CD11c<sup>high</sup> Dendritic Cells Are Essential for Activation of CD4<sup>+</sup> T Cells and Generation of Specific Antibodies following Mucosal Immunization

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CD11c\textsuperscript{high} Dendritic Cells Are Essential for Activation of CD4\textsuperscript{+} T Cells and Generation of Specific Antibodies following Mucosal Immunization\textsuperscript{1}

Linda Fahlén-Yrlid,\textsuperscript{*} Tobias Gustafsson,\textsuperscript{*} Jessica Westlund,\textsuperscript{*} Anna Holmberg,\textsuperscript{*} Anna Strömbeck,\textsuperscript{*} Margareta Blomquist,\textsuperscript{*} Gordon G. MacPherson,\textsuperscript{†} Jan Holmgren,\textsuperscript{*} and Ulf Yrlid\textsuperscript{2,*}

To generate vaccines that protect mucosal surfaces, a better understanding of the cells required in vivo for activation of the adaptive immune response following mucosal immunization is required. CD11c\textsuperscript{high} conventional dendritic cells (cDCs) have been shown to be necessary for activation of naive CD8\textsuperscript{+} T cells in vivo, but the role of cDCs in CD4\textsuperscript{+} T cell activation is still unclear, especially at mucosal surfaces. The activation of naive Ag-specific CD4\textsuperscript{+} T cells and the generation of Abs following mucosal administration of Ag with or without the potent mucosal adjuvant cholera toxin were therefore analyzed in mice depleted of CD11c\textsuperscript{high} cDCs. Our results show that cDCs are absolutely required for activation of CD4\textsuperscript{+} T cells after oral and nasal immunization. Ag-specific IgG titers in serum, as well as Ag-specific intestinal IgA, were completely abrogated after feeding mice OVA and cholera toxin. However, giving a very high dose of Ag, 30-fold more than required to detect T cell proliferation, to cDC-ablated mice resulted in proliferation of Ag-specific CD4\textsuperscript{+} T cells. This proliferation was not inhibited by additional depletion of plasmacytoid DCs or in cDC-depleted mice whose B cells were MHC-II deficient. This study therefore demonstrates that cDCs are required for successful mucosal immunization, unless a very high dose of Ag is administered. The Journal of Immunology, 2009, 183: 5032–5041.

Mucosal immunization is often necessary to generate immunity at mucosal surfaces (1). This protection requires generation of Ag-specific T cells and Abs. To activate Ag-specific T cells, peptides must be displayed on MHC molecules on the surface of activated APCs, particularly dendritic cells (DCs). Indeed, the superior capacity of DCs to activate naive T cells ex vivo following nasal and oral immunizations has been determined in several studies (2–10). Additionally, the strong T cell priming capacity of Ag-loaded DCs has not only been shown after i.v. transfer but also after intratracheal injection (11). A transgenic system has been developed where diphtheria toxin (DTx) administration conditionally ablates CD11c\textsuperscript{high} conventional DCs (cDCs) in vivo (12). Using these CD11c-DTX receptor (CD11c-DTR) mice, the absolute requirement for cDCs to activate naive CD8\textsuperscript{+} T cells following infections and parenteral immunizations has been demonstrated in several reports (12–16).

Despite the requirement for cDCs in naive CD8\textsuperscript{+} T cell activation, the role of cDCs in CD4\textsuperscript{+} T cell activation is not clear. For example, although depletion of CD11c\textsuperscript{high} cells significantly reduces the expansion of adoptively transferred vesicular stomatitis virus (VSV)-specific CD4\textsuperscript{+} T cells following i.v. infection (17), it does not affect the VSV-driven generation of CD4\textsuperscript{+} T cell-derived cytokines (13). CD4\textsuperscript{+} T cell activation is delayed but not abrogated in Mycobacterium tuberculosis-infected cDC-depleted mice (18). Additionally, cDCs are not essential for achieving maximal clonal expansion of CD4\textsuperscript{+} T cells following vaccinia virus infection (19). Finally, a recent study has shown that CD4\textsuperscript{+} T cells in lymph nodes (LN), but not spleen, can be activated in vivo following parenteral administration of protein, even in the absence of cDCs (16). This suggests that cells other than cDCs, such as plasmacytoid DCs (pDCs), can prime CD4\textsuperscript{+} T cells in a tissue- or route-dependent fashion. Despite these data from parenteral immunization systems, the types of APCs required for activation of CD4\textsuperscript{+} T cells in vivo following mucosal immunization have not been investigated.

