells was lower in DT-treated zDCDTR Cx3cr1+/GFP chimeric host mice than in Cx3Cr1+/GFP chimeric host mice, and the degree of this reduction was the same in the GF condition and SPF condition (Supplementary Fig. 3c,d). These data indicated that the composition of APCs in GF mice was less favorable for the induction of pTreg cells than was the composition of APCs in SPF mice and that the remaining APCs in GF hosts depleted of cDCs was not able to improve the blunted induction of pTreg cells observed in zDCDTR Cx3cr1+/GFP SPF mice.

To further reconcile our lineage-based approach with CX3CR1-targeting strategies and address the role of CX3CR1+ APCs in the induction of pTreg cells, we performed experiments in which we adaptively transferred OT-II cells into lethally irradiated C57BL/6 recipients of IlgaxCre × Cx3cr1Lsd−DTR (CX3CR1-DTR) BM to generate ‘CX3CR1-DTR host chimeras’ or Cx3cr1Lsd−DTR (‘wild-type’ control) BM (to generate ‘wild-type host chimeras’) \(^1\). In CX3CR1-DTR mice, expression of Cre recombinase is under control of the Ilgax promoter, while the sequence encoding DTR is preceded by a loxP site–flanked stop cassette under control of the Cx3cr1 promoter, which allows DT-mediated depletion of (CD11c+) CX3CR1+ cells for at least 48 h (ref. 13). As reported before \(^1\), the administration of DT to CX3CR1-DTR chimeric hosts led to depletion of mLN macrophages (CD11c+CD64+ cells), although it also depleted CD11c+CD64− cells (Fig. 4f). The degree of depletion in each APC subset (Fig. 4g) correlated with the frequency of CX3CR1+ cells in these subsets (Fig. 4a–c). In line with that pleiotropic targeting of APCs, the induction of pTreg cells from adaptively transferred naive OT-II cells at 48 h after gavage of OVA was reduced more in DT-treated CX3CR1-DTR chimeric hosts than in DT-treated MM DTR chimeras, relative to that of their respective wild-type (control) counterparts (Figs. 4h and 3c), while the activation and division of OT-II cells were affected only mildly, and the number of CD4+ T cells and engraftment of OT-II cells were not altered, relative to that of their wild-type counterparts (Figs. 4i and Supplementary Fig. 3e,f). However, similar to the results obtained for MM DTR chimeric mice, the frequency of pTreg cells and division of OT-II cells were restored to wild-type levels by day 7.5 after gavage of OVA (Fig. 4j). Together these results suggested that CX3CR1-expressing macrophages were not essential for the generation of pTreg cells by dietary antigen, while cDCs were required for this; they also highlight the merits of a lineage-based approach for delineation of the differential contributions of APCs to the induction of pTreg cells and oral tolerance.

