

Influence of Mucosal Adjuvants on Antigen Passage and CD4⁺ T Cell Activation during the Primary Response to Airborne Allergen¹

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Ag delivery via the nasal route typically induces tolerance or fails to polarize CD4⁺ T cell responses unless an adjuvant is provided. To better understand this process, we assessed the effects of two mucosal adjuvants, *Escherichia coli* LPS and cholera toxin (CT), on Ag passage and T cell activation in the draining lymph nodes (DLN) of BALB/c mice following per nasal administration of the model protein allergen, OVA. We found a range of cell types acquired small amounts of fluorescent OVA in the DLN 4 h after per nasal administration. However, this early uptake was eclipsed by a wave of OVA⁺CD8 α ^{low} dendritic cells that accumulated in the DLN over the next 20 h to become the dominant OVA-processing and -presenting population. Both LPS and CT stimulated increases in CD80 and CD86 expression on OVA⁺CD8 α ^{low} DC. LPS also increased the number of OVA⁺CD8 α ^{low} dendritic cells accumulating in the DLN. When the primary T cell response was examined after adoptive transfer of CD4⁺ T cells from DO11.10 mice, CT and LPS stimulated surprisingly similar effects on T cell activation and proliferation, IL-4 and IFN- γ priming, and memory T cell production. Despite these similarities, T cell recipients immunized with CT, but not LPS, developed lung eosinophilia upon secondary OVA challenge. Thus, we found no bias within the DLN in Ag handling or the primary T cell response associated with the eventual Th2 polarization induced by CT, and suggest that additional tissue-specific factors influence the development of allergic disease in the airways. *The Journal of Immunology*, 2006, 177: 913–924.

Exposure to Ag via the airways can induce tolerance, immunity, or allergy depending on a number of factors, including Ag dose, form, and frequency of exposure. Pulmonary infection with influenza in mice induces strong immunity after a vigorous phase of T cell activation and proliferation in the draining lymph nodes (DLN)⁴ (1, 2), while multiple doses of soluble Ag delivered via the nasal route generally induce tolerance, also after a vigorous T cell response in the DLN (3–8). Allergic sensitization of the airways can be induced by multiple inhaled doses or systemic immunization via the i.p. route (reviewed by Refs. 9 and 10), and while less is known about the primary T cell response, the allergic response appears to depend on the generation

of a large number of lung-homing allergen-specific T cells (11). Thus, tolerance, immunity, and allergy are all consequences of T cell activation in the DLN, however, questions remain as to how the immune system regulates the development of these states.

Dendritic cells (DC) are well-placed to play a crucial role in the immune surveillance of the mucosal surfaces of the airways and in parenchymal lung tissue (reviewed by Ref. 12). They form an extensive network in the airways (13, 14), exhibit a high basal turnover in the respiratory mucosae (15), and respond to local infection and/or inflammation with a rapid increase in their numbers (14, 16, 17). Pulmonary DC are also potent APCs (18–21) that are able to rapidly migrate to the DLN (17, 22, 23), so they are likely to be responsible for the initiation of the immune response against airborne Ags.

It is now widely accepted that DC control immune activation, and thus, have the potential to regulate the differentiation of the immune response. Indeed, there is a growing body of evidence to suggest that different immune states are directed by phenotypically or functionally discrete subsets of DC. For example, CD8 α ⁺/CD11b[−] DC preferentially induce Th1 responses in vivo, while resting CD8 α [−]/CD11b⁺ DC appear to preferentially induce Th2 responses (24, 25). This relationship also appears to hold true for pulmonary DC, because they are largely CD8 α [−] (21, 22) and are associated with preferential induction of Th2 responses (20, 26). However, pulmonary DC can be induced to elicit Th1 responses (20) or tolerance (3), so their influence on the immune response may be shaped by the context in which they acquire and/or present Ag. Additionally, or alternatively, the immune response to airborne Ags may be influenced by other DC populations such as plasmacytoid DC (pDC) in the lungs (27) and CD8 α ⁺ DC in the DLN (28).

To better understand the regulation of the immune response to airborne allergens, and in particular, define the role of specific DC

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⁴ Abbreviations used in this paper: DLN, draining lymph node; DC, dendritic cell; p.n., per nasal; CT, cholera toxin; PTLN, parathymic lymph node; pDC, plasmacytoid DC; BAL, bronchoalveolar lavage.

populations, we developed a mouse model where we could compare the fate of per nasal (p.n.) Ag with primary T cell activation and cytokine priming *in vivo*. We selected OVA to serve as a model protein allergen, and used two distinct mucosal adjuvants, *Escherichia coli* LPS and cholera toxin (CT), to differentially induce Th1 (29–31) and Th2 (32–35) responses, respectively. Fluorescent conjugates of OVA were used to identify the cell populations involved in allergen uptake and presentation in the DLN, while the primary T cell response was visualized using adoptive transfer of OVA-specific CD4⁺ T cells from DO11.10 mice. Our results show that, whether OVA was administered on its own or with either adjuvant, a population of migratory CD8 α^{low} CD205⁺ DC was largely responsible for delivering and presenting OVA in the DLN, leading to T cell activation, cytokine priming, clonal expansion, and memory T cell generation. CT and LPS improved the potency of OVA presentation by up-regulating surface levels of CD80 and CD86 on OVA⁺CD8 α^{low} DC in the DLN, and had similar effects on T cell activation, clonal expansion, memory cell generation, and cytokine priming. Despite the lack of any evidence of a Th2 bias during the primary response, DO11.10 cell recipients immunized with CT developed lung eosinophilia upon secondary OVA challenge, suggesting the presence of strong Th2-mediated immunity. In contrast, DO11.10 cell recipients immunized with LPS never developed lung eosinophilia upon secondary OVA challenge. Thus, these data suggest there is no association between Ag presentation by CD8 α^{low} DC, an early Th2 bias, and the induction of allergic disease in this model.

Materials and Methods

Mice

BALB/c mice were bred specific pathogen-free at the Animal Resource Centre (Perth, Australia) and housed under clean conditions at the Telethon Institute for Child Health Research (TICHR). DO11.10 TCR transgenic mice were purchased from The Jackson Laboratory and bred under clean conditions at the TICHR. All mice used in experiments were females aged 8–12 wk. The TICHR Animal Experimentation Ethics Committee, operating under guidelines set by the National Health and Medical Research Council of Australia, approved all animal experiments.

CFSE labeling and adoptive transfer

Pooled lymph node cells from DO11.10 mice were prepared and made up to 2.5×10^7 /ml in prewarmed GKN (11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na₂HPO₄, 5.5 mM NaH₂PO₄ · 2 H₂O) supplemented with 0.2% BSA. CFSE (Molecular Probes) was added to a final concentration of 10 μ M and incubated for 10 min at 37°C before the labeling reaction was stopped by adding at least 5 volumes of ice-cold GKN-5% FCS. The cells were subsequently washed twice in GKN-0.2% BSA, then twice in PBS, before injecting i.v. $5\text{--}10 \times 10^6$ cells/recipient in 200 μ l of PBS.

Ag and immunization

Chicken egg OVA (Sigma-Aldrich) was prepared in PBS or endotoxin-free saline and run over a polymyxin B column (AffinityPak Detoxi-Gel Column; Pierce) to remove LPS. Stocks (10 mg/ml) were prepared and stored at -20°C . OVA peptide 323–339 (ISQAVHAHAHAEINEAGR) (Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg) was synthesized by Proteomics International and stored at 2 mg/ml after passage over a Pierce AffinityPak Detoxi-Gel Column. Mice were routinely immunized via the nasal route 2–3 days after adoptive transfer, receiving 100 μ g of OVA in 50 μ l of saline (endotoxin-free; AstraZeneca), with or without 1 μ g of CT (Sigma-Aldrich) or 10 μ g of *E. coli* LPS (Sigma-Aldrich), after gradual application to the nares during Halothane anesthesia. We refer to this mode of administration as p.n. to distinguish it from intranasal immunization, because the volume administered cannot be contained in the nasal passages and penetrates the lungs. OVA uptake was tracked *in vivo* using Alexa 488 or Alexa 648 conjugates (Molecular Probes) and OVA processing was tracked using DQ OVA (Molecular Probes). All fluorescent species were prepared in endotoxin-free saline before p.n. administration and 50 μ g was routinely administered in 50 μ l.