A role for DCs in B cell priming has been suggested by both in vitro studies and investigations using adoptively transferred Ag-loaded DCs (20–22). However, a recent study showed that MHC-II molecules on B cells could be rapidly loaded with peptides despite cDC ablation, illustrating that DCs are not required for loading Ag-specific B cells (23). Furthermore, other experiments using CD11c-DTR mice have shown that differentiation of plasma cells in T-independent immune responses is independent of cDCs (24). T-independent Ab responses to VSV infection do not require CD11c\textsuperscript{high} cells, despite the fact that these cells are required for induction of Abs to purified VSV-G protein (17).
Ab responses to usually inert protein Ags are very poor unless the Ag is coadministered with an adjuvant. Mucosal immunization, particularly by the oral route, is hampered by the paucity of useful adjuvants. Cholera toxin (CT) is a strong oral adjuvant that, despite its toxicity to humans, is an important tool for gaining insight into the mechanisms of oral immunization and the design of novel mucosal vaccines. To function as a mucosal adjuvant, CT binds ubiquitously expressed GM1 ganglioside receptors (25). CT also activates dendritic cells after mucosal administration (2, 25). Additionally, cDC depletion abrogates the induction of Ag-specific intestinal IgA responses as well as Ag-specific serum IgG production following oral immunization. When a very high dose of Ag is given, proliferation of CD4+ T cells is detected in cDC-ablated mice. We demonstrate that this is not due to a compensatory role of B cells, pDCs, or recruited CD11b+CD11c−/low myeloid cells. These results therefore show that cDCs are required for mucosally induced adaptive immune responses unless a very high dose of Ag is administered.

Materials and Methods

Mice

CD11c-DTR transgenic (B6.FVB-Tg Igax-DTR/GFP 57Lan/I) mice (12), OT-II (C57BL/6) TCR transgenic mice harboring OVA-specific CD4+ T cells (26), B cell-deficient mice (C57BL/10-Igh6-2m1Cgn) (µM) (27), and MHC-II-deficient mice (B6.129S2-H2−/−/−/−) (MHC-II-KO) (28) were all bred and maintained under specific pathogen-free conditions at the Experimental Biomedicine Animal Facility, University of Gothenburg, Göteborg, Sweden. CD11c-DTR Tg µMt mice were obtained by intercrossing the respective lines, and CD45.1+CD45.2−OT-II mice were obtained from OT-II × C57BL/6-CD45.1 mating. All experiments were performed using protocols approved by the Swedish government’s Animal Ethics Committee and followed institutional animal use and care guidelines.

Bone marrow (BM) chimeras and DC depletions in vivo

Female and male mice aged 6–8 wk were used. Bone marrow (BM) cells were flushed out of femurs and tibias from donor mice and BM was flushed out. CD11c-DTR BM donor cells were used to generate CD11c−DTR BM donor cells at a 4:1 ratio were used to generate mixed CD11c−DTR/MHC-IIB−/− BM cell chimeras, B cell-deficient mice (C57BL/10-Igh6-2m1Cgn) (µM) (27), and MHC-II-deficient mice (B6.129S2-H2−/−/−/−) (MHC-II-KO) (28) were all bred and maintained under specific pathogen-free conditions at the Experimental Biomedicine Animal Facility, University of Gothenburg, Göteborg, Sweden. CD11c-DTR Tg µMt mice were obtained by intercrossing the respective lines, and CD45.1−CD45.2+OT-II mice were obtained from OT-II × C57BL/6-CD45.1 mating. All experiments were performed using protocols approved by the Swedish government’s Animal Ethics Committee and followed institutional animal use and care guidelines.

Adoptive transfer experiments

Spleens and LNs were taken from OT-II mice, and CD4+ T cells were negatively enriched by magnetic separation using an autoMACS (Miltenyi Biotec) and CD4+ T cell isolation kit (Miltenyi Biotec). Cells were then labeled with CFSE (Invitrogen) by resuspending them at 1 × 10⁷/ml in 5 µM CFSE in serum-free PBS for 5 min at 37°C. The reaction was quenched with an equal volume of FCS. Cells were washed, resuspended in PBS, and 3–5 × 10⁶ were injected i.v. in to mice that had, 70 and 2 h earlier, either been given DTx or not. Eighteen hours later the mice were immunized with OVA with or without CT. Three or 5 days later, mice were sacrificed and the single-cell suspensions from lymphoid tissues were stained and analyzed by flow cytometry to determine the frequency of adoptively transferred cells that had entered division.

Measurements of Ag-specific Ab titers in serum and tissues

To determine OVA-specific Ab titers, 96-well plates (Greiner Bioscience) were coated with OVA (20 µg/ml) and then blocked with PBS/BSA. To determine CT subunit B (CTB)-specific Ab titers, 96-well plates (Nunc) were coated with GM-I (0.3 mmol/ml) and, after blocking with PBS/BSA, CTB (0.5 µg/ml) was added for 60 min. Serially diluted serum samples or perfusion-extraction samples (32) from intestines or lungs were added to OVA- and CTB-coated plates and then incubated for 90 min. After washing, goat anti-mouse IgA/HRP (SouthernBiotech) or goat anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories) was added and developed with o-phenylenediamine dihydrochloride before absorbance determination at 450 nm. IgG or IgA titers were defined as the sample dilution giving an OD value of 0.4 above the background value.

