

Gastrointestinal Dendritic Cells Promote Th2 Skewing via OX40L¹

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Mice can be sensitized to food proteins by oral administration with the adjuvant cholera toxin (CT), such that they undergo anaphylaxis when rechallenged with the sensitizing allergen. In contrast, feeding of Ags alone leads to oral tolerance. Our aim was to define the mechanisms by which gastrointestinal dendritic cells (DCs) participate in the deviation of tolerance to allergic sensitization in the gut in response to CT. BALB/c mice were fed with CT or PBS. The impact of CT on DC subsets in the mesenteric lymph node (MLN) was assessed by flow cytometry. Ag presentation assays were performed with DCs isolated from the MLN of PBS- or CT-fed mice, using OVA-specific CD4⁺ T cells as responder cells. Gene expression in MLN DCs was determined by real-time PCR, and neutralizing Abs were used to test the function of OX40 ligand (OX40L) in Th2 skewing. Oral administration of CT induced an increase in the total CD11c⁺ population in the MLN. CT induced a selective increase in migration of the CD11c⁺CD11b[−]CD8α[−] DC subset and the maturation of all DC subsets. Maturation of DCs in vivo enhanced T cell proliferation and cytokine secretion. Oral CT induced up-regulation of Jagged-2 and OX40L by MLN DCs. Neutralizing anti-OX40L Abs completely abrogated the CT-induced Th2 cytokine response. We show that oral CT induces selective DC migration, maturation, and T cell priming activity in the MLN. Th2 skewing is mediated by OX40L, and we speculate that this molecule may be an important inducer of allergic sensitization to food allergens. *The Journal of Immunology*, 2008, 180: 4441–4450.

Food allergy is defined as an immune-mediated adverse reaction to foods. Food allergy encompasses a range of disorders from acute IgE-mediated immediate hypersensitivity reactions to delayed cell-mediated inflammatory disorders affecting the gastrointestinal tract, respiratory tract, and skin. Food allergy affects ~6–8% of infants and young children and, despite the fact that most children outgrow their food allergies, 3.5–4% of the adult population is food allergic (1). The most common food allergens in the pediatric population include cow's milk, eggs, peanuts, tree nuts, soy, wheat, fish, and shellfish, whereas peanuts, tree nuts, fish and shellfish predominate in adults. The mechanisms responsible for the development of allergic sensitization to dietary protein Ags, instead of the normal process of immune tolerance, remain unknown.

The balance between active immunity and tolerance is critical in sites such as the intestine, where mucosal lymphoid tissues are separated from bacterial and food Ags in the gut lumen by a single layer of epithelial cells. A central role for gastrointestinal dendritic cells (DCs)³ in maintaining intestinal homeostasis has been suggested by the finding that DC expansion with Flt3 ligand (Flt3L) enhances both tolerance and humoral immunity to OVA depending

on the context of Ag delivery (in the absence or presence of adjuvant) (2, 3). DCs are not a homogeneous population and different subsets have been defined based on the expression of CD11c, CD11b, CD8α, and B220 surface markers. Mouse Peyer's patches (PP) contain three predominant populations of CD11c⁺ DCs: CD11b⁺, CD8α⁺, and CD11b[−]CD8α[−] "double negative" (DN) cells (4, 5). CD11b⁺ DCs isolated from PP, but not from spleen, produce high levels of IL-10 and promote the differentiation of IL-4- and IL-10-secreting T cells (6). These DCs have also been shown to secrete IL-6 and promote IgA secretion from responder B cells (in the presence of T cells) (7). In contrast, CD8α⁺ and DN DCs produce elevated levels of IL-12p70 following stimulation with CD40 ligand (CD40L) (5). Bilsborough et al. described a CD8α⁺B220⁺ DC subset in the mesenteric lymph node (MLN) that secreted IFN-α following stimulation with CpG motifs and could induce naive T cell differentiation into cells with regulatory activity (8). Taken together, these studies suggest that there is functional specialization of DC subsets in the gastrointestinal tract, although exogenous factors such as adjuvants or inflammatory stimuli could alter the phenotype of the subsets.

Murine models of food allergy have used coadministration of cholera toxin (CT) to induce allergic sensitization to food allergens including milk proteins, peanut proteins, and OVA (9–12). Oral administration of Ags alone does not lead to allergic sensitization and is well-known to induce immunologic tolerance; but, in contrast, the feeding of food allergens with CT leads to allergic sensitization as defined by Ag-specific IgE production, Th2 cytokine production by spleen or MLN T cells, and the induction of anaphylaxis when mice are rechallenged with Ag orally or systemically. Therefore, oral administration of CT induces an "allergenic" milieu, while in the absence of CT a "tolerogenic" milieu predominates.

In the present study, we used CT as a tool to determine the mechanisms by which DCs participate in the deviation of tolerance to allergic sensitization in the gut. We show that CT results in preferential migration and maturation of DC subsets to the MLN

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³ Abbreviations used in this paper: DC, dendritic cell; CT, cholera toxin; DN, double negative; MLN, mesenteric lymph node; OX40L, OX40 ligand; PP, Peyer's patch; pLN, peripheral lymph node.

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Table I. *Primer sequences*

Primer	Forward (5' to 3')	Reverse (5' to 3')
IL-12p35	ATGACCTGTGCCTTGGTAG	CAGATAGCCCATCACCTGT
IL-12p40	AGGTGCGTTTCCTCGTAGAGA	AAAGCCAACCAAGCAGAAGA
IL-23p19	AATAATGTGCCCCGTATCCA	CTGGAGGAGTTGGCTGAGTC
IL-10	TGCTATGCTGCCTGCTCTTA	TCATTTCCGATAAGGCTTGG
Jagged-1	TCCAGGTCTTACCACCGAAC	GGACGCCCTCTGAACCTGAC
Jagged-2	GGCAAAGAATGCAAAGAAGC	GCTCAGCATTGATGCAGGTA
Delta-4	ACCTTTGGCAATGTCTCCAC	TTGGATGATGATTTGGCTGA
OX40L	CCCTCCAATCCAAAGACTCA	ATCCTTCGACCATCGTTTAC

and demonstrate a critical role for OX40L in Th2 skewing by allergenic MLN DCs.

Materials and Methods

Mice

Female BALB/c mice (6–10 wk of age) were obtained from (Frederick, MD). Breeding pairs of DO11.10 OVA TCR transgenic mice were obtained from The Jackson Laboratory and maintained in a specific pathogen-free facility at Mount Sinai School of Medicine (New York, NY) in accordance with approved ethical guidelines. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Mice were administered CT (20 μ g) or PBS by gavage, using a 20-gauge feeding needle (Fisher Scientific).

Reagents

Cells were cultured in RPMI 1640 (Cellgro) with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (all from Invitrogen Life Technologies). OVA_{323–339} peptide was purchased from American Peptide, CT from List Biological Laboratories, and CFSE from Invitrogen Life Technologies. Abs used for flow cytometry were allophycocyanin anti-CD11c, allophycocyanin-Alexa Fluor 750 anti-CD11b, PE-Cy7 anti-CD8 α , PE anti-CD103, allophycocyanin-Alexa Fluor 750 anti-CD4, and PE-Cy5 anti-DO11.10 TCR (KJ1–26) (all from eBioscience), and FITC anti-CD86, FITC anti-I-A/I-E, FITC anti-CD80, PE anti-CD45R/B220, and PE anti-CD11c (from BD Pharmingen). Anti-OX40L neutralizing Ab was purchased from R&D Systems.