Lymph node, main conducting airway, and lung cell preparation

Pooled DLN (consisting parathymic and posterior mediastinal nodes) or non-DLN (inguinal) were collected after mice were killed by CO₂ asphyxiation. The nodes were sliced with a scalpel before grinding between frosted slides to prepare a single-cell suspension for T cell studies. Alternatively, when nodes were collected for DC studies, they were sliced with a scalpel then digested with type IV collagenase (1.5–2 mg/ml; Worthington) and pancreatic type I DNase (0.1 mg/ml; Sigma-Aldrich) in GKN-10% FCS at 37°C for 30 min in a shaking water bath. Lungs and main conducting airways were collected and digested to prepare single-cell suspensions as described previously (21). Briefly, the mice were killed by 100 μ l of phenobarbitone sodium (Lethobarb; Virbec) injected i.p., then the lungs were perfused with at least 5 ml of PBS before the lungs and main conducting airways were collected. Lung tissue was sliced with a McIlwain tissue chopper (Mickle Laboratory Engineering) and the main conducting airways were sliced with a scalpel. The sliced tissue was then digested with type IV collagenase (1.5–2 mg/ml; Worthington) and pancreatic type I DNase (0.1 mg/ml; Sigma-Aldrich) at 37°C for 90 min in a shaking water bath. A second dose of DNase (0.1 mg/ml) was added after 60 min to further reduce tissue clumping. Digested tissue preparations were routinely filtered through cotton wool to remove debris and RBC were lysed by addition of 0.83% ammonium chloride (pH 7.2).

Flow cytometry

OVA-specific CD4⁺ cells were detected in lymph node and lung cell preparations with mAbs against the transgenic TCR (KJ1-26; Caltag Laboratories), CD4 (RM4-5; BD Biosciences), and biotinylated or directly conjugated activation/memory/adhesion markers (CD25-PC61, CD69-H1.2F3, CD49d, CD44-IM7, CD62L-MEL-14, CD103-M290; BD Biosciences) and incubated for 30 min on ice. CD62P binding was measured using a human Ig-fusion protein (BD Biosciences) and biotinylated F(ab')₂ goat anti-human IgG (The Jackson Laboratory). Biotinylated Abs were detected using streptavidin FITC, PE, PerCP, CyChrome, or allophycocyanin (BD Biosciences). APCs were detected using a combination of mAbs against I-A/I-E (2G9 or M5/114.15.2; BD Biosciences), CD11c (HL3 (BD Biosciences) or N418 (BioLegend)), and CD11b (M1/70; BD Biosciences), CD8 α (53-6.7; BD Biosciences), CD205 (DEC-205; Cedarlane Laboratories), CD19 (1D3; BD Biosciences), B220 (RA3-6B2; BD Biosciences), Ly6G/C (RB6-8C5; BD Biosciences), or 120G8 (provided by Drs G. Trinchieri and C. Asselin-Paturel, Schering-Plough, Dardilly, France (36)). Costimulatory molecule expression was assessed using biotinylated mAbs against CD40, CD80, or CD86 (clones 3/23, 16-10A1, and GL1, respectively; BD Biosciences). FcR were routinely blocked using 2.4G2 (BD Biosciences) for 10 min before the addition of phenotyping mAbs. Samples were collected using a four-color FACSCalibur (BD Immunocytometry Systems) and analyzed using FlowJo software (Tree Star).

Cell sorting and T cell stimulation assay

Pooled draining lymph nodes were collected 24 h after p.n. administration of 50 μ g of OVA-Alexa Fluor 488 and digested to yield a single-cell suspension for cell sorting. CD8 α^{low} and CD8 α^{high} DC were sorted on the basis of I-A/I-E and CD11c expression (see Fig. 1B for gates used) using an Epics Elite flow cytometer (Coulter). CD4⁺ T cells were enriched (>95% purity) from the lymph nodes of DO11.10 mice by negative selection using purified rat mAbs against class II MHC (TIB120, lab tissue culture stocks) and CD8 α (53-6.7; BD Biosciences) and sheep anti-rat IgG Dynabeads (Dyna). Cells were prepared in RPMI 1640 with glutamine (Invitrogen Life Technologies) supplemented with 10% FCS, 20 μ g/ml gentamicin, and 20 μ M 2-ME, then 1×10^4 DC were cultured with 1×10^5 T cells in round-bottom 96-well plates in triplicate for 48 h, then pulsed with [³H]thymidine for another 24 h to measure T cell proliferation.

Intracellular cytokine detection

Lung and lymph node cells were prepared in RPMI 1640 plus 5% FCS and cultured 10^7 /ml for 5 h with 10 μ g/ml OVA 323–339 in sterile 12 × 75-mm tubes (Falcon). Brefeldin A (250 ng/ml; Sigma-Aldrich) was added after 1 h. At the end of the culture period, the cells were incubated with mAbs against CD4 and KJ1-26 for 30 min on ice before fixation in formaldehyde (BDH; 0.4% in PBS) for 30 min overnight at 4°C. Cells were washed and briefly incubated in perm wash (0.1% saponin (Sigma-Aldrich), 0.5% BSA, in PBS) before mAbs against IL-2-PE (JES6-5H4; BD Biosciences), IL-4-PE (11B11; BD Biosciences), IL-5-PE (TRFK5; Caltag Laboratories), or IFN- γ -allophycocyanin (XMG1.2; BD Biosciences) were added for 30 min. Cytokine staining was judged by comparison with controls cultured without peptide.

Secondary OVA challenge and bronchoalveolar lavage (BAL)

Secondary OVA challenge was performed with 25 μ g of OVA in 50 μ l of saline p.n. on days 14, 15, 16, 19, and 20 postimmunization. The mice were sacrificed 48 h after the fifth challenge and BAL was performed with 1 ml of PBS/0.2% BSA. Cells were deposited on glass slides by cytospin and stained with DiffQuik (Lab Aids). Differential counts were performed on 300 cells for each slide.

Statistics

The bulk of the experiments presented in this study were typically conducted using pooled tissue from three or more animals, and thus, the experiments were repeated two or more times to gauge reproducibility of the results. Where applicable, we have reported the values or the fold change observed in duplicate experiments. The results for the number of eosinophils in BAL after OVA challenge (see Fig. 9A) were pooled from five experiments and compared using a two-tailed Mann-Whitney *U* test provided in Prism software (GraphPad Software). Values of *p* < 0.05 were considered significant.

Results

CD8 α^{low} DC acquire Ag after p.n. immunization

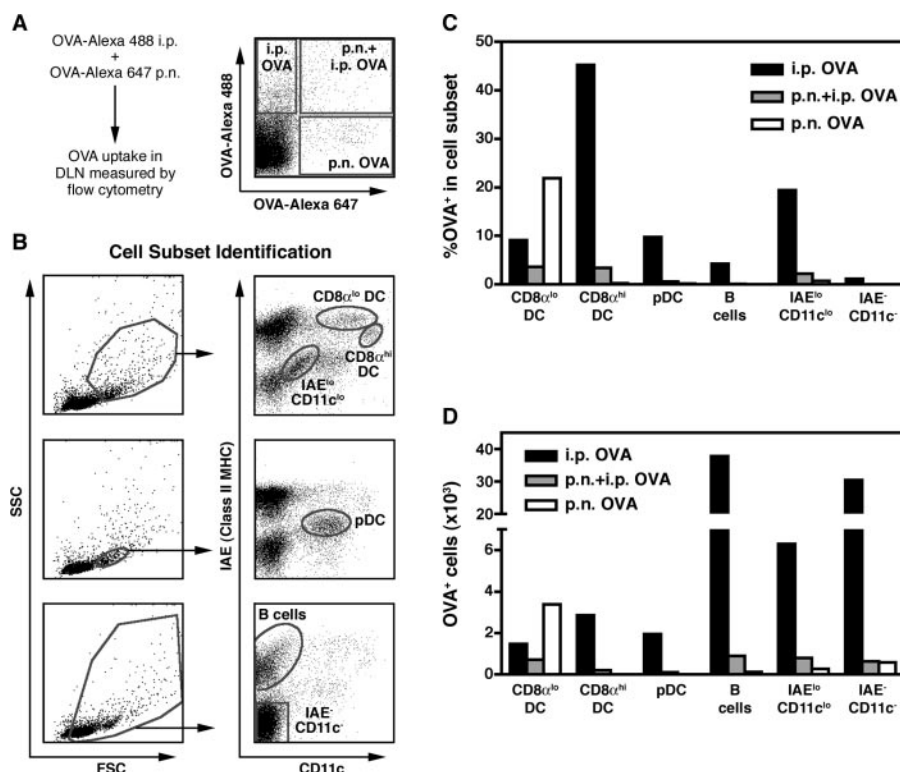
Vermaelen et al. (22) originally used intratracheal instillation of 700 μ g of FITC-conjugated OVA to identify the murine pulmonary DC population that delivers inhaled Ag to the DLN. We wanted to repeat this experiment using a lower dose of OVA and determine whether these findings applied equally to p.n. administration. We also modified our approach to include two fluorescent OVA conjugates so that we could differentiate passive Ag delivery from cell-assisted delivery. Thus, 50 μ g of OVA-Alexa Fluor 647 was delivered to BALB/c mice in 50 μ l of saline p.n.; at the same time, 50 μ g of OVA-Alexa Fluor 488 was administered in 200 μ l of saline i.p. Because the peritoneal cavity and the airways are drained by a common set of nodes, namely the parathymic lymph nodes (PTLN), we were able to differentiate cells exposed to Ag via the different routes by comparing OVA-Alexa Fluor 488⁺ (i.p. OVA⁺) cells with OVA-Alexa Fluor 647⁺ cells (p.n. OVA⁺) (Fig. 1A).