In vitro proliferation assay

Splenocytes from CD11c-DTR mice given DTx i.p. 24, 48, or 72 h previously or PBS-treated controls were seeded 5 × 10⁵ in 96-well plates. Splenocytes were pulsed in six identical wells with titrated amounts of OVA protein or peptide (323–339) for 2 h. Remaining proteins and peptides were washed away and 1 × 10⁴ MACS-purified CD4+ OT-II T cells were added to each well. DTx was added to half of the wells to give a final concentration of 200 ng/ml. Cells were cocultured for 64 h, pulsed for 8 h with [3H]thymidine, and then incorporation into cellular DNA was measured.
Results
Efficient depletion of cDCs in mucosal and lymphoid tissues in CD11c-DTR/WT following DTx administration

Our aim was to determine the role of cDCs in activating CD4^+ T cells in vivo following mucosal Ag administration. We thus took advantage of CD11c-DTR Tg mice, which allow conditional ablation of cDCs upon administration of DTx (12). However, injection of DTx leads to death of CD11c-DTR mice within a week (29) in a process mediated by nonhematopoietic cells. The use of CD11c-DTR^3 WT BM chimeras avoids this problem, and chimeric mice can be given multiple injections of DTx without any adverse effects (29). CD11c-DTR^3 WT BM chimeras (CD11c-DTR/WT) were generated and used throughout this study unless otherwise stated.

To determine the chimerism of DCs, flow cytometric analysis was performed. A small frequency of GFP-negative resident CD11c^high DCs were present in chimeric CD11c-DTR/WT mice (Fig. 1A, upper left plot). Importantly, an identical population of CD11c^high-GFP^+ DCs was observed in the spleen and mesenteric LN of (nonchimeric) CD11c-DTR mice (Fig. 1A, upper middle and right plots), demonstrating that this is background staining or that the penetrance of GFP expression in cDCs of CD11c-DTR mice is not 100%. Furthermore, irradiation experiments using congenic mice confirmed the lack of remaining cDCs of recipient origin in the chimeras (data not shown). To ensure complete ablation of cDCs, the mice received two injections of DTx. This treatment depleted 85–98% of donor GFP^+ cDCs from spleen, mucosal tissues, and draining lymphoid tissues (Fig. 1).

Histochemical analysis confirmed the depletion of CD11c-expressing cells and showed retention of lymphoid follicles with no

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FIGURE 1. Efficient ablation of cDCs in CD11c-DTR and CD11c-DTR/WT following DTx administration. A, Flow cytometric analysis of GFP vs CD11c expression by 7AAD^-CD3^-CD19^- splenocytes or cells from MLN of CD11c-DTR/WT and CD11c-DTR mice treated with or without DTx. B, Flow cytometric analysis of GFP vs CD11c expression by 7AAD^-CD3^-CD19^- cells from cervical LN (CLN), nasal-associated lymphoid tissue, PP, or intestinal lamina propria (LP) of CD11c-DTR/WT mice treated with or without DTx.

FIGURE 2. Ablation of cDCs in CD11c-DTR/WT retains lymphoid follicles and does not effect baseline recruitment, retention, or activation of adaptively transferred CD4^+ T cells. A, Histological analysis of tissue sections from mice given DTx (+DTx) or not (−DTx) stained with anti-CD11c (green) and B220 (red) (×20 magnification). B and C, Flow cytometric analysis of CD45.1 and CD4 for detection of adaptively transferred OT-II cells in CLN cells from mice given DTx (+DTx) or not (−DTx). OT-II cells were transferred at the same time as the second DTx treatment and the tissues were taken 18 (B) or 90 h (C) later. B and C, Left panel, The number in the dot plot indicates the frequency of gated cells among total viable cells. C, Right panels, Expression of CD45.1 and CD69 by CD45.1^-CD4^-OT-II T cells.
FIGURE 3. cDCs are required for activation of CD4+ T cells following OVA feeding. Flow cytometric analysis of 7AAD−/Vα2+/CD4+ cells from PP (A) or MLN (B) of CD11c-DTR/WT mice adoptively transferred with CFSE-labeled OT-II cells treated with DTx or not and fed PBS (control) or OVA as indicated. Cells were analyzed 72 h after OVA feeding. The histograms represent 7AAD−/Vα2+/CD4+ cells gated as shown. The number in the histograms indicates the frequency of transferred T cells that have entered division taking into account the expansion of divided cells (undivided cells are indicated with a gate in the histogram). Plots are of three pooled mice and are representative of at least three experiments. The statistical analysis (C and D) was performed using Student’s t test where *p < 0.05 and **p < 0.01. The bars show the SEM.