**Differential molecular signatures of cDCs**

Published studies have described pTreg cell–inducing DC populations in mouse gut and gut-draining lymph nodes \(^6,8,10,16\). Additionally, large transcriptome databases for specific cDC populations have been made available \(^24–26\). However, those studies did not compare IRF4− versus IRF8−dependent DC populations (distinguishable by CD8α–CD11b+ or CD8α–CD11b− staining, respectively) among resident (CCR7+ or MHCIImed) and migratory (CCR7− or MHCIIfp) DC populations. To obtain a higher resolution of the cDC subsets and to link them with their lineage of origin, we performed RNA sequencing (RNA-seq) of cDCs present in the mLNs. We analyzed four CD11c+CD64− subsets: mLN-resident MHCIImed (CD8α+CD11b+CD103+ or CD8α−CD11b−CD103−) subpopulations and migratory MHCIIfp (CD11b+CD103+ or CD11b+CD103−) subpopulations. Plasmacytoid DCs (pDCs) were not evaluated due to their very low abundance and the fact that these cells are not targeted in zDCDTR chimeras after the administration of DT. Of note, we...
observed that the relative frequencies of cDC and lymphocyte subsets were similar in the various lymph nodes draining the proximal to distal gastrointestinal tract (Supplementary Fig. 4a–m), and therefore we subjected cDCs sorted from total mLNs pools to expression profiling. Unsupervised principle-component analysis revealed that resident and migratory cDC subsets were the populations most segregated (Supplementary Fig. 4n), while IRF8- and IRF4-dependent subsets followed a hierarchical pattern in their tolerance-inducing gene expression. Thus, migratory, IRF8-dependent CD103+CD11b− DCs had the highest expression of Aldh1a2 (which encodes the retinaldehyde dehydrogenase RALDH2), as well as Tgfb2 (which encodes the cytokine TGF-β2), Ilbs (which encodes the integrin β9) and several other genes encoding products associated with generation of Treg cells and homing to the gut (Fig. 5a–c and Supplementary Fig. 4o,p), This gene signature was progressively lost in the CD8α− and CD103+CD11b+ populations, which instead expressed genes encoding other isoforms of TGF-β and RALDH; CD11b+ DCs had the lowest expression of these genes (Fig. 5a–c and Supplementary Fig. 4o,p). This hierarchy was confirmed by the activity of RALDHs in cDCs freshly isolated from total mLNs (Fig. 5d). In contrast, CD103+CD11b− DCs did not express genes encoding typical pro-inflammatory products, although Il12b (which encodes
Figure 5 Characterization of mLN cDC subpopulations. (a) RNA-seq analysis of genes (right margin) expressed most differentially in CD11b+ DCs (relative to their expression in CD103+CD11b+ DCs) in various cDC subsets (above plots) from the mLNs of C57BL/6 mice. (b, c) RNA-seq analysis of gene clusters encoding products involved in the synthesis of TGF-β (b) or RA (c) in various cDC subsets (key) from the mLNs of C57BL/6 mice. (d) Mean fluorescence intensity (MFI) of fluorescein isothiocyanate–positive (FITC+) aminoacetate (indicative of ALDH activity on the substrate aminoacetaldehyde) in various mLN cDC populations (key) as in b, c, pre-incubated with the ALDH inhibitor DEAB (+) or not (−) and assessed by flow cytometry 30 min after the addition of substrate. (e) RNA-seq analysis of gene expression in various cDC subsets in mLNs as in b, c. Each symbol (b–d) indicates an individual mouse. *P < 0.05, **P < 0.01 and ***P < 0.005 (two-tailed t-test). Data are representative of one experiment (a–c); average ± s.e.m. of n = 3 biological replicates in b, c; n = 3 biological replicates in e) or four independent experiments (d); average ± s.e.m. of n = 4 biological replicates.)
the cytokine IL-12p40) was ‘preferentially’ expressed in these cells relative to its expression in the other DC populations (Supplementary Fig. 4q). However, transcripts of Il12a (which encodes IL-12p35) or Il23a (which encodes IL-23p19) were not present in the absence of inflammatory triggers (Supplementary Fig. 4q). While CD11b+ DCs had the lowest expression of the pTreg cell–inducing gene signature, they had the highest expression of genes encoding products associated with pathogen sensing and the initiation of inflammatory responses, including TLRs and TLR-signaling molecules (Fig. 5a,e), and they had a distinct cytokine-expression profile (Supplementary Fig. 4q). This expression profiling indicated that IRF8-dependent cDCs were the main tolerogenic subsets in the mLN.

**Effect of Zbtb46-driven targeting of Irf8 on pTreg cell induction**

Several strategies have been devised in the past to ablate CD11b− DCs dependent on the transcription factors IRF8 and BATF3 (refs. 27–30), but some approaches have led to defects beyond those caused by the loss of cDC subsets. Additional cDC-specific genetic approaches have been reported and they have revealed the reliance of helper T cell polarization on certain DC populations. However, whether a specific cDC subset is necessary for the induction of pTreg cells and oral tolerance has not been addressed. We therefore generated a mouse strain expressing Cre under control of the endogenous Irf8 exon 2 (Irf8fl/fl), which encodes the DNA-binding domain of IRF8, to generate mice with cDC-specific Irf8 deficiency (zDC(ΔIrf8) mice). The zDC(ΔIrf8) mice were viable, were born at normal frequencies and had a normal lymphoid organ architecture. However, compared with control mice, zDC(ΔIrf8) mice had significantly fewer cDCs in the mLNs and other tissues, which was confirmed by flow cytometry (Fig. 3a). This reduction was accompanied by a significant decrease in the number of CD103+CD11b−, CD103+CD11b+ and CD103− cells among MHCII+CD11c+ migratory cDCs, CD11c+MHCIIint (resident) cDCs and total small intestine of 10-week-old IRF8fl/fl and zDC(ΔIrf8) mice. Numbers adjacent to outlined areas indicate percent cells in each. (d,e) Frequency of CD103−CD11b−, CD103−CD11b+ and CD103− cells among MHCII+CD11c+ cells (d) or CD11c+CD64− cells (e) as in c. (f,g) Frequency of CD8+ or CD4+ cells T cells (CD3+) (f) and CD4+CD62L+ or CD4+CD44+ T cells and CD4+Foxp3+ Treg cells (g) in the mLNs or lamina propria of the SI LP of 10-week-old IRF8fl/fl and zDC(ΔIrf8) mice. Each symbol (b,d,g) indicates an individual mouse. *P < 0.05, **P < 0.01 and ***P < 0.005 (two-tailed t-test). Data are representative of two independent experiments (average ± s.e.m. n = 4 mice per group in b,d,g).