Preliminary experiments revealed that OVA uptake peaked between 24 and 48 h following p.n. immunization (data not shown), so we collected the PTLN 36 h after simultaneous p.n. and i.p. immunization. OVA was detected in at least six cell subsets based on variations in forward scatter, side scatter, and class II MHC (hereafter referred to as IAE) and CD11c expression, as detailed in Fig. 1B. Four of these subsets were identified as B cells (B220⁺, CD19⁺), CD8 α^{low} DC (hereafter referred to as CD8 α^{low} DC), CD8 α^{high} DC, and pDC (B220⁺, Ly6G/C⁺, 120G8⁺) by subsequent phenotyping experiments (Fig. 2C and data not shown), while the other two (IAE^{low}CD11c^{low}, IAE^{low}CD11c⁺) could not be identified as comprising a single or known cell type. All together, these six subsets accounted for 75% of p.n. OVA⁺ cells and 50% of i.p. OVA⁺ cells recovered from the PTLN 36 h after immunization, as shown in Table I.

All six of the subsets described above acquired i.p. OVA 36 h after immunization, as shown in Fig. 1C. There were differences in the proportion of i.p. OVA⁺ cells among each subset (Fig. 1C), the total number of i.p. OVA⁺ cells (Fig. 1D), and to some extent, the amount of OVA incorporated (data not shown). More than 40% of CD8 α^{high} DC were i.p. OVA⁺ compared with <10% of B cells (Fig. 1C), yet there were 10-fold more i.p. OVA⁺ B cells than i.p. OVA⁺CD8 α^{high} DC (Fig. 1D) due to the vast difference in the total number of B cells and CD8 α^{high} DC recovered from the PTLN. A smaller proportion of CD8 α^{low} DC and pDC acquired i.p. OVA (Fig. 1C), while the largest number of i.p. OVA⁺ cells were contained in the IAE^{low}CD11c⁺ subset (Fig. 1D).

Compared with the number of i.p. OVA⁺ cells, there was a considerably smaller number of p.n. OVA⁺ cells in the DLN (Fig. 1D). Most of the p.n. OVA⁺ cells were CD8 α^{low} DC, though a small proportion (<1%) of CD8 α^{high} DC, pDC, and IAE^{low}CD11c^{low} cells acquired p.n. OVA (Fig. 1C). However, most of the p.n. OVA⁺ CD8 α^{high} DC, pDC, and IAE^{low}CD11c^{low} cells were also i.p. OVA⁺ (Fig. 1C), suggesting they were located in the PTLN when they acquired p.n. OVA. In contrast, only a

FIGURE 1. Identification of cells that acquire p.n. OVA in the PTLN. **A**, Thirty-six hours after simultaneous administration of 50 μ g of OVA-Alexa Fluor 647 p.n. and 50 μ g of OVA-Alexa Fluor 488 i.p., three OVA⁺ populations were detected in the PTLN by flow cytometry, representing OVA acquired via the i.p. route (i.p. OVA⁺), the p.n. route (p.n. OVA⁺), or both routes (p.n. + i.p. OVA⁺). **B**, p.n. and i.p. OVA was associated with at least six cell subsets in the PTLN, identified on the basis of cell size, side scatter, IAE (class II MHC), and CD11c expression. **C**, Using these gates, the uptake of i.p. OVA, p.n. + i.p. OVA, and p.n. OVA was determined for each of the six cell subsets, along with **D** the total number of i.p. OVA⁺, p.n. + i.p. OVA⁺, and p.n. OVA⁺ cells. Results were collected from one experiment using DLN pooled from five mice. The gates used to classify i.p. OVA⁺, p.n. + i.p. OVA⁺, and p.n. OVA⁺ cells were set using negative controls for each OVA conjugate. The values plotted in **D** were derived from a total number for the pool of mice used and divided by the number in the pool to yield a per mouse equivalent.



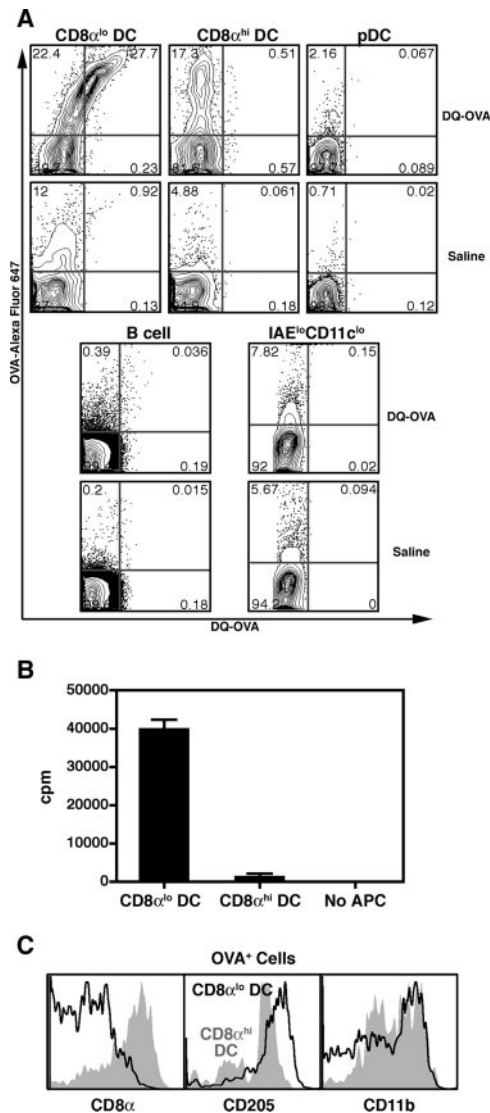


FIGURE 2. CD8 α^{low} DC are the major Ag-processing population in the DLN. **A**, DQ OVA was used to identify cell subsets capable of processing OVA in the DLN pooled from five mice 24 h after p.n. administration of 50 μ g of OVA-Alexa Fluor 647. OVA processing was identified on the basis of an increase in green fluorescence when compared with DLN from mice immunized with OVA-Alexa Fluor 647 alone (Saline). CD8 α^{low} DC, CD8 α^{high} DC, pDC, B cells, and IAE ^{low} CD11c ^{low} cells were investigated using the gating strategy shown in Fig. 1B. OVA-Alexa Fluor 647 uptake was determined by comparison with cells from the inguinal lymph nodes. Results are shown for one of two experiments, with <10% variation between experiments. **B**, A group of 10 mice were administered 50 μ g of OVA-Alexa Fluor 488 p.n., and 24 h later, the DLN were collected and pooled for cell sorting. CD8 α^{low} and CD8 α^{high} DC were purified using the gating strategy shown in Fig. 1B and cultured with purified CD4 $^{+}$ T cells from DO11.10 mice for 72 h without any exogenous Ag. T cell proliferation was measured by thymidine incorporation and compared with T cells cultured alone. Results are presented for one experiment of three performed, with similar results in each experiment. CD8 α^{low} DC were ~45% OVA $^{+}$ and CD8 α^{high} DC were ~15% OVA $^{+}$ on the basis of postsort analysis for the experiment shown. **C**, Phenotypic analysis of OVA $^{+}$ DC was conducted 24 h after p.n. administration of 50 μ g of OVA-Alexa Fluor 488. DLN were collected from five mice, pooled, and CD8 α^{low} and CD8 α^{high} DC were identified using the gating strategy shown in Fig. 1B. The expression of CD8 α (left panel), CD205 (middle panel), and CD11b (right panel) was determined for OVA $^{+}$ CD8 α^{low} DC (open histograms) and OVA $^{+}$ CD8 α^{high} DC (filled histograms). Results are presented for one of three experiments performed, with similar results in each experiment.

small proportion (<15%) of p.n. OVA $^{+}$ CD8 α^{low} DC were i.p. OVA $^{+}$ (Fig. 1C), suggesting most of these cells had acquired p.n. OVA outside the PTLN. Thus, these results identify CD8 α^{low} DC as the major, if not sole, cell type that delivers OVA to the DLN after p.n. immunization, in agreement with the earlier studies of Vermaelen et al. (22). However, our results also suggest that up to one-third of the OVA $^{+}$ cells in the DLN may have acquired OVA passively (Table I), presumably from the draining lymph.