cDCs are essential for priming naive CD4+ T cells following mucosal immunization

CFSE-labeled OT-II T cells were adoptively transferred into CD11c-DTR/WT mice 48 h after receiving DTx or PBS. DTx-treated mice received a second injection of DTx shortly after the transfer. Eighteen hours later both groups of mice were fed different doses of OVA, and cells from Peyer’s patches (PP) and MLNs were analyzed for proliferation of the adaptively transferred T cells 3 days later (Fig. 3, A and B). Little, if any, OT-II T cell expansion was detected in either organ of mice receiving DTx before feeding OVA, while significantly more proliferation was readily observed in mice not receiving DTx (Fig. 3, C and D).

To determine the impact of a mucosal adjuvant on the expansion of transferred T cells, CT was given together with OVA. Again, a significant reduction in the proliferation of labeled CD4+ T cells was found in the draining LNs after oral (Fig. 4, A and C) or nasal (Fig. 4, B and D) administration to DTx-treated mice compared with controls without receiving DTx treatment. Expression of CD69 by the transferred OT-II T cells was detected among undivided OT-II cells in both DTx-treated mice and untreated controls following oral immunization with OVA and CT (Fig. 4E). In the absence of DTx treatment, CD69 expression was down-regulated upon cell division. The same CD69 expression pattern was detected on OT-II T cells from cervical LN 5 days after nasal immunization with OVA (Fig. 4F). Restimulation of OT-II T cells from these animals resulted in Ag-specific proliferation irrespective of DTx treatment before immunization (data not shown). This suggests that the observed lack of OT-II T cell division following DTx treatment (Figs. 3 and 4) was not due to a delayed response in the transferred cells and that these cells were not anergic to subsequent stimulation. These results hence show that cDCs are required for priming of CD4+ T cells following nasal and oral administration of protein both in the absence and presence of CT.

cDCs are required for efficient generation of specific Abs following mucosal administration of Ag and CT

To investigate the role of cDCs in generating Ag-specific Abs after oral and nasal immunization, CD11c-DTR/WT mice were fed OVA and CT. Before feeding, half of the mice were depleted of cDCs, as described above, and Ag-specific Ab titers were measured. The IgG titers in serum (10–12 days postadministration) and IgA in intestinal tissues (3 wk postadministration) were determined by ELISA. Anti-OVA and anti-CTB IgG titers in serum, following oral (Fig. 5, A and B) and nasal immunization (Fig. 5, D and E), were abrogated in CD11c-DTR/WT mice given DTx compared with controls. Although no significant intestinal anti-OVA IgA could be detected regardless of the dose of OVA given orally and irrespective of cDC-depletion (data not shown), anti-CTB IgA was clearly detected and completely lost in CD11c-DTR/WT mice given DTx (Fig. 5C). These results show that cDCs are required for the generation of OVA- and CTB-specific serum IgG after mucosal administration, and of CTB-specific intestinal IgA after feeding mice OVA with CT.

Immunization of cDC-depleted mice with very high doses of Ag results in proliferation of transferred CD4+ T cells

A very high dose of protein Ag is sometimes required to initiate immune responses at mucosal surfaces. The abundance of Ag has also been suggested to affect activation of the adaptive immune response, possibly because additional APC populations access the Ag in peripheral or lymphoid tissues (17). To test this, we mucosally immunized cDC-depleted mice with a very high dose of OVA, 30-fold more than that required to detect proliferation of Ag-specific CD4+ T cells in WT mice after mucosal immunization (Fig. 6, A and B). OVA administration at 300 mg orally or 3 mg nasally in the presence of CT resulted in extensive proliferation of adaptively transferred OT-II T cells in control mice not given DTx. However, treatment with DTx had no significant effect on the frequency of cells that entered division (Fig. 6, A, B, D, and E). To determine whether this was due to the route of immunization, a very high dose of OVA (0.3 mg) was injected i.v.

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and titrated amounts of OVA were given i.p. (Fig. 6G) and then the proliferation of the transferred cells in the spleen was measured. As observed with mucosal immunization, increasing the dose of OVA to a very high dose resulted in similar proliferation of the transferred cells in cDC-depleted mice and in animals not treated with DTx (Fig. 6, F, G, and H). This is in contrast to cDC-dependent proliferation where lower doses of Ag are used (Fig. 6H). These results show that immunizing mice depleted of cDCs with a very high dose of Ag results in proliferation of CD4⁴/K11⁰¹ T cells.