**Figure 6** Analysis of steady-state cDC and lymphocyte populations in the mLNs and lamina propria of zDC(ΔIrf8) and IRF8fl/fl mice. (a) Flow cytometry of cells from the mLNs of IRF8fl/fl and zDC(ΔIrf8) mice. Numbers adjacent to outlined areas indicate percent cells in each. (b) Frequency of CD11c+ cDCs, CD11c+MHCIIhi (migratory) cDCs, CD11c+MHCIIint (resident) cDCs and total CD45+ cells as in a. (c) Flow cytometry of DCs in the lamina propria of the small intestine of 10-week-old IRF8fl/fl and zDC(ΔIrf8) mice. Numbers adjacent to outlined areas indicate percent cells in each. (d,e) Frequency of CD103−CD11b−, CD103−CD11b+ and CD103− cells among MHCII+CD11c+ cells (d) or CD11c+CD64− cells (e) as in c. (f,g) Frequency of CD8+ or CD4+ cells T cells (CD3+) (f) and CD4+CD62L+ or CD4+CD44+ T cells and CD4+Foxp3+ Treg cells (g) in the mLNs or lamina propria of the SI LP of 10-week-old IRF8fl/fl and zDC(ΔIrf8) mice. Each symbol (b,d,g) indicates an individual mouse. *P < 0.05, **P < 0.01 and ***P < 0.005 (two-tailed t-test). Data are representative of two independent experiments (average ± s.e.m. n = 4 mice per group in b,d,g).
Mendelian ratios, and exhibited normal weights and no visible pathology up to the age of 12 weeks (data not shown). This strategy led to the loss of CD8α+ and CD11b− cDCs in the mLNs, lamina propria (Fig. 6a–e) and spleen (Supplementary Fig. 5a–c) of zDC(Δirf8) mice in contrast to the presence of these cells in those tissues in Irf8fl/fl mice, with no major effects on the frequency of T cells or B cells (Fig. 6f and data not shown). We noticed that excision of irf8 (ref. 35) led to variable loss of cDCs; however, since the degree of loss of CD8α+ and CD11b− DCs was the same in lethally irradiated wild-type recipients of zDC(Δirf8) BM as in the donor of the BM used (data not shown), BM transplantation preserved the degree of niche depletion and permitted the generation of large and uniform cohorts of mice deficient in CD8α+ and CD11b− cDCs.

We therefore transplanted BM from zDC(Δirf8) or Irf8fl/fl mice into lethally irradiated wild-type host mice to assess the importance of IRF8-dependent cDCs in oral tolerance (Fig. 1b, excluding DT injection). We found that ear swelling and titers of IgG1 and IgG2c in the serum were suppressed to a similar degree in OVA-fed zDC(Δirf8) or Irf8fl/fl chimeras compared with such results obtained for their PBS-fed counterparts (Fig. 7a–c). We observed this apparently intact tolerance in zDC(Δirf8) chimeras even when we immunized them with OVA plus CFA 24 h after the first oral antigen encounter, to blunt potential compensatory population expansion of pTreg cells (data not shown).