Flow cytometry

MLN cells were isolated and stained with specific Abs according to standard techniques. Cells ($2.5\text{--}3 \times 10^6$ per sample) were acquired on a LSRII flow cytometer (BD Biosciences). Dead cells (identified by propidium iodide staining) were excluded from analysis. For Ag presentation assays, proliferation was determined by the CFSE dilution method using CD4⁺ and KJ1–26⁺-gated cells. Analysis of flow cytometric data was performed using Flow Jo software (Tree Star). Sorting of DC subsets was performed after triple staining with PE-CD11c, allophycocyanin-Cy7-CD11b, and PE-Cy7-CD8 on a FACSVantage cytometer (BD Biosciences) using CellQuest software. Alternatively, CD11c⁺ subsets were sorted based on the expression of CD103.

DC isolation

DCs were isolated from the MLN 16 and 24 h after feeding mice with PBS or CT. A single cell suspension was prepared and cells were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotec) and selected on MACS separation columns. The DC preparations obtained by this technique had a purity of >90% as determined by flow cytometry.

Analysis of CD4⁺ T cell proliferation and cytokine production (Ag presentation assays)

CD4⁺ T cells from the MLN and spleen of DO11.10 OVA TCR transgenic mice were purified by negative selection (StemCell Technologies) according to the manufacturer's instructions. CD4⁺ T cells (>90% pure) were CFSE-labeled using a CellTrace CFSE cell proliferation kit (Invitrogen Life Technologies). CD4⁺ cells (10^5) were cultured together with $10^3\text{--}10^4$ DCs purified from the MLN of PBS- or CT-fed mice. Cells were cultured with OVA_{323–339} peptide (1–1000 ng/ml) or medium alone. After 4 days, T cell proliferation was assessed by flow cytometry (CFSE dilution method). The division index, which is the average number of cell divisions in the entire population, was calculated as division index = proliferation

index $\times$ percentage divided, where the proliferation index is the average number of divisions of the dividing cells. Data were then normalized to the PBS-fed controls and expressed as fold increase. To assess cytokine secretion, CD4⁺ T cells were restimulated with 3 μ g/ml plate-bound anti-CD3 mAb (eBioscience) plus 3 μ g/ml soluble anti-CD28 (eBioscience). Cells were cultured in complete medium for 3 days and supernatants were collected for the detection of cytokines by ELISA (all from eBioscience).

In vivo Ag presentation assay

CD4⁺ T cells from the MLN and spleen of DO11.10 OVA TCR transgenic mice were purified by negative selection as described above and $3\text{--}5 \times 10^6$ CD4 T cells were transferred i.v. to BALB/c mice. Twenty-four hours after transfer, mice were fed with PBS, OVA (50 mg), or OVA plus CT. Seventy-two hours after feeding the mice were sacrificed and cells were isolated from the MLN. Cells (2×10^5) were cultured with OVA_{323–339} (1000 ng/ml). After 3 days, cells were restimulated with 3 μ g/ml plate-bound anti-CD3 mAb plus 3 μ g/ml soluble anti-CD28. Cells were cultured in complete medium for 3 days and supernatants were collected for detection of cytokines by ELISA.

RT-PCR

DCs were purified from the MLN as outlined above. RNA was isolated using an RNeasy Micro kit (Qiagen). RNA was reverse transcribed with SuperScript reverse transcriptase (Invitrogen Life Technologies) and cDNA was amplified in a real-time thermocycler (Applied Biosystems 7300) using SYBR green master mix (Invitrogen Life Technologies) and primers from Invitrogen Life Technologies (Table I). Data were expressed as fold increase compared with levels measured in PBS-fed mice by using $\Delta\Delta C_T$ threshold cycle method of calculation. GAPDH was used as the housekeeping gene.

Statistical analysis

Data were analyzed by one-way ANOVA followed by posthoc testing with the Bonferroni test. A value of $p < 0.05$ was considered significant.

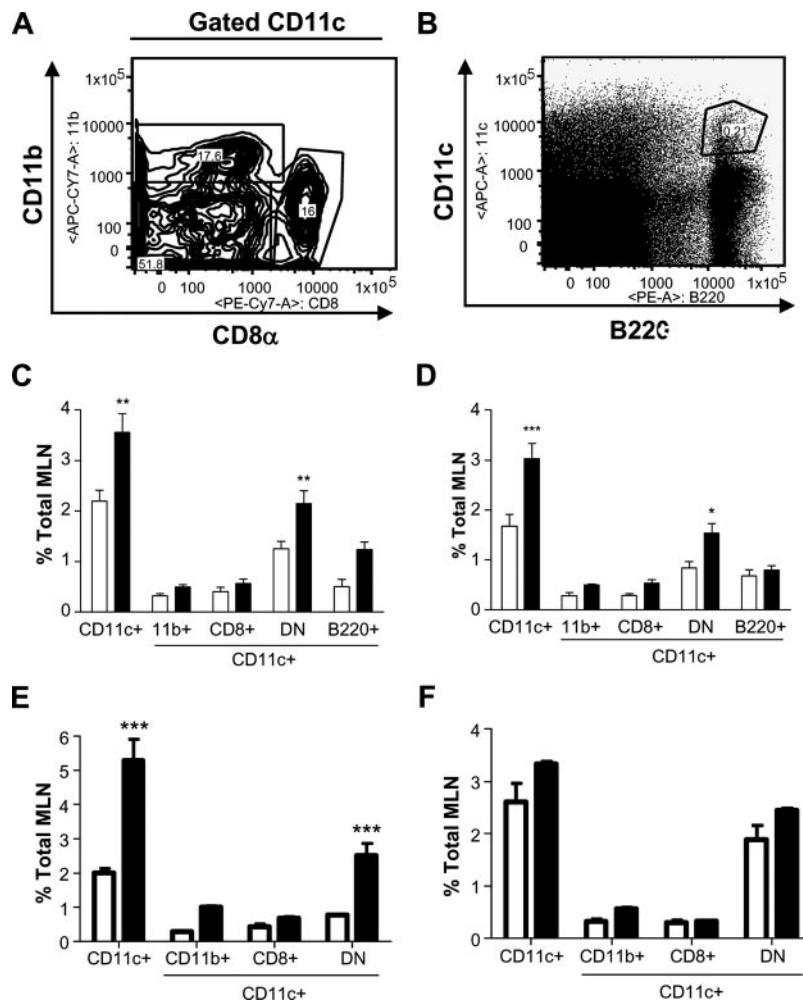
Results

Oral administration of CT induces the expansion of a subset of CD103⁺ DCs within the MLN

We first analyzed the phenotype of DCs in the MLN by flow cytometry using the cell surface markers CD11c, CD11b, CD8 α , and B220. The composition of DC subsets in MLN was very similar to that described in the PP (5). Normal MLN preparations contained 1.5–2% CD11c⁺ cells, of which ~15% were CD11b⁺CD8 α ⁺, 18% were CD11b⁺CD8 α ⁺, and 56% were CD11b⁺CD8 α ⁺ or DN (Fig. 1A). Plasmacytoid DCs (B220⁺) account for ~22% of DCs (Fig. 1B), of which a small fraction (5%) are CD8 α ⁺ as has been described by Bilsborough et al. (8).