CD11b⁴/KDC11c⁻/⁻ cells cannot compensate for cDC depletion by activating CD4⁺ T cells

A possible explanation for CD4⁺ T cell activation in DTx-treated chimeric mice given a very high dose of Ag is that other APCs accumulate in tissues following DTx-induced cell death of DTR⁺/⁺ DCs. The cellular composition of lymphoid organs from CD11c-DTR/WT mice treated twice with DTx was therefore analyzed. After giving one or two injections of DTx, CD11b⁺/⁺CD11c⁻/⁻ cells accumulated in lymphoid tissues (Fig. 7A). Multicolor flow cytometry of the splenic CD11b⁺/⁺CD11c⁻/⁻ cells accumulated in lymphoid tissues (Fig. 7A). Multicolor flow cytometry of the splenic CD11b⁺/⁺CD11c⁻/⁻ cells revealed a decrease in frequency of Ly6C⁺/⁺/⁶G⁺/⁺ cells and an increase in Ly6C⁺/⁺/⁶G⁻/⁻ cells, representing monocytes and neutrophils, respectively (33) (Fig. 7B). The increase of Ly6C⁺/⁺/⁶G⁻/⁻ cells was

**FIGURE 4.** cDCs are essential for proliferation of CD4⁺ T cells following mucosal administration of OVA and CT. Flow cytometric analysis of 7AAD⁻/Vo²⁺/CD4⁺ cells from (A and E) MLN or (B and F) CLN of CD11c-DTR/WT mice adoptively transferred with CFSE-labeled OT-II cells treated with or without DTx. Mice were then given OVA plus CT (A and E) orally, (B) nasally, or (F) OVA nasally. Cells were analyzed (A, B, and E) 72 h or (F) 96 h after OVA administration. A and B, Histograms represent 7AAD⁻/Vo²⁺/CD4⁺ cells gated as shown. The number in the histograms indicates the frequency of transferred T cells that have entered division taking in to account the expansion of divided cells (undivided cells are indicated with a gate in the histogram). E and F, Expression of CD69 and CFSE by CD45.1⁺/CD4⁺ OT-II T cells. The numbers in the dot plot indicate the frequency of CD69-expressing cells among the transferred CD45.1⁺/CD4⁺ OT-II T cells. Plots are of three pooled (A, E, and F) or individual mice (B). Data are representative of three separate experiments. Statistical analysis (C and D) was performed using Student’s t test where ***, p < 0.01 and ****, p < 0.001. The bars show the SEM.

**FIGURE 5.** cDCs are essential for efficient generation of Ag-specific Abs following mucosal administration of OVA and CT. The indicated amounts of OVA and CT were administered (A–C) orally or (D and E) nasally to CD11c-DTR/WT mice. The graphs show log₁₀ titers of anti-OVA- (A and D), anti-CTB- (B and E) specific serum IgG or anti-CTB-specific intestinal IgA (C) in CD11c-DTR/WT mice treated with DTx (open bars) or not (filled bars). DTx was administered 72 and 18 h before the immunizations. Serum samples were collected 10–12 days postimmunization and intestines were collected 3 weeks postimmunization. The IgG or IgA titer was defined as the sample dilution giving an OD value of 0.4 above the background value in ELISA. Unimmunized controls showed a titer <10 for IgA and IgG. Error bars show the SEM and statistical analysis was performed using Student’s t test where *, p < 0.05, **, p < 0.01, and ***, p < 0.001.
detected very rapidly and at all time points when cDCs were efficiently ablated. In contrast, no increase of these cells was observed in DTx-treated WT mice (data not shown). Recruitment of neutrophils is thus an effect of the DTx-induced ablation in CD11c-DTR/WT mice.

The depleted splenic cDC population in DTx-treated mice is restored 6 days following treatment, during which time the observed neutrophil accumulation occurs. Therefore, we wondered whether cells (precursor or inflammatory cells) recruited to the tissue during this period of cDC absence could contribute to the activation of CD4<sup>+</sup>/H<sub>11001</sub> T cells. To address this, depletion of CD11<sup>chigh</sup> cells from the splenocytes was conducted in vitro (12) so that no recruitment of cells was possible. Initial experiments showed that 0.2 µg/ml DTx efficiently depleted CD11<sup>chigh</sup> cells from splenocyte cultures (0.4% compared with 23% CD11<sup>chigh</sup> cells among nonlymphocytes with or without DTx, respectively). There was no increase in CD11<sup>bhigh</sup>/CD11c<sup>−/low</sup> cells (data not shown). Addition of DTx, followed by a pulse with OVA peptide or protein, abrogated the proliferative response of cocultured