Next, we directly assessed the importance of IRF8-dependent cDCs for the polarization of pTreg cells after the administration of oral antigen in vivo. We adoptively transferred naive OT-II cells into zDC(Δirf8) and Irf8fl/fl chimeras and treated them as described above (Fig. 3a, excluding injection of DT). At 48 h after first oral encounter with OVA, the induction of pTreg cells among adoptively transferred naive OT-II cells was lower in zDC(Δirf8) chimeric hosts than in Irf8fl/fl chimeric hosts, while the division of OT-II cells was significantly greater in zDC(Δirf8) chimeric hosts than in Irf8fl/fl chimeric hosts (Fig. 7d–f). This phenomenon persisted in the mLNs, spleen and other peripheral lymph nodes at 7.5 d after mice were fed OVA (Fig. 7g and data not shown). OT-II cells did not produce substantial amounts of the cytokines IFN-γ.

Figure 8 In vitro characterization of the pTreg cell-induction potential of cDC subpopulations in the mLNs. (a) RNA-seq analysis of genes (right margin) expressed most differentially in OT-II cells cultured for 24 h with OVA peptide plus CD11b+ DCs (relative to the expression in OT-II cells cultured with CD103+CD11b− DCs), among various cDC subsets (above plots) from the mLNs of C57BL/6 mice (n = 3 per group, with DCs from three independent donor pools). (b) RNA-seq analysis of gene clusters encoding products involved in Treg cells and proliferation (vertical axes) in OT-II cells cultured together with various cDC subsets (key) from the mLNs of C57BL/6 mice (n = 3 per group), as determined in a. (c) Frequency of Foxp3+ cells and division index of sorted naive CD45.1+ OT-II cells cultured for 72 h together with various cDC subsets (key; three independent DC donor pools) from the mLNs of C57BL/6 mice (n = 4 (resident) or n = 6 (migratory) mice per group), along with OVA peptide, plus anti-IL-4 and anti-IFN-γ, assessed by flow cytometry. (d) Frequency of Foxp3+ cells among sorted naive CD45.1+ OT-II cells cultured for 72 h with anti-IL-4 and anti-IFN-γ, together with various cDC subsets (key; three independent DC donor pools) obtained from the mLNs of mice (n = 4–6 mice per group) fed an OVA-rich diet for 48 h, assessed by flow cytometry. Each symbol (b–d) indicates an individual mouse. *P < 0.05, **P < 0.01 and ***P < 0.005 (two-tailed t-test). Data are representative of one experiment (a,b; average ± s.e.m. in b) or three independent experiments (c,d; average ± s.e.m.).
IL-5 or IL-17a under these conditions, and this was not greater in zDC(ΔIRF8) chimeric hosts than in Irf8−/− chimeric hosts (data not shown). These findings were in line with loss of the migratory and resident mLN DC subsets with the most tolerogenic signatures. However, the impairment in the induction of pTreg cells was less severe in zDC(ΔIRF8) chimeric hosts (Fig. 7d,g) than in zDCΔDTR chimeric hosts (Fig. 3c). These results demonstrated that the targeting of IRF8-dependent cDCs partially prevented the generation of pTreg cells in vivo but did not affect oral tolerance, which indicated that the remaining cDC pools and pTreg cells could have compensated for loss of the IRF8-dependent DC lineage during the development of oral tolerance.

**Hierarchical roles of cDCs in pTreg cell induction**

To gain insight into early changes ‘imprinted’ on T cells by different DC populations, we isolated cDC subsets from the mLNs of C57BL/6 mice, then cultured OT-II cells together with the cDCs and OVA peptide and performed RNA-seq analysis of the activated OT-II cells after 24 h of co-culture. Unsupervised principle-component analysis indicated that OT-II cells stimulated by resident and migratory cDC subsets were the populations most segregated (Supplementary Fig. 6), analogous to the clustering of DC subsets (Supplementary Fig. 4n). The various cDC subsets induced a hierarchical pTreg cell pattern in OT-II cells (Fig. 8a). Consistent with the cDC gene profile (Fig. 5a–c), and with a process initiated by the TGF-β and RA pathways6–8,16,17,36, naive T cells upregulated genes encoding several pTreg cell- and gut homing–associated products when exposed to CD103+CD11b− DCs; CD103+CD11b+ and CD8α+ DCs induced an intermediary phenotype; and CD11b+ DCs were the least able to trigger this signature (Fig. 8a,b). The pTreg cell–polarizing capacity of mLN cDC subsets was confirmed in co-culture experiments8–10,16,17,36, with the process initiated by the TGF-β and RA pathways6–8,16,17,36, naive T cells upregulated genes encoding several pTreg cell- and gut homing–associated products when exposed to CD103+CD11b− DCs; CD103+CD11b+ and CD8α+ DCs induced an intermediary phenotype; and CD11b+ DCs were the least able to trigger this signature (Fig. 8a,b). The pTreg cell–polarizing capacity of mLN cDC subsets was confirmed in co-culture experiments8–10,16,17,36, with the process initiated by the TGF-β and RA pathways6–8,16,17,36