To determine the impact of the allergenic adjuvant CT on gastrointestinal DCs, we fed mice with 20 μ g of CT or PBS as control and, after 16–24 h, cells were isolated from MLN and analyzed by flow cytometry (Fig. 1, C and D). CT induced a significant increase in the proportion of CD11c⁺ cells in the MLN at both time points without having any significant effect on absolute numbers in the MLN (PBS = $11.52 \times 10^6 \pm 1.162 \times 10^6$ cells/mouse; CT = $11.68 \times 10^6 \pm 2.579 \times 10^6$ cells/mouse), showing that the total number of CD11c⁺ cells increases. As shown in Fig. 1, C and D, CT feeding of mice led to a marked increase in the number of

FIGURE 1. Oral CT induces selective expansion of DC subsets in the MLN and this expansion is restricted to CD103⁺ cells. **A**, Flow cytometry analysis of cell suspensions isolated from MLN of normal BALB/c mice. Three DC subsets (gated on CD11c⁺) were defined by the expression of CD8 α and CD11b as shown. **B**, B220⁺ DCs were defined by the coexpression of CD11c and B220. **C** and **D**, Cells were isolated from MLN of control (□) and CT-fed (■) mice at 16 h (**C**) and 24 h (**D**) after feeding mice PBS or CT. Staining was performed with allophycocyanin anti-CD11c, allophycocyanin-Cy7 anti-CD11b, PE-Cy7 anti-CD8 α , and PE anti-CD45R/B220. Results are expressed as mean ($\pm$ SEM) percentage of total cells per MLN ($n = 8$ independent experiments for 16 h and $n = 4$ independent experiments for 24 h). **E** and **F**, Cells were isolated from the MLN of PBS (□) or CT-fed (■) mice and first gated on CD103⁺ (**E**) or CD103⁻ (**F**) cells before analysis of DC subsets as described above. Results are expressed as means ($\pm$ SEM) percentage of cells per MLN ($n = 3$ independent experiments). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with PBS-fed mice.



CD11b-CD8 α - (DN) DCs in the MLN. In contrast, there was no significant increase in the number of CD11b⁺, CD8 α ⁺, or B220⁺ DCs (Fig. 1, **C** and **D**).

To determine whether the expansion induced by CT was due to the migration of DCs from the lamina propria to the MLN, we used the integrin α -chain CD103 (α_E), which has been shown to be a marker of lamina propria-derived DCs (13). We analyzed the expression of CD103 on DCs isolated from the MLN of PBS- or CT-fed mice (Fig. 1, **E** and **F**). CT induced a significant increase in the CD103⁺ population of DN DCs (Fig. 1E) with no significant effect on CD103⁻ DCs (Fig. 1F). There was no reduction in CD103⁻ cells, which would be the case if CT was inducing local CD103 up-regulation by DCs within the MLN. These results are consistent with CT inducing the selective homing of DN DCs from the lamina propria to the MLN.

CT induces maturation of gastrointestinal DCs in vivo

In vitro studies have shown that CT can induce up-regulation of both CD80 and CD86 on DCs (3) and selective up-regulation of CD86 on bone marrow-derived macrophages (14). Recently, Anjuère et al. showed that oral CT induced the expansion and maturation of MLN DCs in mice (15). We examined the impact of CT on the expression of maturation markers in the MLN. CT induced an up-regulation of CD86 expression on CD11c⁺ cells. Consistent with the effect of CT on DC numbers, this up-regulation was highest in the DN DC population (Fig. 2A) but was also clearly present in the CD11b⁺ and CD8 α ⁺ populations of DCs. CD80 and MHC class II expression showed a minor up-regulation by CT in vivo

(data not shown). Up-regulation of CD86 was observed on the CD103⁺ (lamina propria-derived) DCs but not on CD103⁻ DCs of the MLN (Fig. 2, **B** and **C**), consistent with a local effect of CT on DCs within the lamina propria before migration. To determine whether this effect of CT was specific to gut-associated lymphoid tissues, we analyzed the effect of CT on the phenotype of DCs from spleen or from pooled peripheral lymph nodes (pLN), including superficial cervical nodes, deep cervical nodes, axillary nodes, and inguinal nodes (Fig. 3). Although CT up-regulated CD86 expression on CD11c⁺ cells in the MLN (Fig. 3A), CT did not have any effect on this population in the pLN (Fig. 3B) or in spleen (Fig. 3C), suggesting that the effect of orally administered CT on DCs is restricted to gut-associated lymphoid nodes.

MLN DCs induced by CT in vivo induced a robust proliferation and cytokine response from T cells

Oral administration of CT with allergen to mice results in allergen-specific Th2 cytokine responses, which we hypothesized are mediated by gastrointestinal DCs. To determine the functional outcome of in vivo DC modulation by CT on the T cell response, we performed Ag presentation assays. CD11c⁺ cells were isolated from the MLN after feeding mice CT or PBS as control. Their ability to stimulate Ag-specific T cell proliferation was assessed using CFSE-labeled CD4⁺ T cells isolated from DO11.10 mice. Cell proliferation was assessed by flow cytometry after 96 h of coculture. MLN DCs from CT-fed mice induced more T cell proliferation from responder cells (Fig. 4). This increase in proliferation was observed at all DC:T cell ratios examined.