**FIGURE 7.** CD11<sup>bhigh</sup>/CD11c<sup>−/low</sup> cells with very poor CD4<sup>+</sup> T cell activation capacity accumulate in DTx-treated CD11c-DTR mice. A, Flow cytometric analysis of CD11b vs CD11c expression by 7AAD<sup>+</sup> CD3<sup>+</sup>/H<sub>9251</sub> splenocytes or cells from MLN of CD11c-DTR/WT mice treated with or without DTx. B, Flow cytometric analysis of Ly6C/Ly6G expression by 7AAD<sup>+</sup> CD3<sup>+</sup>/H<sub>9251</sub> CD11<sup>bhigh</sup>/CD11c<sup>−/low</sup> splenocytes. C, In vitro proliferation of OT-II cells cocultured with CD11c-DTR splenocytes pulsed for 2 h with titrated amounts of OVA protein (left) or peptide (right) in the presence (●) or absence (○) of DTx. After 72 h the cocultures were pulsed with [3H]thymidine and incorporation was measured after 6 h. D, In vitro proliferation of OT-II cells cocultured with splenocytes from CD11c-DTR mice given PBS (○) or DTx i.p. 24 (●), 48 (●), or 72 h (●) earlier and subsequently pulsed for 2 h with titrated amounts of OVA protein (left) or peptide (right). After 72 h the cocultures were pulsed with [3H]thymidine and incorporation was measured after 6 h. One representative experiment out of two is shown.

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OT-II cells unless very high peptide or protein concentrations were used (Fig. 7C).

Having shown that DCs were required to induce proliferation of CD4⁺ T cells in vitro, we next assessed whether the cells recruited to the spleen after DTx treatment could activate CD4⁺ T cells. CD11c-DTR mice were therefore given DTx before sacrifice. Splenocytes from CD11c-DTR mice given DTx 72, 48, or 24 h earlier were pulsed with OVA protein or peptide and cultured with OT-II cells. While proliferation was observed in PBS-treated controls, little proliferation was detected when splenocytes from CD11c-DTR mice given DTx in vivo were used (Fig. 7D). Taken together, these results show that CD11chigh cells are efficiently ablated by DTx treatment in lymphoid tissues of CD11c-DTR/WT mice. Concomitantly, CD11bhighCD11c⁻/⁻ neutrophils are recruited to tissues, but these cells have a very poor capacity to present peptides to CD4⁺ T cells in vitro.

**Expansion of CD4⁺ T cells following administration of high doses of Ag in mice with MHC-II-deficient B cells and ablated cDCs**

Having shown that the recruited CD11bhighCD11c⁻/⁻ cells are not capable of inducing proliferation of CD4⁺ T cells after Ag exposure, we next addressed if other APCs could be responsible for the observed activation of OVA-specific CD4⁺ T cells after administration of a high dose of OVA to cDC-ablated mice. To determine whether B cells were capable of priming T cells in the absence of cDCs, we crossed the DTR Tg mice with B cell-deficient μMT mice, generating DTR Tg;μMT mice. Similar to μMT mice, these mice display a multitude of architectural defects in the spleen, including absence of follicular DCs, marginal zone macrophages, and metallophilic macrophages (34), as well as differences in DC function (35). We therefore made mixed BM chimeras (CD11c-DTR/MHC-II⁻/⁻) in which the B cell compartment is normal in number but MHC-II is deficient, as described by Crawford et al. (30). Seven weeks after engraftment, ~80% of splenic cDCs (CD11c⁺B220⁻ cells) from CD11c-DTR/MHC-II⁻/⁻ mice were MHC-II⁺ and expressed DTR (i.e., were GFP⁺) (Fig. 8A). Importantly, the remaining DCs (GFP⁻) and all B cells were MHC-II⁺. These mice were then adoptively transferred with CFSE-labeled OT-II cells and 1 day later given OVA at a high dose nasally or at titrated amounts of OVA i.p. Although expansion of the transferred T cells was cDC-dependent when lower doses of Ag were administered i.p. (Fig. 8C, left panel), cDCs were not essential for OT-II T cell division when a high dose of Ag was given nasally or i.p. (Fig. 8B, middle panel). Because the DTx-treated mice had a MHC-II⁻/⁻ deficient B cells, the observed T cell proliferation must have been initiated by cells other than B cells.