Since the provision of antigen in vitro might obscure differences in antigen capture and processing that occur in vivo, we also assessed the polarization of pTreg cells in co-cultures with Cx3cr1 reporter mice and lineage-depletion strategies, we determined the contribution of macrophages and cDC subsets to the CX3CR1+ pools in mLN and lamina propria. We observed deletion of both macrophages and CX3CR1-expressing cDCs in the mLNs of CX3CR1ΔDTR chimeras upon administration of DT. Regardless of the apparent targeting differences, we did not find sustained impairment in the induction of pTreg cells in CX3CR1ΔDTR chimeras given injection of DT, in line with our observation that monocyte-derived APCs were not strictly needed for oral antigen–mediated induction of pTreg cells. However, whether CX3CR1-expressing cells have a specific role in the maintenance of pTreg cells and oral tolerance or, alternatively, whether CX3CR1 expression is required for the functional and anatomical properties of cDCs are issues that remain to be resolved.

**Lineage-based intersectional genetics also provided information about the hierarchy of cDC subsets in their pTreg cell–induction potential.** IRF8-dependent cDCs and the CD8α+ or CD103+CD11b+ cDC populations43,44 were highly efficient in the induction of pTreg cells. Our transcriptomics results confirmed and expanded published analyses24,25, identifying much higher expression of genes related to the TGF-β and RA pathways in migratory CD103+CD11b− DCs. Furthermore, while our data have confirmed published observations that CD103+ DCs are efficient inducers of pTreg cells via these pathways, they also identified the subpopulation among the three CD103+ DC populations in the mLNs (CD8α+CD11b−CD103+, CD11b−CD103+ and CD11b+CD103+) that governs this phenomenon. Parallel RNA-seq of T cells and cDCs also revealed cDC subset–specific gene signatures that closely mirrored early gene programs induced in T cells, which further reinforced the conclusion of a role for these pathways in the generation of pTreg cells24,6,8.
In line with published studies addressing the role of IRF4 and the receptor Notch2 in the development of cDCs, we observed that CD11b+ DCs subsets were rather inefficient in the induction of pTreg cells, while they showed a superior capacity (relative to that of the other DC subpopulations) for inducing the population expansion and maintenance of CD4+ T cells and polarization toward helper T cells. The ‘preference’ of particular cDC subsets in inducing the differentiation of pTreg cells versus effector T cells was not absolute, as demonstrated by our Irf8-targeting approach that resulted in reduced generation of pTreg cells but intact oral tolerance. Our observations support those of published reports indicating a ‘division of labor’ among APC subsets. However, depletion of the microbiome, which has been shown to boost the migratory capacity of intestinal APCs, did not ‘rescue’ the induction of pTreg cells in mice depleted of cDCs. Additionally, although migratory IRF8-dependent cDCs seemed to have the greatest capacity to induce pTreg cell differentiation in the mLNs, this subset was underrepresented in the lamina propria. That observation, along with the known cell-free antigen distribution through the intestinal draining lymph, raises the possibility that antigen sampling and carriage might have only a limited effect compared with that of cell-independent pathways of antigen distribution; instead, what determines the degree of pTreg cell induction might be the composition of cDCs in the mLNs that capture antigen in situ. Therefore, characterization of the relative contributions of APC-mediated antigen delivery versus cell-free antigen delivery is a logical next step for such studies.