CD8 α^{low} DC exhibit ongoing Ag processing in the DLN

Having identified the range of cell types that could acquire OVA in the DLN after p.n. immunization, we used a novel Ag-tracking strategy to determine whether any of these cells were capable of processing OVA for presentation to T cells. DQ OVA (Molecular Probes) is prepared by heavily labeling OVA with BODIPY FL dye to the point where the fluorescence is quenched; upon proteolytic cleavage, the OVA fragments begin to fluoresce as the intramolecular quenching is relieved, and cells containing OVA fragments can be identified by flow cytometry. To control for variations in OVA uptake between the different cell types, we combined DQ OVA with OVA-Alexa Fluor 647 and delivered 50 μ g of each conjugate in 50 μ l of saline p.n. and collected the DLN 24 h later. The cells were stained for class II MHC and CD11c expression and five subsets (CD8 α^{low} DC, CD8 α^{high} DC, pDC, IAE ^{low} CD11c ^{low} cells, B cells) were assessed for OVA processing in comparison with mice immunized with OVA-Alexa Fluor 647 alone.

As shown in Fig. 2A, all five subsets acquired OVA-Alexa Fluor 647 and they could be ranked according to amount of OVA uptake: CD8 α^{low} DC > CD8 α^{high} DC > IAE ^{low} CD11c ^{low} > pDC > B cells. CD8 α^{low} DC were the only cells to contain any significant amount of processed DQ OVA, where 50% or more OVA-Alexa Fluor 647 $^{+}$ cells contained DQ OVA fragments. Small amounts of DQ OVA fragments were detected in CD8 α^{high} DC, and B cells, indicating a potential for processing that was perhaps limited by the amount of OVA acquired. To test the relationship between DQ OVA processing and OVA presentation, we sorted CD8 α^{low} DC and CD8 α^{high} DC from the DLN 24 h after immunization with OVA and incubated them with purified CD4 $^{+}$ T cells from DO11.10 mice. In this experiment, no OVA was added to the cultures so any T cell proliferation could be directly attributed to the amount of OVA that had been processed for presentation by the sorted DC population. CD8 α^{low} DC were able to stimulate large amounts of T cell proliferation, while CD8 α^{high} DC could only stimulate a small amount of proliferation (Fig. 2B). The difference in proliferation was far greater than the difference in OVA uptake by the two DC populations (which was 3-fold in this experiment, data not shown), but correlated well with the amount of DQ OVA fragments detected. Thus, CD8 α^{low} DC appear to be the most important cell population to acquire, deliver, and present airborne OVA in the DLN.

Phenotype of OVA $^{+}$ DC entering the DLN

To better classify the two OVA $^{+}$ DC populations, we compared the expression of CD8 α , CD205, and CD11b on OVA $^{+}$ CD8 α^{low} and OVA $^{+}$ CD8 α^{high} DC. Fig. 2C shows that OVA $^{+}$ CD8 α^{low} DC expressed high levels of CD205 and could be split into two populations on the basis of CD11b. OVA $^{+}$ CD8 α^{high} DC also expressed CD205 and could also be split into two populations on the basis of CD11b expression (Fig. 2C). Based on these results, the OVA $^{+}$ CD8 α^{low} CD11b $^{+}$ DC can be classified as interstitial DC and the OVA $^{+}$ CD8 α^{low} CD11b $^{-/low}$ DC as epithelium-derived DC (CD11b $^{-/low}$) (37, 38). Among the OVA $^{+}$ CD8 α^{high} DC, the

Table I. Recovery of administered OVA conjugates in described cell subsets

	p.n. OVA ^a	i.p. OVA ^a	p.n. + i.p. OVA ^a
Total OVA ⁺ cells ^b	4.83×10^3	157×10^3	5.06×10^3
OVA ⁺ cells in subsets ^c	4.38×10^3	80.8×10^3	3.24×10^3
% OVA ⁺ cells described by subsets	90.7	51.5	64.0

^a Mice were simultaneously administered 50 μ g of OVA-Alexa Fluor 647 p.n. and 50 μ g of OVA-Alexa Fluor 488 i.p., and three patterns of OVA uptake were detected in the PTLN 36 h later, as shown in Fig. 1A.

^b The total number of cells associated with each pattern of OVA uptake was calculated for the pool of mice used and divided by the number in the pool to yield a per mouse equivalent.

^c The total number of CD8 α^{low} DC, CD8 α^{high} DC, pDC, B cells, IAE $^{\text{low}}$ CD11c $^{\text{low}}$, and IAE $^{\text{low}}$ CD11c $^{\text{low}}$ cells associated with each pattern of OVA uptake was calculated for the pool of mice used, summed, then divided by the number in the pool to yield a per mouse equivalent.

CD11b $^+$ cells can be classified as lymphoid DC (38), while the CD11b $^+$ cells have not been previously classified.

LPS increases the number of OVA⁺ APCs in the DLN

Having identified the cell subsets that could acquire p.n. OVA, we immunized BALB/c mice with 50 μ g of OVA-Alexa Fluor 647 and compared the effects of 10 μ g of *E. coli* LPS or 1 μ g of CT on the number of OVA⁺ cells in the DLN at 4 and 24 h. Fig. 3 shows that compared with OVA alone, LPS induced a large increase (2.1-, 8.1-, and 8.6-fold in three experiments) in the number of OVA⁺CD8 α^{low} DC at 24 h, as well as smaller increases in OVA⁺ B cells and pDC (1.5-, 3.0-, and 7.7-fold for B cells, and 1.3-, 3.4-, and 12.9-fold for pDC in three experiments). All of these increases could be attributed to migration of OVA⁺ cells into the DLN, because LPS had no effect on the number of OVA⁺ cells

at 4 h. Similarly, CT induced a small increase (1.1-, 3.5-, and 3.5-fold in three experiments) in the number of OVA⁺ B cells at 24 h, while CD8 α^{high} DC and IAE $^{\text{low}}$ CD11c $^{\text{low}}$ cells were not affected by either adjuvant.

LPS and CT increase CD80 and CD86 expression on CD8 α^{low} DC

To further investigate the effect of LPS and CT on Ag presentation, we measured the expression of CD40, CD80, and CD86 on OVA⁺CD8 α^{low} DC (as identified by OVA-Alexa Fluor 488) in the DLN 24 h after p.n. immunization. Fig. 4, A and B, shows that, compared with immunization with OVA alone, both CT and LPS stimulated an increase in CD80 and CD86 expression on CD8 α^{low} DC. LPS appeared to be more potent than CT, inducing larger increases in CD80 (LPS: 2.8- and 3.4-fold in duplicate experiments vs CT: 0.9- and 2.3-fold in duplicate experiments) and CD86 (LPS: 1.9- and 2.3-fold in duplicate experiments vs CT: 1.0- and 1.8-fold in duplicate experiments). Interestingly, CT induced down-regulation of CD40 expression (0.3- and 0.5-fold in duplicate experiments), while LPS had no effect on CD40 levels. The effect of CT was largely confined to OVA⁺CD8 α^{low} DC, because there was little or no change in the levels of CD40, CD80, and CD86 on OVA $^-$ CD8 α^{low} DC (Fig. 4C). Similarly, there was little or no change in CD40 and CD80 expression on OVA $^-$ CD8 α^{low} DC after immunization with LPS (Fig. 4D); however, there was an increase in CD86 expression, presumably as part of a general effect of LPS on pulmonary DC maturation and migration to the DLN. Importantly, CT and LPS did not influence CD40, CD80, and CD86 expression on OVA⁺CD8 α^{high} DC (Fig. 4, E and F, respectively).