**Activation of CD4⁺ T cells and induction of serum Ab responses in mice depleted of both cDCs and pDCs**

Our data so far show that neither B cells nor recruited myeloid cells are responsible for CD4⁺ T cell proliferation in DTx-treated mice given a very high dose of OVA. We thus determined if pDCs contribute to this CD4⁺ T cell expansion by administering the pDC-depleting Ab 120.G8 (31) to DTx-treated CD11c-DTR/WT mice before administering OVA (Fig. 9). This combined treatment resulted in a complete loss of cDCs (CD11c⁺B220⁻ cells) and pDCs (CD11c⁺B220⁻mPDCA-1⁻) in the spleen (Fig. 9A). As shown above, cDC depletion had a relatively minor effect on the proliferation of transferred OT-II CD4⁺ T cells in CD11c-DTR/WT mice given 3 mg of OVA nasally (Fig. 9B) or i.p. (Fig. 9C). Depleting both pDCs and cDCs had little additional effect, and significant expansion could still be observed in the pDC/cDC-depleted mice.

We next measured the OVA-specific IgG response in CD11c-DTR/WT mice primed with OVA plus CT nasally in the presence or absence of DCs (cDCs or cDCs/pDCs) and boosted with OVA i.p. with either DTx or 120.G8 treatment (Fig. 9D). This immunization regime led to a high anti-OVA IgG titer in the serum of CD11c-DTR/WT mice that was significantly reduced by DTx treatment. However, there was no significant effect of additional pDC depletion. To confirm that the pDC depletion was functional, depleted mice were immunized with OVA and CpG, as pDCs are essential for CpG-induced activation of cDCs (36). Only the combined cDC and pDC ablation led to a significant reduction in serum anti-OVA IgG titers upon OVA plus CpG immunization (Fig. 9D). These results show that a high dose of OVA results in CD4⁺ T cell proliferation even in mice depleted of both cDCs and pDCs. Finally, depletion of pDCs in addition to cDCs at the time of priming with OVA and CT via the nasal route did not further reduce the anti-OVA Ab response compared with cDC depletion alone.
Discussion

To generate vaccines that protect mucosal surfaces, a better understanding of the cells required in vivo for activation of the adaptive immune response following mucosal immunization is required. To determine the role of DCs in this regard, we analyzed the activation of CD4^+ T cells following oral and nasal immunization of mice depleted of cDCs in vivo. Our results show that cDCs are required for the activation of naive OVA-specific CD4^+ T cells in vivo and for the generation of mucosal and systemic Ag-specific Abs after both oral and nasal administration of OVA and CT, unless a very high dose of OVA is used.

Our results with high doses of Ag show that an APC population present in, or recruited to, the tissue of DTx-treated CD11c-DTR mice possesses the capacity to present peptides, but only when Ag is abundant. DC depletion resulted in an immediate recruitment of cells to lymphoid tissues, most of which express a high level of CD11b, Ly6C, and Ly6G, but no, or very low level of, CD11c. These cells are likely neutrophils, but the recruited population could also contain other cells potentially capable of differentiating into APCs (33). However, this appears unlikely, as splenocytes from CD11c-DTR mice treated with DTx 3 days earlier (a time frame that should allow for differentiation) could not activate CD4^+ T cells. This result is identical to that observed with splenocytes following DC depletion in vitro where no recruitment was possible.

The appearance of neutrophils in tissues of CD11c-DTR mice after DTx-induced ablation of DCs has not previously been reported, even though this Tg mouse has been used in several studies (12–17, 19, 24, 29, 37–39). Although our results show that the recruited cells do not have the capacity to efficiently present peptides on MHC-II ex vivo, these results emphasize the need for a cautious approach when using DTx-treated CD11c-DTR mice to study the role of inflammatory cells and secretion of soluble factors in the absence of DCs.

Furthermore, we could not detect a significant role for B cells in the activation of T cells following immunization with a high dose of Ag in the absence of cDCs. Our results therefore support a previous report showing that CD4^+ T cell activation in LN could still be observed in a B cell-deficient animal where cDCs had been ablated (16). Importantly, our results also extend this observation, as we used mice in which B cells are present but are MHC-II deficient. Using this system, we ensured that the observed MHC-II presentation was not due to changes in LN cellularity or altered structure of peripheral lymphoid tissues, which is a caveat when using B cell-deficient mice (34, 35). Our results could seem contradictory to a study using mice with MHC-II−/− B cells, which showed that B cells provide extra Ag presentation capacity above that provided by DCs (30). This could be because different doses of Ag were used in that study compared with the doses used by us, and/or that Crawford et al. performed all immunizations in the presence of alum (30). It remains possible, however, that B cells may collaborate with cDCs during CD4^+ T cell activation.

Our study shows that immunization of mice depleted of both cDCs and pDCs with a very high dose of OVA still resulted in activation of CD4^+ T cells, comparable to that seen when only cDCs were depleted. It has been reported that OVA-specific CD4^+ T cells can be activated by pDC-specific Abs carrying OVA, and also in LNs of cDC-depleted mice following s.c. immunization with OVA (16). Sapoznikov et al. (16) found no significant depletion of pDCs after DTx treatment, while we consistently observe a partial depletion, which has also been reported by others (13, 17). It is therefore possible that this partial depletion is sufficient to deplete mucosal pDCs that have a capacity to activate CD4^+ T cells. However, only after combining DTx treatment with the pDC-depleting Ab could we significantly inhibit the generation of OVA-specific Abs in mice that had been given Cpg and OVA mucosally. This shows that pDCs remaining after DTx treatment may collaborate with cDCs during CD4^+ T cell activation.