METHODS

Accession codes. GEO: RNA-seq data, GSE77305.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.E. designed the study, performed the experiments (unless stated otherwise below) and wrote the manuscript; J.L. generated the zDCCre mice and backcrossed them onto the Irf8−/−background, carried out their hematopoietic characterization (Fig. 1) and substantially contributed to the design of experiments; M.L. and V.J. contributed substantially to the establishment of oral-tolerance protocols and to the oral-tolerance experiments; T.Y.O. performed the bioinformatics analysis of the RNA-seq experiments; and D.M. initiated, designed and supervised the study, performed experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 CD45.1 (B6.85-L-Prrca Pech/B0y) and CD45.2 (C57BL/6), Lyz2<sup>Cre/+</sup> (B6.129P2-Lyztm1(cre)Hfe/J), Cx3cr1<sup>YFP</sup> (B6.129P(Cg)-Pprca Cx3cr1tm1Litt/Litt), Cx3cr1<sup>YFP</sup> (B6.129P2-Cx3cr1tm1(Litt)/Litt), CD11c<sup>Cre</sup> (B6.Cg-Tg(Itgax-cre)1-1Reiz/J), CD11c<sup>DTR</sup> (B6.FVB-Tg(Itgax-DTR/EGFP)23Lan/J) and Irf8<sup>fl/fl</sup> (B6.Cg-Ybrm1.1HmJ) mice were purchased from the Jackson Laboratories, and CD45.1 OT-II mice were originally purchased from Taconic Farms and maintained in our facilities. Cx3cr1<sup>YFP</sup> (B6.Cg-Ybrm1.1HmJ) and Irf8<sup>fl/fl</sup> (B6.Cg-Ybrm1.1HmJ) mice were purchased from the Jackson Laboratories, and determined the most stomach-proximal lymph nodes surrounded by chyle, indicative of duodenal drainage, 2 h after gavage. Non-chylous stomach-proximal lymph nodes (celiac and pancreatic) were excluded from total mLN analyses.

Cell sorting. Cells were sorted using a FACSAria cell sorter flow cytometer (Becton Dickinson). For mesenteric DC sorting, cells were pre-enriched using anti-CD11c MACs beads (Miltenyi Biotec) and LS MACs Separation Columns (Miltenyi Biotec). DCs were sorted as Aqua<sup>CD45.1</sup>L<sup>−</sup> (CD3<sup>B20</sup>-NK1.1<sup>CD19</sup>)<sup>CD11<sup>)</sup>Ch<sup>−</sup> and the subpopulations further as MHCI<sup>CD8</sup>ε<sup>CD11<sup>)B</sup>B<sup>CD8</sup>ε<sup>CD</sup>8<sup>CD11<sup>)B</sup>B</sup>-, MHCI<sup>CD8</sup>ε<sup>CD8</sup>ε<sup>CD11<sup>)B</sup>B</sup>-, MHCI<sup>CD8</sup>ε<sup>CD</sup>8<sup>ε</sup>CD11<sup>−</sup> and MHCI<sup>CD8</sup>ε<sup>CD</sup>8<sup>ε</sup>CD11<sup>−</sup>. Naive CD4<sup>T</sup> cells were pre-enriched by negative selection using biotinylated anti-CD8<sup>ε</sup>, anti-CD25, anti-CD11c, anti-CD11b, anti-TER-119, anti-NK1.1 and anti-B220 (Supplementary Table 1) and anti-biotin MACs beads (Miltenyi Biotec) and sorted as Aqua<sup>CD11<sup>C</sup>B6</sup> OT-II<sup>DTR</sup> and MMDTR chimeric mice; and finally, because depletion type mice; next, we determined the frequency of each subpopulation in DT-treated wild-type mice; and immediately mixed with an equal volume of 0.1 N HCl containing 125I-iodine labeling of ovalbumin and 125I-OVA uptake and clearance analysis. OVA grade VII (A7641; Sigma) was administered under a sterile hood and re-imported into sterile isolators. Mice tested negative for cDC- (or MM-) targeted to the wild-type levels to draw pie charts. For studies comparing Irf8<sup>fl/fl</sup> and DT administration. The first dose of DT (D0S64; Sigma) was delivered intraperitoneally as 500 ng in 200 µl PBS per 20 g body weight following by one or two doses of 100 ng in 200 µl PBS per 20 g body weight, as indicated. Depletion efficiency was monitored in each animal by the serum Flt3L surge upon cDC depletion (zDC<sup>DTR</sup> chimeras) and the loss of blood monocytes 24 h after DT administration in MM<sup>DTR</sup> chimeric mice.