LPS and CT increase OVA-specific CD4⁺ T cell activation in the DLN

The results from the experiments presented above indicated that LPS and CT both enhanced the potency of OVA presentation in the DLN by increasing the number of OVA⁺CD8 α^{low} DC and/or up-regulating expression of CD80 and CD86. To test this hypothesis, we used adoptive transfer of CFSE-labeled OVA-specific CD4⁺ T cells from DO11.10 mice to characterize the effect of 10 μ g of LPS and 1 μ g of CT on the T cell response to p.n. administration of 100 μ g of OVA.

When BALB/c recipients were administered OVA alone, around 10% of transferred OVA-specific CD4⁺ T cells (identified by expression of the transgenic TCR, hereafter referred to as CD4⁺TgTCR⁺ cells) in the DLN expressed CD25 at 36 h, compared with 7–15% and 13–25% when CT and LPS were added, respectively (Fig. 5A, left panels). Thus, LPS, and to a lesser extent CT, improved T cell activation in the DLN. LPS also stimulated an early increase T cell proliferation, seen as an increase in the proportion of CFSE $^{\text{low}}$ cells (Fig. 5A, right panels). The early increase

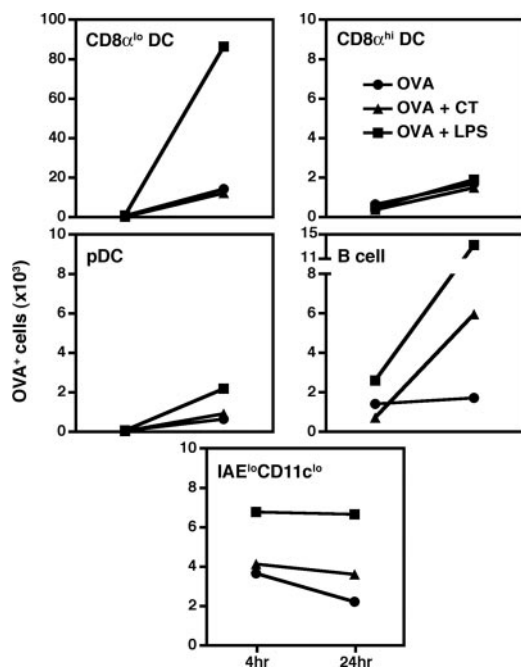


FIGURE 3. Influence of LPS and CT on OVA passage to the DLN. Three groups of 10 mice received 50 μ g of Alexa-Fluor 647 p.n., with or without 10 μ g of *E. coli* LPS or 1 μ g of CT, in 50 μ l of saline. At 4 and 24 h postadministration, DLN were collected from five mice in each group, pooled, and prepared for flow cytometry. OVA uptake was determined for CD8 α^{low} DC, CD8 α^{high} DC, pDC, B cells, and IAE $^{\text{low}}$ CD11c $^{\text{low}}$ cells after comparison with mice administered saline. The total number of OVA⁺ cells in each cell subset was calculated for the pool in each group then divided by the number in the pool to yield a per mouse equivalent. Results are shown for one of five experiments performed. See the text for detail on the range of increases stimulated by CT and LPS. Note the difference in scale used to present the results for CD8 α^{low} DC.

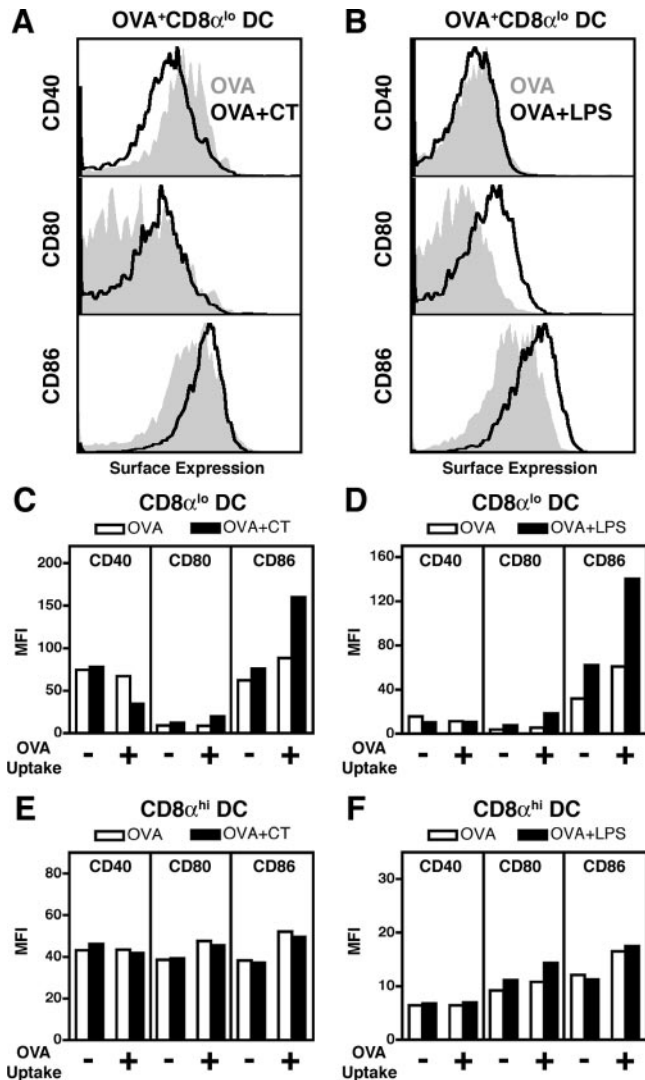


FIGURE 4. CT and LPS up-regulate CD80 and CD86 expression on CD8 α^{low} DC. Mice were administered 50 μg of OVA-Alexa Fluor 488 p.n., with or without 1 μg of CT (A, C, and E) or 10 μg of *E. coli* LPS (B, D, and F), in 50 μl of saline. Twenty-four hours later, the DLN were collected from three to five mice, pooled, and prepared for flow cytometry. A and B, CD8 α^{low} DC were identified as shown in Fig. 1B, gated on OVA $^{+}$ cells after comparison with a saline control, and the surface expression of CD40 (upper panels), CD80 (middle panels), and CD86 (lower panels) was determined. The gray histograms represent mice that received OVA-Alexa Fluor 488 alone, the dark outline represent mice that received OVA-Alexa Fluor 488 with CT (A) or LPS (B). C and D, The geometric means for CD40, CD80, and CD86 are plotted for OVA $^{-}$ and OVA $^{+}$ CD8 α^{low} DC from DLN of mice that received OVA-Alexa Fluor 488 with CT (C) or LPS (D) (■) compared with mice that received OVA-Alexa Fluor 488 alone (□). E and F, CD8 α^{high} DC were identified as shown Fig. 1B, and the geometric means for CD40, CD80, and CD86 were determined for OVA $^{-}$ and OVA $^{+}$ CD8 α^{high} DC from DLN of mice that received OVA-Alexa Fluor 488 with CT (E) or LPS (F) (■) compared with mice that received OVA-Alexa Fluor 488 alone (□). Results are shown for two experiments (one each to measure the effects of CT and LPS) of four performed. See the text for detail on the range of up-regulation stimulated by CT and LPS. The results for CT and LPS cannot be directly compared because they were measured in separate experiments.

in T cell activation was translated into an increase in clonal expansion on day 3, where there were up to 3-fold more CD4 $^{+}$ TgTCR $^{+}$ cells in the DLN of mice administered CT or LPS (Fig. 5B). There were no further increases in any of the groups on