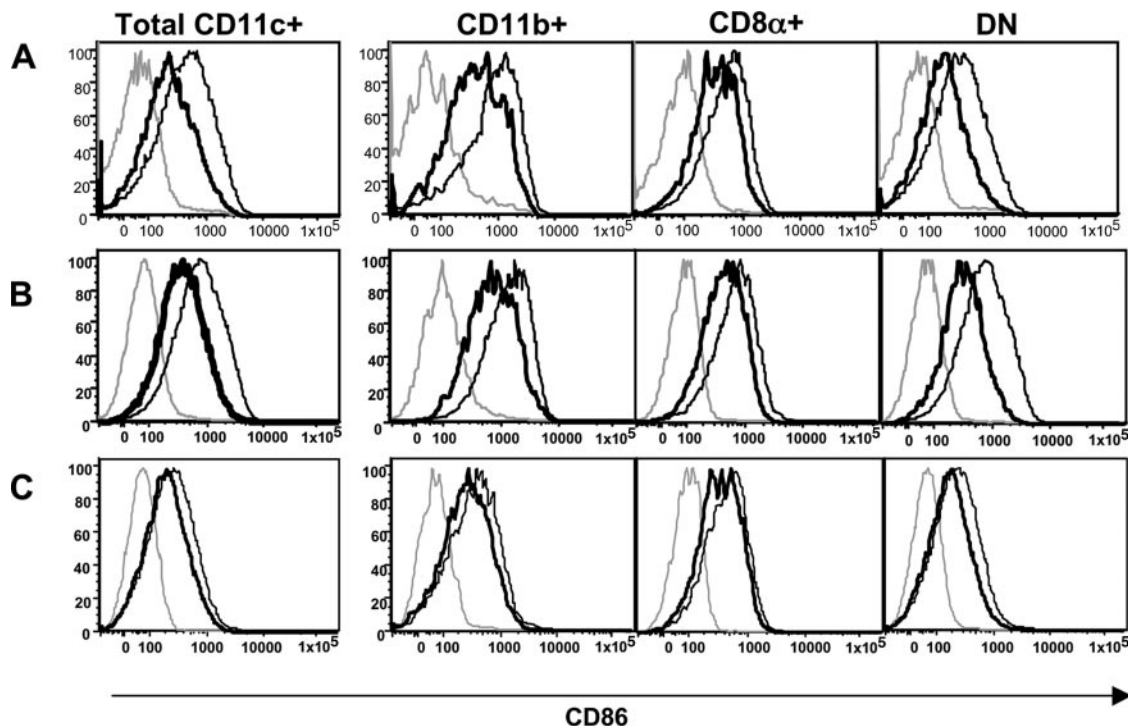


FIGURE 2. Up-regulation of CD86 on CD103⁺ MLN DCs in response to oral CT. A single cell suspension was prepared from MLN of control and CT-fed mice and stained with cell surface marker Abs. Histograms show CD86 expression as follows: gray lines correspond with isotype controls, black bold lines with PBS-fed mice, and black lines with CT-fed mice. *A*, Total CD11c⁺ cells and DC subsets. *B*, CD103⁺CD11c⁺ cells, and DC subsets. *C*, CD103⁻CD11c⁺ cells and DC subsets. These data are representative of three independent experiments.

We next analyzed cytokine secretion from responder T cells by ELISA. DCs isolated from CT-fed mice induced significantly greater levels of the Th2 cytokines IL-4 and IL-13 compared with PBS-fed mice (Fig. 5, *A* and *B*). Consistent with the ability of CT to induce comprehensive mucosal immunity, including IgG Ab responses and cytolytic T cell responses, CT also significantly increased IL-17 and (to a lesser extent) IFN- γ secretion (Fig. 5, *C* and *D*), although this up-regulation was less consistent than the Th2 up-regulation. These findings show that DCs migrating to the lymph node after CT administration are able to induce a strong proliferation and cytokine response, including a robust Th2 cytokine response that is thought to be critical for allergic sensitization.

We next performed *in vivo* Ag presentation assays by transferring DO11.10 cells to naive BALB/c mice 24 h before feeding PBS, OVA plus PBS, or OVA plus CT. After oral administration of OVA plus CT, primed DO11.10 cells produced greater amounts

of the Th2 cytokines IL-4 (Fig. 6*A*) and IL-13 (Fig. 6*B*) compared with mice primed with PBS or OVA alone, and, consistent with the *in vitro* data (Fig. 5), they also produced higher levels of IL-17 (Fig. 6*C*) and IFN- γ (Fig. 6*D*). However, the highest fold change was observed for Th2 cytokines (IL-4 and IL-13) followed by IFN- γ and IL-17 (Fig. 6*E*). *In vitro* and *in vivo* findings demonstrate that CT strongly enhances the T cell response and promotes Th2 differentiation via DCs.

Because different subpopulations of DCs have been shown to preferentially induce Th1, Th2, or regulatory T cell responses after CD40 ligand (CD40L) stimulation, we analyzed the *in vivo* effect of CT on DC subsets by sorting subsets before coculture with responder T cells. CD11b⁺, CD8 α ⁺, or DN DCs were cultured with 10⁵ CD4⁺ T cells isolated from OVA TCR transgenic mice and cytokine secretion was analyzed by ELISA. Consistent with the effects on DC maturation and in contrast to the effects on DC

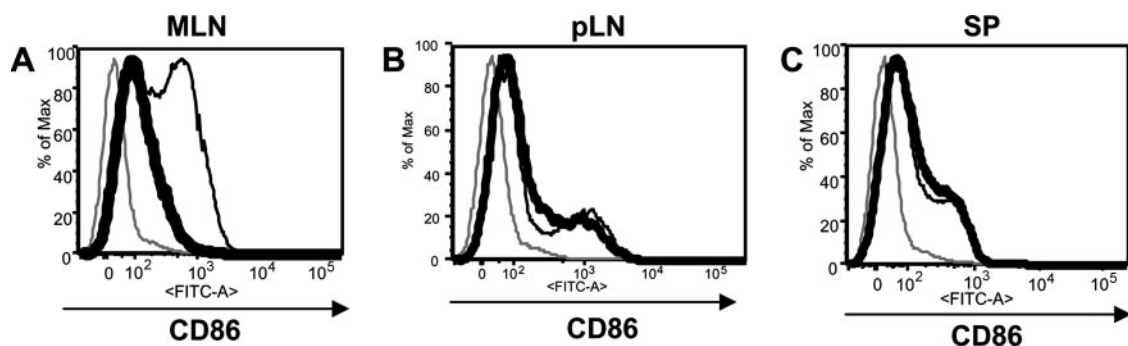


FIGURE 3. Oral CT induces maturation of CD11c⁺ cells locally. A single cell suspension was prepared from MLN (*A*), pooled peripheral lymph nodes (pLN) (*B*), and spleen (SP) (*C*) of control and CT-fed mice and stained with cell surface marker Abs. Histograms show CD86 expression as follows: gray lines correspond with isotype controls, black bold lines with PBS-fed mice, and black lines with CT-fed mice. These data are representative of three independent experiments.

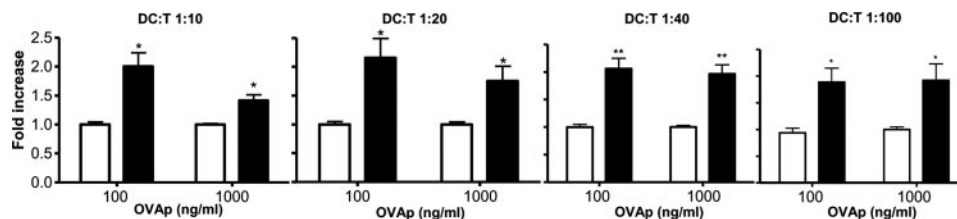


FIGURE 4. MLN DCs primed with CT in vivo induce increased T cell proliferation. CFSE-labeled DO11.10 (OVA-TCR transgenic) CD4⁺ T cells (10^5 cells per well) were stimulated with CD11c⁺ cells isolated from the MLN of PBS (□) or CT-fed (■) mice in the presence or absence of the peptide OVA_{323–339} (OVAp). DC:T ratios from 1:10 to 1:100 were used as shown. After 96 h, proliferation was determined by flow cytometry. Live CD4⁺KJ1–26⁺ cells were gated and the division index was calculated as stated in *Materials and Methods*. Data were normalized to PBS-fed controls. Results are expressed as means ($\pm$ SEM) of five independent experiments. *, $p < 0.05$; and **, $p < 0.01$.

migration, CT feeding induced an increase in priming ability in all three DC subsets. As shown in Fig. 7, priming of CD4⁺ T cells by CD11b⁺, CD8 α ⁺, and DN DCs from the MLN of mice fed CT led to increased IL-13 (Fig. 7A) and IL-4 (Fig. 7B) secretion compared with DC subsets from control mice. This was also true for IFN- γ and IL-17 (data not shown) as compared with corresponding DC subsets from PBS-fed mice. These results suggest that environmental stimuli, rather than functional specialization of DC subsets, are predominantly responsible for differential immune responses to luminal Ags.