Lymphocyte and APC isolation from lymph nodes and spleen. Tissues were dissected into cold HBSS supplemented with Mg<sup>2+</sup> and Ca<sup>2+</sup>, finely chopped and incubated in 400 U/ml Collagenase D (Roche) in HBSS for 25 min at 37°C, 5% CO<sub>2</sub>. Collagenase was quenched on ice by addition of final 10% FCS. Single-cell suspensions were extracted from connective tissue by taking up and resuspending the digests five times. Erythrocytes were lysed by incubation in erythrocyte lysis buffer (Sigma) for 7 min at RT.

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Antibodies and cell staining. Antibodies are described in Supplementary Table 1. Aqua LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, L-34965, was purchased from Life Technologies. Horseradish peroxidase conjugated Streptavidin (Jackson ImmunoResearch Laboratories, Inc.) was used as control (ex germ-free) SPF mice.

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OVA in 1M glycine, pH 9.5, 100 mM NaCl (2.26 μM OVA, equivalent to an iodine/OVA molar ratio of 3:1), and the solution was incubated at RT for 10 min. Unincorporated iodine was removed by purification of the sample on two consecutive NAP25 columns (GE Healthcare). 123I-OVA was eluted in H2O and kept at 4 °C in 1x PBS by appropriate addition of 10x PBS. Amount of labeling was determined on a Packard Cobra gamma counter and an estimated dilution to be 95,000 c.p.m./μg OVA. Protein purity and integrity were confirmed on a Coomassie-stained gel and specific labeling of OVA was confirmed by exposure of the gel to autoradiography film. To determine OVA uptake and clearance, mice were fasted for 3 h and then gavaged at 12 p.m. with 50 μg OVA grade III in 200 μl PBS and 300,000 counts per minute of 125I-OVA (about 4 μg OVA). Submandibular blood and urine were collected in 5 μl aliquots; whole organs were taken at indicated time points and weighed. Radioactivity was measured using on a Packard Cobra gamma counter.

CFA immunization and subcutaneous ear challenge. Eight days after oral OVA gavage, CFA-OVA was administered subcutaneously between the shoulder blades as an emulsion of 100 μl CFA and 100 μl PBS containing 300 μg endotoxin free OVA (EndoGrade Endotoxin-free Ovalbumin; 321000; HYGLOYS) under isoflurane gas anesthesia. The first ear challenge was performed 14 d after immunization. 2.5 mg/ml endotoxin free OVA was heat-aggregated in PBS at 2 h at 65 °C, and 30 μl of OVA (left ear) or PBS vehicle (right ear) were injected subcutaneously. Ear thickness was measured using a caliper at 24 h after injection under isoflurane gas anesthesia. The first ear challenge was performed 14 d after CFA immunization and subcutaneous ear challenge. Ear thickness was measured using on a Packard Cobra gamma counter.

Bronchoalveolar lavage, lung histology and infiltrate analysis by flow cytometry. Mice were anesthetized with intraperitoneal injection of 100 μl Avertin (Sigma). The trachea was cannulated and lungs were lavaged once with 0.5 ml and then 1.0 ml PBS. Total BAL1 cells were counted after erythrocyte lysis and were stained for flow cytometry (antibodies, Supplementary Table 1). Lungs were perfused via the right ventricle with 10 ml saline to remove residual blood. One lobe was digested in 400 U/ml collagenase D/HBSS and processed for flow cytometry. Eosinophils were determined as CD45+SSA+MHCI+CD11b+Ly6G+SiglecF+. Another lobe was fixed with 4% phosphate-buffered formalin and embedded in paraffin. Tissues sections were stained with hematoxylin and eosin to determine cellular inflammation. Sections were assessed by a blinded fashion. An score was created for infiltration observed in a 1-cm2 section: 0 = no infiltration, 1 = very mild infiltration, 2 = mild infiltration, 3 = locally severe infiltration, 4 = uniformly severe infiltration, 5 = uniformly very severe infiltration.