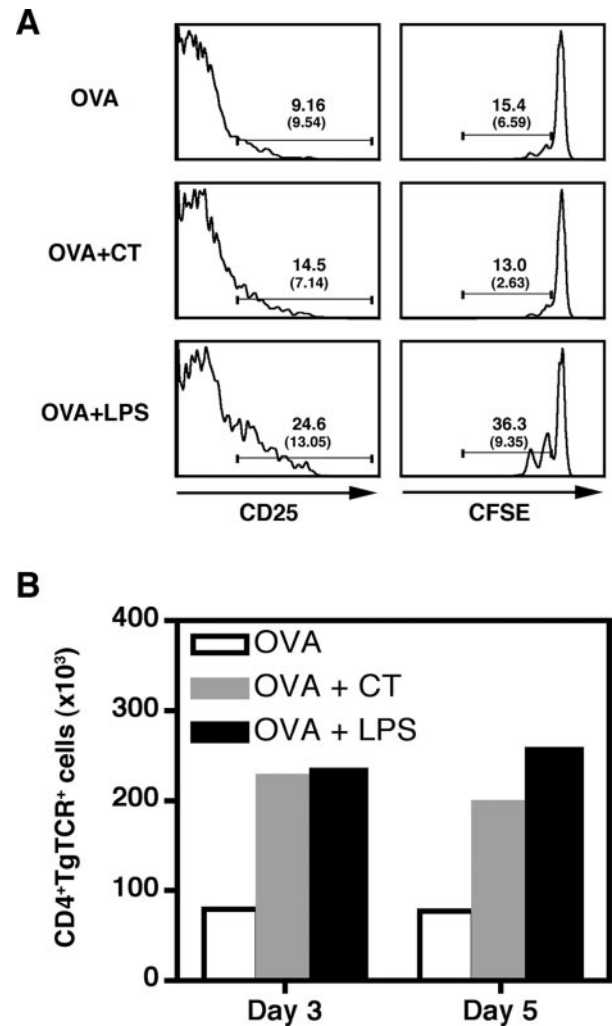


FIGURE 5. CT and LPS enhance T cell activation and clonal expansion in the DLN. Three groups of two to five mice were administered 100 μg of OVA p.n., with or without 1 μg of CT or 10 μg of *E. coli* LPS, in 50 μl of saline at least 2 days after adoptive transfer of CFSE-labeled lymph nodes cells from DO11.10 mice. A, T cell activation (left panels) and proliferation (right panels) was determined in the DLN 36 h after OVA administration by gating on CD4 $^{+}$ cells expressing the transgenic TCR. Results shown in parentheses were obtained in a duplicate experiment. B, The number of CD4 $^{+}$ cells expressing the transgenic TCR (CD4 $^{+}$ TgTCR $^{+}$ cells) was enumerated 3 and 5 days after OVA administration. Results are shown for one of four experiments performed. Compared with mice administered OVA alone, CT stimulated a 2.2- and 2.9-fold increase in CD4 $^{+}$ TgTCR $^{+}$ numbers on day 3, while LPS stimulated a 2.9- and 3.1-fold increase in duplicate experiments. The number of CD4 $^{+}$ TgTCR $^{+}$ cells plotted was derived from the total number calculated for the pool of mice and divided by the number in the pool to yield a per mouse equivalent.

day 5 (Fig. 5B), suggesting that the adjuvants did not significantly prolong the phase of clonal expansion in the DLN.

Both LPS and CT enhance IL-4- and IFN- γ -production by OVA-specific CD4 $^{+}$ T cells

We used intracellular cytokine detection after 5 h restimulation with OVA peptide in vitro to determine the proportion of CD4 $^{+}$ TgTCR $^{+}$ cells that were primed to produce IL-4 and IFN- γ in the DLN 36 and 72 h after OVA administration (Fig. 6). We consistently observed an early and short-lived peak of IL-4 and IFN- γ priming around 36 h after immunization with OVA (Fig. 6A, upper panels) that diminished with clonal expansion (Fig. 6B).

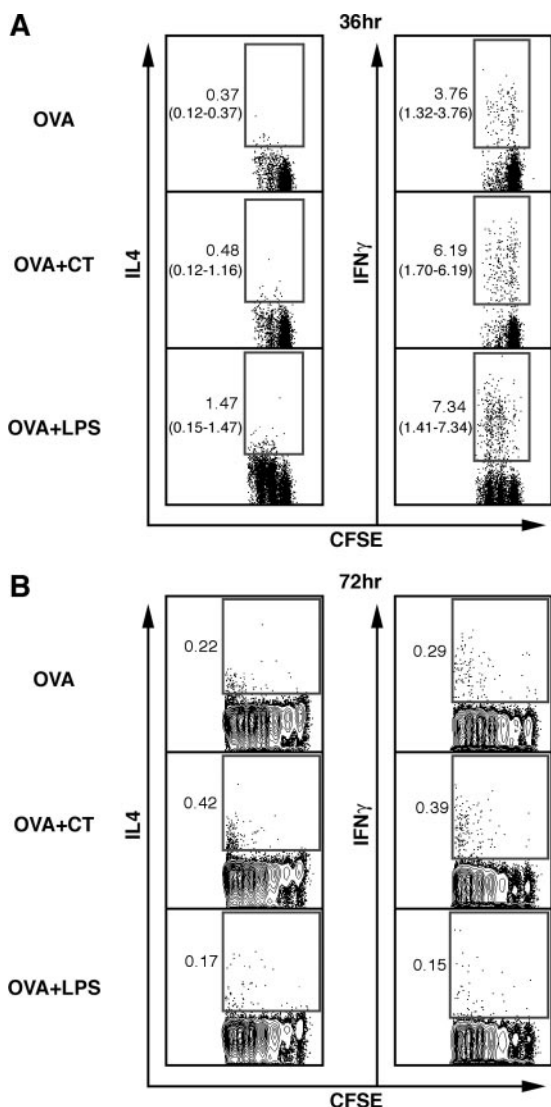


FIGURE 6. CT and LPS enhance cytokine priming in the DLN. Three groups of mice were administered 100 μ g of OVA p.n., with or without 1 μ g of CT or 10 μ g of *E. coli* LPS, in 50 μ l of saline at least 2 days after adoptive transfer of CFSE-labeled lymph nodes cells from DO11.10 mice. The DLN were harvested from five mice in each group 36 h (A) or 72 h (B) after OVA administration, pooled, and restimulated with OVA peptide for 5 h in vitro. Brefeldin A was added for the last 4 h of culture to facilitate intracellular cytokine detection. Cells were gated on CD4⁺ cells expressing the transgenic TCR and IL-4 (left panels) and IFN- γ (right panels) production were determined after comparison with unstimulated controls. Results are shown for two of four experiments performed. The values shown in parentheses in A represent the range of values collected from four duplicate experiments. There was no significant difference between any of the groups.

Up to 10-fold more CD4⁺TgTCR⁺ cells were primed to produce IFN- γ than IL-4 (Fig. 6A, upper panels), and dual staining revealed very few cells that were able to simultaneously produce both cytokines (data not shown). LPS, and to a lesser extent CT, appeared to increase IL-4 and IFN- γ priming at 36 h (Fig. 6A, middle and lower panels), though these increases were not statistically significant. Neither adjuvant acted to prolong cytokine-priming in the DLN (Fig. 6B, middle and lower panels) nor did they affect the ratio of IL-4- to IFN- γ -producing cells.

OVA-specific CD4⁺ T cells enter the lungs after activation in the DLN

Having characterized the early T cell response in the DLN, we went on to investigate the lungs for evidence of a tissue response. Two days after OVA administration, we detected a small population of transferred CD4⁺TgTCR⁺ cells (Fig. 7A), but there was no evidence of activation or proliferation despite the presence of large amounts of OVA (data not shown). By day 3, however, the number of CD4⁺TgTCR⁺ cells increased dramatically, coinciding with the appearance of a population of CD4⁺TgTCR⁺ cells that had divided several times and contained a small proportion of cells primed to produce IL-5 or IFN- γ (Fig. 7B, upper panels). CT and LPS both increased the number of CD4⁺TgTCR⁺ cells (Fig. 7A)