FIGURE 9. Activation of CD4^+ T cells and induction of serum Ab responses in mice depleted of both cDCs and pDCs after administration of a very high dose of Ag. A, Flow cytometric analysis of mPDCA-1 and CD11c expression by 7AAD−CD3−CD19− splenocytes from CD11c-DTR/WT treated with PBS, DTx, or DTx plus 120.G8. The mAb 120.G8 was given for 3 consecutive days before immunization. The numbers indicate the frequency of cells among gated 7AAD−CD3−CD19− cells. B, Flow cytometric analysis of 7AAD−CD2−CD4^+ cells from the cervical LN or spleen from CD11c-DTR/WT mice adoptively transferred with CFSE-labeled OT-II cells treated with DTx, DTx plus 120.G8, or untreated and then given OVA nasally (B) or i.p. (C). Analysis was performed 72 h after OVA administration. Histograms represent 7AAD−Vv2−CD4^+ cells, gated as shown. The number in the histograms indicates the frequency of transferred T cells that have entered division (determined from three separate experiments) taking into account the expansion of divided cells (undivided cells are indicated with a gate in the histogram). Plots are of individual mice and are representative of three separate experiments. D, Log_{10} titers of anti-OVA-specific IgG in serum from CD11c-DTR/WT mice treated with DTx, DTx plus 120.G8, or untreated before nasal immunization with OVA plus CT or OVA plus Cpg. Ten to 14 days later these mice and unimmunosized controls (filled gray bars) received OVA i.p. Serum was collected 1 wk later and analyzed for OVA-specific IgG titers by ELISA. Tiers were defined as the sample dilution giving an OD value of 0.4 above the background value. Error bars show the SEM and statistical analysis was performed using Student’s t test where *p < 0.05 and n.s., not significant.
The ag is titrated. Finally, it is also possible that pDCs in skin are functionally different from those in the spleen and mucosal tissues. For example, we have not been able to detect pDCs in afferent lymph from mucosal tissues (40), while this has been reported in lymph draining the skin (41).

CT holotoxin is the most potent oral adjuvant, but the mechanism behind its adjuvanticity is not fully known. Ab responses to both OVA and CT following mucosal coadministration have been shown to be completely dependent on CD4⁺ T cell help (42). The effect of CT could therefore be to make DCs more potent activators of CD4⁺ T cells. Alternatively, CT could act on B cells, rendering them capable of activating naive T cells. Our results show that coadministering CT and OVA mucosally did not overcome the essential role of cDCs for activation of CD4⁺ T cells and induction of intestinal IgA and serum IgG. Detection of anti-OVA IgA responses in mucosal tissues required that the mice be immunized a second time with OVA and CT. However, ablation of cDCs before both immunizations creates a problem, as plasma cells in the spleen express CD11c and thereby become sensitive to DTx treatment (24). Indeed, in preliminary experiments we have found that the number of CT- and OVA-specific IgA-secreting cells in the intestine are reduced after DTx treatment (OVA, 120 ± 50, CT, 3515 ± 1889 per million cells (~DTx) compared with OVA, 18 ± 13, 275 ± 129 per million cells (~DTx)). In these experiments DTx was only given 1 wk after the second immunization with OVA and CT, making it unlikely that the effect of DTx was on DCs.

DTx treatment of CD11c-DTR mice has also been reported to ablate marginal zone macrophages and their sinusoidal counterparts in LN when using the same dose of DT used by us in this study (43). This makes it unlikely that these cells contribute to the activation of CD4⁺ T cells following immunization with a very high dose of Ag. Additionally, removal of macrophages using chloroquine-containing liposomes increases, rather than reduces, the amount of Ab-secreting cells following immunization with a T cell-dependent Ag (44). The mucosal epithelium has also been suggested to present Ags on MHC-II (45). However, in preliminary experiments using chimeric mice, we have been unable to find a role for nonhematopoietic cells (including epithelial cells) for the activation of CD4⁺ T cells when a very high dose of Ag is administered. During the revision of this manuscript it has been shown that basophils purified from blood can, when loaded with peptides, activate the CD8⁺ T cells. However, the essential role of cDCs shown in this study suggests that targeting of the vaccine to DCs would be a useful way to ensure successful mucosal vaccination.

Disclosures
The authors have no financial conflicts of interest.

References