Anti-OVA IgG1 and IgG2c ELISA. High-binding ELISA 96-well plates (Corning, 3690) were coated with 50 μl of 2 μg/ml anti-IgE (Supplementary Table 1) (Invitrogen) in 100 mM Na2CO3 overnight at 4 °C. Plates were washed three times PBS plus 0.05% Tween 20 (PBS-T; Sigma), and blocked with 1 μl 1% BSA in PBS-T for 2 h at RT. Mouse serum was diluted in blocking buffer (typically 1:2 and 1:20) and added as 25 μl/well in duplicates. A standard curve for IgE isotype-matched control antibody (Supplementary Table 1) was applied at concentrations from 4 to 256 ng/ml. Standards and test serum were incubated for 2 h at RT and the plates were washed three times in wash buffer. Biotinylated anti-IgE (Supplementary Table 1) was added as 50 μl at 33 ng/ml, then plates were incubated for 1 h and washed four times. HRP-Streptavidin was added as 50 μl at 100 ng/ml and the plates were incubated for 45 min at RT and then washed five times. 50 μl HRP substrate TMB was added and the reaction was stopped by the addition of 10 μl 1 N H2SO4. Plates were ‘read’ as absorbance at 450–570 nm. Absolute concentrations of serum IgE were determined from the standard curve.

Adoptive T cell transfer. Sorted naive CD45.1+ OT-II T cells were labeled using the Cell Trace Violet Cell Proliferation Kit (C-34557; Life Technologies). 2 × 106 or 6 × 106 cell were transferred by retro-orbital injection under isoflurane gas anesthesia for analyses of cells 48 h or 7.5 d after gavage of OVA, respectively.

In vitro T cell and DC co-culture. Sorted and Cell Trace–stained naive OT-II cells were cultured for 3 d in flat-bottom 96-well plates in the presence of sorted mLN DC subpopulations, 1 μg/ml anti-IL4 and 1 μg/ml anti-IFN-γ (antibodies, Supplementary Table 1). Antigen was either provided as 1 μg/ml of OT-II OVA peptide (chicken OVA amino acids 323–339, sequence ISQAVHAAHAEINEAGR, made by the Rockefeller University Proteomics Resource Center), or by in vivo loading of DCs by maintenance of donor mice on a 20% OVA diet for 2 d and gavaging with 50 μg OVA h before mLN isolation. Cells were co-cultured at a 5:1 T cell/DC ratio (100,000/20,000 or 50,000/10,000).

RALDH activity assay. RALDH activity was determined using the Aldefluor kit (STEMCELL Technologies) according to the manufacturer’s protocol, with a few optimizing modifications: 3 × 106 cells were incubated at 1 × 106 cells/ml, one l PBS and 300,000 counts per minute of 125I-OVA (about 2.26 μg/g OVA). Protein purity and integrity were confirmed on a non-parametric Mann-Whitney test was used. A unpaired Student’s t-test was used. Data was analyzed by application of one-way analysis of variance or two-tailed unpaired Student’s t-test where necessary. For analysis of histological scores, a non-parametric Mann-Whitney test was used. A P value of less than 0.05 was considered significant.

RNA preparation and RNA-seq. RNA from sorted cell pellets was extracted and column-purified using the Arcturus PicoPure RNA Isolation kit (Applied Biosystems). Genomic DNA was removed by on-column digest with DNASE I (Qiagen), according to the PicoPure RNA Isolation kit manual. RNA libraries were prepared using the SMARTer Ultra Low Input RNA for Illumina Sequencing kit (Clontech Laboratories) and sequenced by 50-bp paired-end reading on a HiSeq 2500 instrument (Illumina). The reads were aligned using the STAR version 2.3.0 software that permits unique alignments to Mouse Ensembl genes. Differential expression was determined by use of the cufflinks software with default settings. Heat maps were generated by comparison of the expression profile CD11b+ and CD103+CD11b– DCs, or of OT-II T cells cultured with CD11b+ and CD103+CD11b– DCs. Gene clusters were determined using Gene Set Enrichment Analysis (GSEA v2.2.2) software. Principle-component analysis was performed using R software.

Statistics. Statistical analysis was performed in GraphPad Prism software. Data was analyzed by application of one-way analysis of variance or two-tailed unpaired Student’s t-test where necessary. For analysis of histological scores, a non-parametric Mann-Whitney test was used. A P value of less than 0.05 was considered significant.