CT modulates the expression of Th2 skewing factors by DCs

Our aim was to define proallergic pathways induced by CT as a means for examining the mechanisms of allergic sensitization to food proteins. We next examined whether CT could modulate the expression level of DC-expressed genes that are known to influence T cell cytokine skewing. We analyzed the expression of factors that are involved in Th1, Th2, regulatory T cell, or Th17 differentiation including cytokines (IL-12p35, IL-12/IL-23p40, IL-23p19, and IL-10), Notch ligands (Jagged-1, Jagged-2, and Delta-4), and OX40L. Delta expression by APCs has been shown to prime naive T cells for Th1 responses, while Jagged and OX40L

prime for Th2 responses (16–20). CD11c⁺ cells were isolated from the MLN of CT-fed mice or PBS-fed mice, and real-time RT-PCR was performed. The expression of the cytokines IL-10, IL-12 (p35/p40), and IL-23 (p19/p40) by MLN DCs was not significantly changed by CT feeding (Fig. 8A). In contrast, CT feeding induced a significant up-regulation of the Th2-skewing factors Jagged-2 and OX40L expression by CD11c⁺ cells (Fig. 8B). Because we have demonstrated that CT exclusively induced the maturation of CD103⁺ DCs, we examined gene expression in sorted CD103⁺ or CD103[−] subsets. Real-time RT-PCR data showed that oral CT induced a significant up-regulation of OX40L and Jagged-2 expression by CD103⁺ cells (Fig. 8, C and D). In contrast, CT feeding did not have a significant effect on the expression of those two Th2-skewing factors by CD103[−] cells, demonstrating that oral administration of CT selectively up-regulated the expression of the Th2-skewing factors OX40L and Jagged-2 by the CD103⁺ DC population within the MLN.

Up-regulation of OX40L on DCs is critical for the priming of Th2 cells

Our results showed that in vivo administration of CT induced the up-regulation of Jagged-2 and OX40L on CD11c⁺ cells of

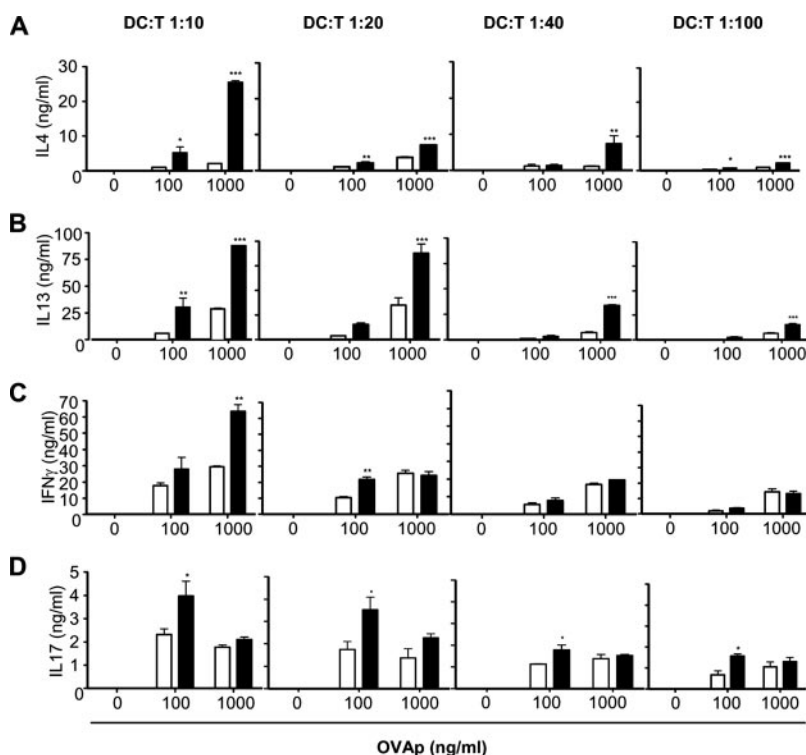
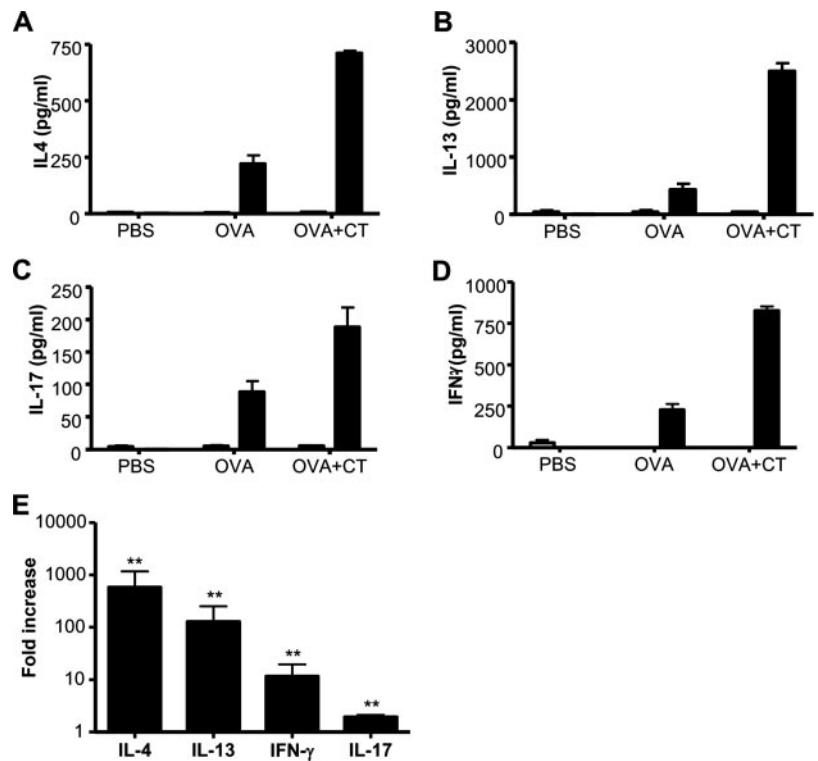


FIGURE 5. MLN DCs primed with CT in vivo induce enhanced T cell cytokine secretion. DO11.10 (OVA-TCR transgenic) CD4⁺ T cells (10^5 cells per well) were stimulated with CD11c⁺ cells isolated from the MLN of PBS (□) or CT-fed (■) mice in the presence or absence of the peptide OVA_{323–339} (OVAp). DC:T cell ratios from 1:10 to 1:100 were used as shown. After 72 h, T cells were restimulated with anti-CD3 and anti-CD28 Abs. After 72 h, supernatants were harvested and cytokines were measured by ELISA. IL-4 (A), IL-13 (B), IFN- γ (C), and IL-17 (D) levels were measured by ELISA in triplicate cultures. Results are expressed as means ($\pm$ SEM) of five independent experiments. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