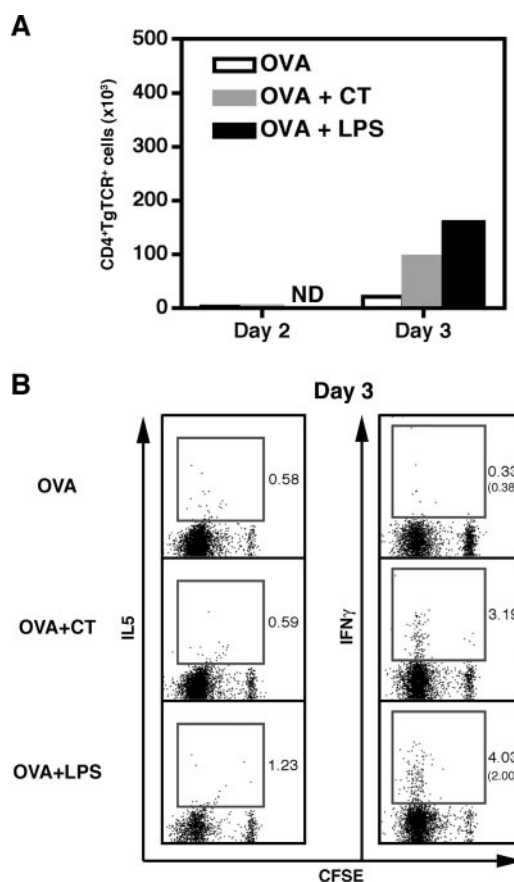


FIGURE 7. CT and LPS increase T cell numbers and cytokine priming in the lung. Three groups of mice were administered 100 μ g of OVA p.n., with or without 1 μ g of CT or 10 μ g of *E. coli* LPS, in 50 μ l of saline at least 2 days after adoptive transfer of CFSE-labeled lymph nodes cells from DO11.10 mice. The lungs were collected from two mice in each group, pooled, and prepared for flow cytometry and restimulation in vitro. A, The number of CD4⁺ cells expressing the transgenic TCR (CD4⁺TgTCR⁺ cells) was determined 2 and 3 days after OVA administration. Compared with mice administered OVA alone, CT stimulated a 1.3- and 4.3-fold increase in CD4⁺TgTCR⁺ numbers on day 3, while LPS stimulated a 2.0- and 7.4-fold increase in duplicate experiments. B, Samples from day 3 were restimulated with OVA peptide for 5 h in vitro. Brefeldin A was added for the last 4 h of culture to facilitate intracellular cytokine detection. Cells were gated on CD4⁺ cells expressing the transgenic TCR and IL-5 (left panels) and IFN- γ (right panels) production were determined after comparison with unstimulated controls. Results are shown for two of four experiments performed. The values plotted in A were derived from the total number calculated for the pool of mice and divided by the number in the pool to yield a per mouse equivalent. The values shown in parentheses in B were obtained in a duplicate experiment.

and the proportion of cytokine-producing cells (Fig. 7B, *middle* and *lower panels*) detected in the lungs on day 3 without prolonging the duration of IFN- γ or IL-5 priming (data not shown). These results suggest a T cell population migrates out of the DLN to enter the lungs ~ 3 days after OVA administration, and the size of this population appears to be related to the magnitude of primary T cell response in the DLN.

Because there was evidence of T cell migration into the lungs, we investigated the expression of some adhesion molecules on the divided $CD4^+TgTCR^+$ cells found in the lungs on days 3 and 5 after immunization. We found a large proportion ($>50\%$) of lung $CD4^+TgTCR^+$ cells expressed high levels of CD49d, a much smaller proportion ($<10\%$) expressed CD103 or could bind CD62P (data not shown), and there was no indication that CT or LPS had a differential effect on the expression of these adhesion molecules (data not shown).

Persistence of memory T cells in DLN and lungs

After the peak of clonal expansion on day 3, the number of $CD4^+TgTCR^+$ cells slowly declined (up to 6-fold over 18 days, data not shown) in the DLN and lungs. By day 21, most of the $CD4^+TgTCR^+$ cells remaining in the nondraining inguinal nodes, DLN, main conducting airways (trachea) and lung had divided several times, with the proportion of undivided cells varying between the tissue sites examined (for example, there were fewer undivided cells in the draining nodes compared with inguinal lymph nodes, data not shown). The DLN contained the largest number of $CD4^+TgTCR^+$ cells, though a large number of $CD4^+TgTCR^+$ cells was also found in the lungs of mice administered LPS (Fig. 8A). CT and LPS both increased the number of $CD4^+TgTCR^+$ cells persisting in the host by a factor of 2- to 3-fold (Fig. 8A), of which 80% or more were $CD44^{high}$ and $CD62L^-$ (Fig. 8B). In contrast, only $\sim 20\%$ of $CD4^+TgTCR^+$ cells persisting in the inguinal nodes were $CD44^{high}$ and $CD62L^-$ (Fig. 8B). Thus, p.n. administration of OVA, with or without an adjuvant, generated a persistent population of memory T cells that were concentrated around the site of Ag exposure. CT and LPS both increased the size of the memory T cell pool to a similar degree, though there appeared to be differences in the localization of the memory T cells.

Mice immunized with LPS do not develop eosinophilia upon OVA challenge

To test for the development of allergic disease in our adoptive transfer model, we used a challenge protocol originally developed by Eisenbarth et al. (29). BALB/c mice received DO11.10 lymph node cells in adoptive transfer 2–3 days before p.n. administration of 100 μ g of OVA, with or without 1 μ g of CT or 10 μ g of LPS, then 14 days later, they were challenged with five doses of 25 μ g of OVA in 50 μ l of saline p.n. The first three doses were given 24 h apart before a 2-day break then the final two doses were administered 24 h apart. Forty-eight hours after the final challenge, BAL was performed. In this assay, an increase in the number of eosinophils in the BAL suggested allergic disease.

Fig. 9 shows that the challenge protocol did not elicit eosinophilia in mice immunized with saline alone, whereas mice immunized with OVA and CT consistently developed elevated numbers of eosinophils in BAL fluid. In contrast, mice immunized with OVA and LPS never developed eosinophilia. Interestingly, an eosinophilic response could be elicited in mice immunized with OVA, except it was inconsistent and weaker compared with mice immunized with OVA and CT (Fig. 9). When control experiments were performed in BALB/c mice that did not receive any DO11.10 cells, eosinophilia was never detected in mice immunized with

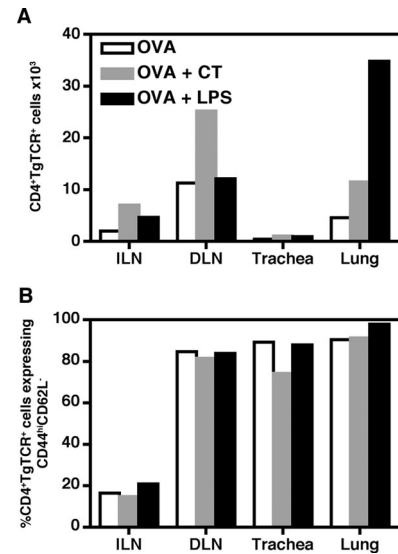


FIGURE 8. CT and LPS increase the number of long-lived memory T cells. Three groups of mice were administered 100 μ g of OVA p.n., with or without 1 μ g of CT or 10 μ g of *E. coli* LPS, in 50 μ l of saline at least 2 days after adoptive transfer of CFSE-labeled lymph nodes cells from DO11.10 mice. A, Twenty-one days after OVA administration, the inguinal lymph nodes (ILN), DLN, main conducting airways (trachea), and lungs were collected from six mice in each group, pooled, and the number of $CD4^+$ cells expressing the transgenic TCR ($CD4^+TgTCR^+$ cells) in each tissue was enumerated. Compared with mice administered OVA alone, CT stimulated a 2.2- and 2.9-fold increase in $CD4^+TgTCR^+$ numbers in the DLN, and a 2.5- and 125-fold increase in the lung in duplicate experiments; LPS stimulated a 1.1- and 1.2-fold increase in the DLN, and a 7.6- and 16-fold increase in the lung in duplicate experiments. B, The proportion of $CD4^+TgTCR^+$ cells that were $CD44^{high}$ and $CD62L^-$ was determined by flow cytometry for each tissue sample. Results are shown for one of two experiments performed, with similar results. The values plotted in A were derived from the total number calculated for the pool of mice and divided by the number in the pool to yield a per mouse equivalent.

OVA alone, while mice immunized with OVA and CT consistently developed eosinophilia (data not shown). Thus, CT promoted the development of allergic disease in our model, presumably via strong a Th2-mediated response, while LPS opposed the development of allergic disease.

Discussion

Over the last decade, a number of murine models have been developed to study allergic disease in the airways (reviewed by Refs. 9 and 10). These models have allowed researchers to systematically examine the role of various cell types and soluble mediators, identify novel therapeutic targets, and test new treatment strategies. Although we have learned a great about how the allergic response proceeds in the airways, we know very little about the events that prime the airways for allergic disease. In this study, we have characterized the first two steps in allergen sensitization via the airways, namely Ag presentation and $CD4^+$ T cell activation. By using two distinct mucosal adjuvants that differ in their ability to prime for allergic disease, we were able to compare the APCs and the T cells involved in allergic sensitization of the airways with those undergoing a nonallergenic immune response.