FIGURE 6. In vivo primed DO11.10 T cells become Th2 biased upon oral administration of OVA plus CT (OVA+CT). DO11.10 (OVA-TCR transgenic) CD4⁺ T cells were isolated and transferred to BALB/c mice by i.v injection and then fed with PBS, OVA (50 mg), or OVA (50 mg) plus CT (20 μ g). After 72 h, cells were isolated from the MLN and cultured with OVA_{323–339} for 72 h followed by restimulation with anti-CD3/CD28. IL-4 (A), IL-13 (B), IL-17 (C), and IFN- γ (D) levels were measured by ELISA in triplicate cultures. Results are expressed as means ($\pm$ SEM) of triplicate cultures and data are representative of three independent experiments. E, Fold increase in cytokine secretion of OVA plus CT-fed mice ($\log_{10}$ scale). Data were normalized to OVA-fed controls. Results are expressed as means ($\pm$ SEM) of triplicate cultures from three independent experiments. **, $p < 0.01$.



the MLN, but it was not clear whether these factors played a role in the development of Th2 differentiation or whether they were selective for the priming of Th2 cells. To address this

issue, neutralizing anti-OX40L Ab was added into the Ag presentation assay and cytokine output from the DO11.10 T cells was measured by ELISA. As shown in Fig. 9, A–D, neutralizing

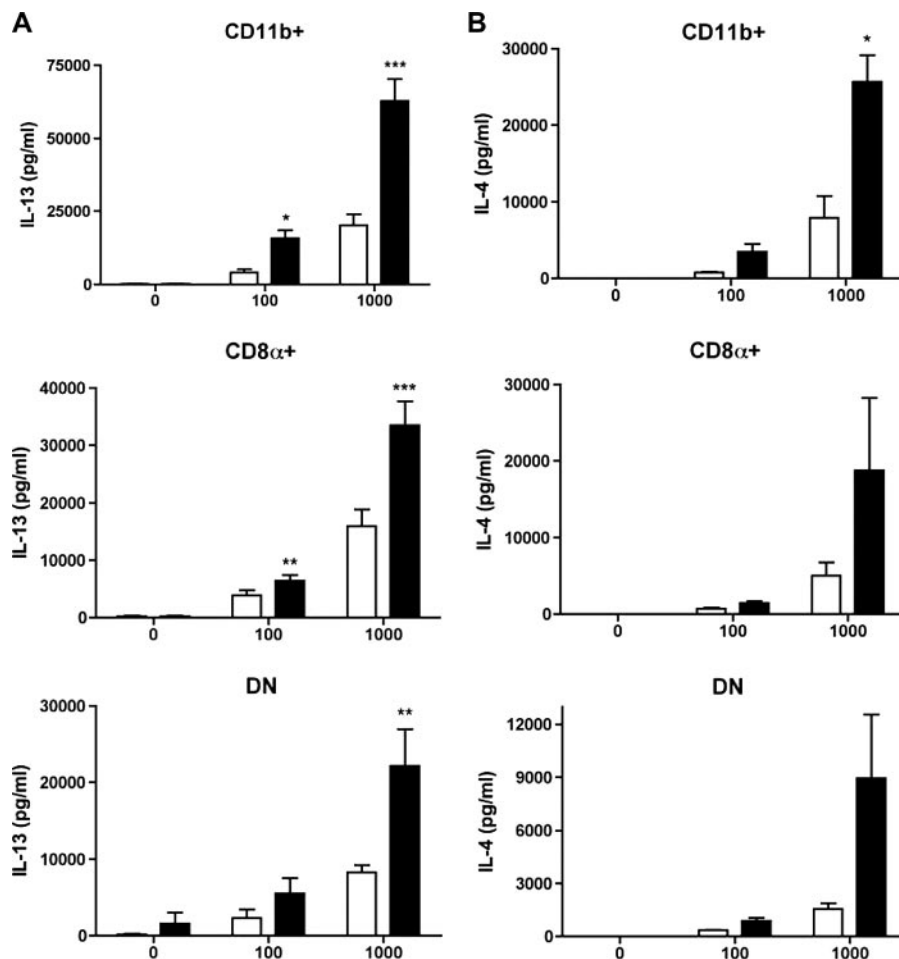


FIGURE 7. Oral administration of CT enhances the T cell stimulatory activity of all DC subsets as shown by sorting. MLN DC subsets (CD11b⁺, CD8 α ⁺, and DN) were isolated from mice by sorting 19 h after the feeding of either PBS ($\square$) or CT ($\blacksquare$). DCs were cultured with DO11.10 T cells at a ratio of 1:10 to 1:20 DC:T cells, with OVA_{323–339}. 72 h after coculture, T cells were restimulated with anti-CD3 and anti-CD28 Abs. Supernatants were harvested after 72 h and IL-13 (A) and IL-4 (B) levels were measured by ELISA. Pooled MLN from six PBS-fed mice and four CT-fed mice were used per experiment. Results are expressed as means ($\pm$ SEM) of duplicate cultures from two independent experiments. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

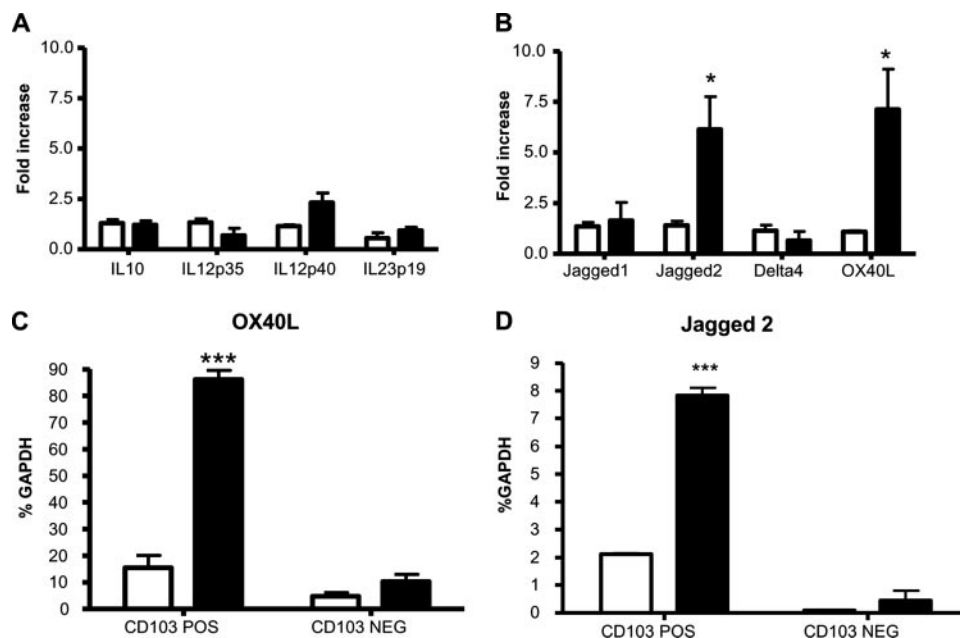


FIGURE 8. Oral CT induces up-regulation of Jagged-2 and OX40L expression by MLN DCs. *A* and *B*, CD11c⁺ cells were purified from the MLN of PBS- (□) or CT-fed (■) mice, and RNA was isolated. Real-time RT-PCR was performed for IL-10, IL-12 subunits (p35 and p40), IL-23 subunits (p40 and p19) (*A*) and for Notch ligands (Jagged-1, Jagged-2, and Delta-4) and OX40L (*B*). Fold increase in gene expression was calculated using the $\Delta\Delta C_T$ threshold cycle method with GAPDH as the housekeeping gene. *C–D*, CD103⁺ (CD103 POS) and CD103[−] (CD103 NEG) DC subsets were isolated by FACS sorting from MLN CD11c⁺ cells of PBS- (□) or CT-fed (■) mice. Real-time RT-PCR was performed for OX40L and Jagged-2. Data are expressed as the percentage of the housekeeping gene GAPDH. Results are expressed as means ($\pm$ SEM) of at least three separate experiments. *, $p < 0.05$; and ***, $p < 0.001$.

anti-OX40L Ab dose-dependently suppressed IL-4 and IL-13 secretion from T cells stimulated with DCs from CT-fed but not PBS-fed mice. At a dose of 10 μ g/ml anti-OX40L, the enhancement in Th2 priming induced by CT was almost completely

abolished. In contrast, anti-OX40L did not suppress the IFN- γ or IL-17 priming induced by CT (Fig. 9, *E* and *F*), demonstrating that OX40L on gastrointestinal DCs selectively mediates CT-induced Th2 development.

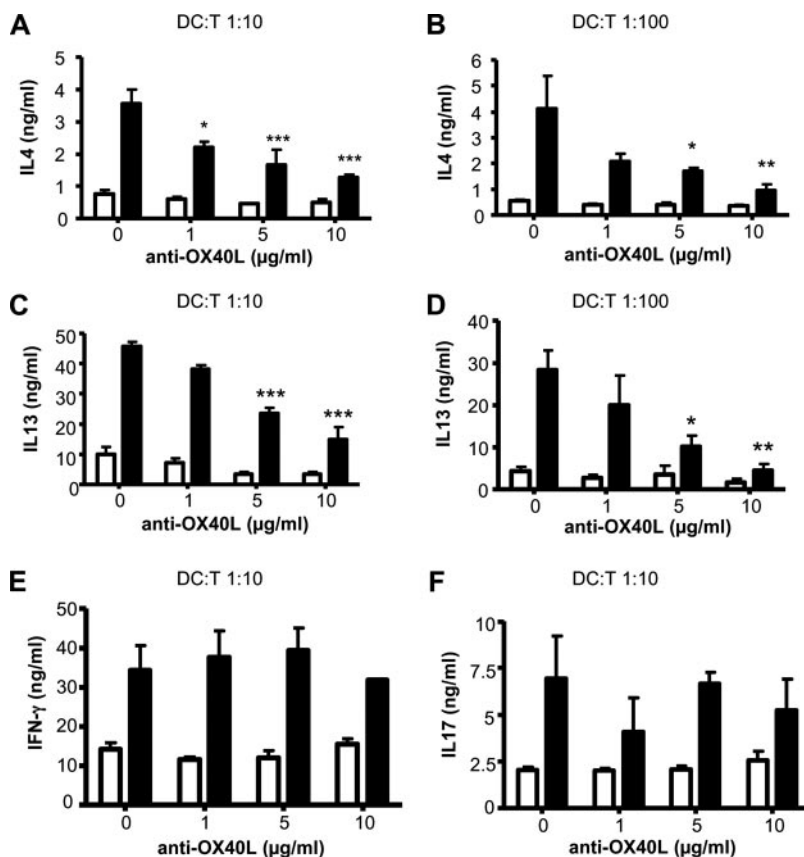


FIGURE 9. Effect of anti-OX40L neutralizing Ab on Th2 cytokine secretion. DCs (10^3 – 10^4) isolated from MLN of PBS- (□) or CT-fed (■) mice were cocultured with 10^5 DO11.10 CD4⁺ T cells. Neutralizing anti-OX40L Ab was added at a range from 1 to 10 μ g/ml. After 72 h, T cells were restimulated with anti-CD3 and anti-CD28 Abs. Supernatants were harvested after 72 h and IL-4 (*A* and *B*), IL-13 (*C* and *D*), IFN- γ (*E*), and IL-17 (*F*) levels were measured by ELISA. Only the 1:10 DC:T cell ratio is shown for IFN- γ and IL-17 as that was the ratio at which CT-induced priming was observed for these two cytokines. Results are expressed as means ($\pm$ SEM) of three different experiments. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Discussion

The normal response to soluble dietary proteins is the induction of systemic immunologic hyporesponsiveness known as oral tolerance (21). This induction of tolerance is critical not only for the prevention of inappropriate immune reactivity to food allergens but also to commensal flora. The mechanisms responsible for generating tolerance rather than immunity (including both allergenic or inflammatory responses) remains unclear, although the development of spontaneous colitis in murine knockout mice has highlighted the importance of multiple immune pathways in the maintenance of tolerance to the commensal flora. In this study we examined the mechanisms by which gastrointestinal DCs participate in the generation of allergic sensitization. CT has been widely used as an adjuvant to generate murine (10–12) and swine (22) models of food allergy. Therefore, we used CT as a tool to generate an allergenic milieu *in vivo* and examined the influence of CT on gastrointestinal DCs. We show that the Th2-skewing effects of CT can be isolated from other immunogenic effects and are dependent on OX40L expressed by DCs migrating to the MLN.

Mice responded to oral CT with a significant expansion in the total number of CD11c⁺ cells in the MLN. Not all DC populations were affected, and only the CD11c⁺CD11b[−]CD8α[−] (DN) population was significantly expanded. These results are consistent with the findings of Anjuère et al. that showed a selective migration of a DC subset that they described as CD8 intermediate (15). In that article, the CD8-intermediate population appears to be the same population described as double-negative by Iwasaki et al. (5) and that we have also called double-negative.

The population expansion was observed only in those DCs bearing the lamina propria marker CD103, suggesting an active migration induced by CT. Although we cannot rule out the possibility that CT could directly or indirectly modulate CD103 expression in the MLN, we did not observe any decrease in the CD103[−] population, arguing against a shift in expression from negative to positive. Induction of DC migration by CT has been demonstrated by Shreedhar et al. (23), who observed that fluorescent microsphere-loaded DCs in the subepithelial dome relocated to T cell areas after feeding CT. MacPherson and colleagues have directly addressed the migration of DCs from the intestinal lamina propria by collecting lymph over time in cannulated rats (24–26). They have shown that DC migration from the lamina propria to the MLN occurs constitutively and that this migration can be modulated by TLR agonists, in particular TLR4 and TLR7/8 agonists (24, 25). However, they recently reported that another bacterial toxin with adjuvant activity (heat-labile enterotoxin from *Escherichia coli*) did not influence DC migration (26). They did find an increase in Ag-bearing DCs leaving the lamina propria via the lymph and observed a relocation of the migrating DCs to the T cell areas of the MLN. They reported an increase in CCR7 expression on draining DCs, which we speculate may also be a mechanism for the expansion of DCs in the MLN that we observe in response to oral CT. CCR7 has been shown to be necessary for the constitutive traffic of DCs from the lamina propria to the MLN (27) and has been shown to be critical for the induction of tolerance responses to soluble Ags (28). Traffic of DCs from the lamina propria to the MLN is necessary for the induction of both tolerance and immunity; therefore, the increase in DC traffic alone is unlikely to explain the induction of immunity or allergic sensitization rather than tolerance.

We found that oral CT induced the up-regulation of CD86 on DCs of the MLN *in vivo*, confirming the findings of Anjuère et al. (15). In addition, we show that this up-regulation selectively

occurs on CD103⁺ DCs, suggesting that the matured DCs are derived from the lamina propria. The change in the DC phenotype in response to CT was restricted to the GALT and was not observed in spleen or non-GALT pLN. Similar results of DC maturation have been reported in response to CT applied nasally, although both CD86 and CD80 were up-regulated on draining DCs in that report (29). Heat-labile *E. coli* was also shown to mature draining DCs in the rat (shown by up-regulation of CD25) (26). We speculate that the preferential up-regulation of CD86 over CD80 may contribute to Th2 skewing in response to CT. It has been shown in experimental allergic encephalomyelitis models that CD86 and CD80 preferentially induce Th2 and Th1 responses, respectively (30). Therefore, up-regulation of CD86 on DCs may be one factor that promotes allergic sensitization.

In the current studies we did not examine the effect of CT on DCs of the PP. Studies by Iwasaki et al. have shown that the DC subsets identified in the patch are also present in the MLN (4, 5), although the functional studies comparing DC subsets have been done primarily with PP vs spleen DCs (5, 6). Shreedhar et al. studied the *in vivo* effect of CT on PP DCs and showed that DCs that had previously taken up fluorescent microparticles migrated from the subepithelial dome to the T cell areas in response to CT administration (23), but no further studies were done to look at DC subsets or functional outcome of CT administration on T cell activation. Migration is necessary for the induction of tolerogenic immune responses in the MLN (28) but may also required in the PP, where the relocation of Ag-sampling DCs from the subepithelial dome to the T cell area is necessary for an efficient T cell response. Migration of DCs from the LP to the MLN (and subsequent Ag presentation) has been shown to be CCR7 dependent (28), but it is not known whether CCR7 is also required for efficient localization of DCs to the T cell areas and subsequent Ag presentation in the PP. Studies examining T cell activation in the GALT in response to OVA feeding have shown that T cell proliferation can be observed in both the MLN and the PP (31, 32). Soluble Ags (such as OVA or HRP) can readily be taken up by epithelial cells (33–36) and delivered to the MLN by DCs migrating from the lamina propria (28). In contrast, particulate Ags are preferentially taken up by M cells overlying the PP (37, 38). Therefore, sensitization to food allergens such as the peanut, which has been shown to traffic via M cells (39), may be preferentially induced in the Peyer's patch. Several studies using OVA as a tolerogen have shown that PP and MLN are overlapping inductive sites, such that low-dose (active) tolerance can be induced in the absence of PP but not in the absence of both MLN and PP (28, 40–42). Studies are needed to determine whether the same overlap of MLN and PP functions is observed in allergic sensitization. In summary, although there is evidence for significant overlap in the cellular makeup and immune function of the MLN and PP, in the absence of data on PP DCs we cannot conclude whether CT has similar or distinct effects on PP DC subsets as those observed in the MLN.

Sensitization of mice with food allergens using CT as a mucosal adjuvant leads to the induction of a mixed Ag-specific cytokine response, including the Th2 cytokines IL-4 and IL-13, IFN-γ (10–12, 43), and the cytokine IL-17 (F. Roth-Walter, M. C. Berin, and L. Mayer, unpublished data). We show by Ag presentation assays that DCs from the MLN can mediate this effect of CT. Naive T cells cultured with MLN DCs from CT-fed mice secreted significantly more IL-4, IL-13, IFN-γ, and IL-17 than T cells cultured with MLN DCs from PBS-fed mice. The nature of this cytokine response was the same whether the Ag presentation occurred *in vitro* or *in vivo*. These results underscore the

fact that CT has adjuvant activities beyond allergic sensitization and is a potent inducer of IgA and IgG responses as well as cytotoxic T cell responses. Sorting of MLN DC subsets showed that all DC subsets could be modulated by CT to prime IL-4, IL-13, IFN- γ , and IL-17 secretion by responder T cells. These results argue against the functional specialization of DC subsets in allergic sensitization and suggest that the environment in which presentation occurs is a more dominant factor in the resulting T cell response.

One of the mechanisms by which Th2 differentiation may be induced by DCs is the differential expression of the ligands Jagged and Delta (16). Delta was shown to promote Th1 differentiation, and Jagged promoted naive CD4⁺ T cells to differentiate into the Th2 lineage (16). CT could up-regulate the expression of Jagged-2 on bone-marrow-derived DCs (16) in vitro and, therefore, we speculated that a similar mechanism could underlie the Th2 skewing in response to CT in our model. Another factor shown to promote the development of Th2 cells is OX40L, which is a TNF family member expressed on B cells, DCs, and endothelial cells (17, 44, 45). The receptor is OX40 (CD134) expressed on activated CD4⁺ T cells (46, 47). OX40L has been shown to induce Th2 skewing both in vitro (17, 48) and in vivo (18, 19) and mediates Th2 skewing induced by the thymic stromal lymphopoietin (TSLP) (20). We found a significant up-regulation of both Jagged-2 and OX40L expression by CD11c⁺ cells isolated from the MLN of CT-fed mice, specifically by CD103⁺ cells. Neutralizing Abs demonstrated that OX40L alone appeared to be sufficient for the selective induction of Th2 cytokines by MLN DCs in response to CT. Our finding that anti-OX40L Abs could abolish Th2 skewing without affecting IFN- γ or IL-17 suggests that OX40L on gut DCs may be the critical differentiating point between the induction of immunity/inflammation vs allergic sensitization to luminal Ags.

We have not addressed mechanisms responsible for the induction of Th1 or Th17 cells by CT. We did not observe an up-regulation of the IL-12 or IL-23 subunits or the Notch ligand Delta-4 in response to CT. However, CD11b⁺ DCs from PP have been shown to produce IL-6 and promote IgA production by B cells (7). IL-6 and TGF- β are also inducers of Th17 cells (49, 50); therefore, CT may promote IgA responses and other components of protective mucosal immunity by similar mechanisms (51).

We have used CT as a tool to dissect mechanisms of allergic sensitization to food proteins, but this bacterial toxin is not likely to be involved in human food allergy. Other triggers that may be directly involved in human disease pathogenesis are the Ags themselves. For example, Shreffler et al. have recently shown that the peanut allergen Ara h 1 can mature human DCs and promote Th2 skewing via binding to the lectin DC-SIGN (DC-specific ICAM-grabbing non-integrin) (DC-SIGN) (52). Similar results have been shown for other allergens (53), but the OX40L pathway has not yet been addressed in this T cell skewing by allergens. There is a clear genetic susceptibility to allergic disease, so examining genetic polymorphisms in OX40L (or potentially Jagged-2) may provide further clues to the pathogenesis of food allergy.

In summary, our data indicate that an allergenic trigger in the gastrointestinal tract induces an increase in migration of CD11c⁺CD11b⁺CD8 α ⁺CD103⁺ DCs to the MLN. Maturation of gastrointestinal DCs induces the differentiation of Th2 cytokine-producing CD4⁺ T cells via an OX40L-dependent mechanism. In human food allergic disease, genetic modifications of OX40L or external factors that modulate OX40L expression may be critical for the development of allergic sensitization rather than tolerance to food proteins.

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Disclosures

The authors have no financial conflict of interest.

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