It is widely understood that the immune response against an Ag is initiated in the secondary lymphoid organ draining the site of entry. Thus, we used a variety of fluorescent conjugates to identify the APCs that can acquire OVA in the DLN after p.n. administration. We compared the fate of p.n. OVA with i.p. OVA in an

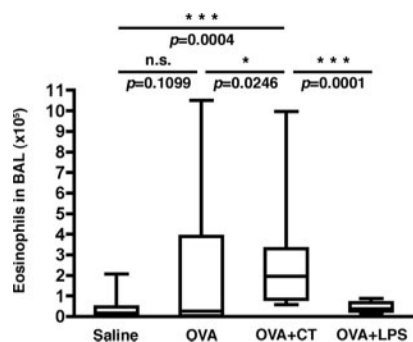


FIGURE 9. Secondary OVA challenge elicits lung eosinophilia in mice administered OVA and CT but not LPS. Three groups of mice were administered 100 μ g of OVA p.n., with or without 1 μ g of CT or 10 μ g of *E. coli* LPS, in 50 μ l of saline at least 2 days after adoptive transfer of lymph nodes cells from DO11.10 mice. A fourth group of DO11.10 recipients received 50 μ l of saline p.n. Two weeks later, each group was challenged with five doses of 25 μ g of OVA p.n. as described in *Materials and Methods*. BAL was performed 48 h after the final challenge and the number of eosinophils present was determined. Results were pooled from six experiments comprising different combinations of the groups shown. The total number of mice included in each group were: saline, $n = 9$; OVA, $n = 19$; OVA + 1 μ g of CT, $n = 19$; OVA + 10 μ g of LPS, $n = 10$. The mean, upper, and lower interquartiles, and spread of values are displayed for each group by a standard box and whiskers plot. n.s., Not significant; *, $p < 0.05$; **, $p < 0.01$, as determined by a two-tailed Mann-Whitney *U* test.

attempt to identify the cells that were responsible for delivering inhaled Ag to the DLN and found that the bulk of p.n. OVA was associated with a single population that we identified as CD8 α^{low} DC based on their expression of class II MHC, CD11c and CD8 α . This population of DC not only acquired large amounts of OVA, they also processed it for presentation, as detected by DQ OVA and T cell stimulation ex vivo. Thus, CD8 α^{low} DC appeared to have acquired p.n. OVA in the airways and/or lungs before migrating to the DLN where they could present the processed allergen to T cells. Additional phenotyping experiments revealed most of the OVA⁺CD8 α^{low} DC expressed CD205, confirming their identity as tissue-derived DC. These cells could be split into two populations on the basis of CD11b expression, which may represent epithelium-derived DC (CD8 α^{low} CD205⁺CD11b^{−/low}) and interstitial DC (CD8 α^{low} CD205⁺CD11b⁺) according to current schema (37, 38). Our results compare well with the earlier work of Vermaelen et al. (22) that identified migratory pulmonary DC in the DLN as CD8 α^{low} , CD205⁺, and CD11b^{−/+} and noted their resemblance to airway and lung DC, which are rarely CD8 α^{high} (21, 22). In a recent report, Sung et al. (39) used CD103 and CD11b to split IAE^{high} DC into two populations and presented evidence for their differential localization in the lung. These authors went on to speculate on different roles for each DC, suggesting that CD11b[−] DC would be more mobile than CD11b⁺ DC. Our results indicate both populations are able to migrate to the DLN, though further work is required to determine whether there is a difference over the entire course of Ag delivery to the DLN.

A variety of other cell types, including CD8 α^{high} DC, pDC and B cells, were able to acquire p.n. OVA in the DLN, albeit at lower levels and in smaller numbers. We believe a large proportion of the OVA⁺ cells in these populations acquired OVA from the lymph, rather than delivering it from the airways and lungs, because the majority of these cells also acquired i.p. OVA when it was simultaneously administered with p.n. OVA. Similarly, a proportion of resident CD8 α^{low} DC could have acquired p.n. OVA from the lymph, and indeed, a small proportion of CD8 α^{low} DC were found

in the DLN with both p.n. and i.p. OVA. However, our data does not allow us to rule out the possibility that all of the p.n. OVA⁺ cells in the DLN (whether or not they were i.p. OVA⁺) had migrated from the airways and lungs. CD8 α^{high} DC, pDC, and B cells are present in the airways and/or lungs and may have acquired i.p. OVA after arriving in the DLN with i.n. OVA. In this instance, these cell types may have been more likely to acquire i.p. OVA due to their rate of migration, localization in the DLN, and/or their ability to capture i.p. OVA. We do not believe this is likely because a variety of soluble proteins have been shown to permeate pulmonary tissue to enter blood and lymph within an hour of inhalation, albeit at a fraction of the administered dose (40–42). Vermaelen et al. (22) failed to detect OVA in CD8 α^{high} DC in the DLN at any time point after intratracheal administration, though this may have been related to sensitivity and/or permeability of the tracking compound used (FITC vs Alexa Fluor 488). Belz et al. (28) found that viral Ags were presented by two populations of DC in the DLN 3 days after pulmonary infection: one was a population of CD8 $\alpha^{\text{−}}$ DC derived from the lungs, and the other was a resident population of CD8 $\alpha^{\text{+}}$ DC. In this instance, the authors attributed Ag presentation by the local CD8 $\alpha^{\text{+}}$ DC population to Ag transfer from CD8 $\alpha^{\text{−}}$ DC rather delivery via the lymph (28). Thus, there may be more than one mechanism to account for the dispersal of Ag among different cell subsets in the DLN.

Our results suggest there were two phases of OVA delivery to the DLN: an early phase of passive Ag delivery (within 4 h), presumably via the lymph draining the airways and lungs, where a small number of CD8 α^{low} DC, CD8 α^{high} DC, pDC, B cells, and IAE^{low}CD11c^{low} cells acquired OVA within 4 h; and a late phase Ag delivery (peaking at 24–48 h) by CD8 α^{low} DC arriving in the DLN from the airways and lungs. This second phase of OVA delivery by migratory CD8 α^{low} DC outnumbered other OVA⁺ cells in the DLN by 10-fold or more by 24 h. Itano et al. (43) observed a similar phenomenon while investigating Ag uptake and presentation in the DLN following s.c. administration of a soluble Ag. The authors were able to formally demonstrate an early, rapid phase of passive Ag uptake by resident DC and a later phase of Ag delivery by DC migrating from the skin. However, Itano et al. (43) did not consider the range of cell types that could acquire Ag, but their results demonstrate that lymph nodes are equipped to collect free Ag as well as migrating cells. Indeed, two detailed studies have recently shown s.c. Ag enters the DLN via the reticular network before the arrival of migrating cells (44, 45), though this mechanism only operates for low m.w. molecules (44). Taken together, it seems likely that any Ag that permeates the lung tissue will enter the DLN where it will be endocytosed by cells located near the reticular conduits of the lymph node.

Before considering the effects of CT and LPS on Ag presentation, it is worth noting that p.n. administration of OVA alone lead to significant Ag delivery to the DLN and marked T cell activation. Indeed, almost half of the CD8 α^{low} DC in the DLN were OVA⁺ 24 h after p.n. immunization, which lead to significant T cell activation and clonal expansion by 48 h. A small proportion of activated CD4⁺TgTCR⁺ cells were primed to produce IL-4 or IFN- γ at 36 h, and a population of proliferating CD4⁺TgTCR⁺ cells entered the lungs by 72 h. After day 3, the number of CD4⁺TgTCR⁺ cells slowly declined to leave a small population of memory CD4⁺TgTCR⁺ cells in the DLN, main conducting airways, and lungs on day 21. A single p.n. dose of OVA was sufficient to sensitize around 25% of DO11.10 cell recipients, as revealed by OVA challenge 14 days after immunization, suggesting that the OVA preparation we used, and dose administered, was nontolerogenic.

Both LPS and CT clearly improved the potency OVA presentation in the DLN. LPS stimulated a marked increase in the number of OVA⁺CD8 α ^{low} DC in the DLN at 24 h, along with smaller increases in the number of OVA⁺pDC and OVA⁺B cells. CT did not appear to be as potent as LPS, stimulating only small increases in the number of OVA⁺B cells without having any effect on the number of OVA⁺CD8 α ^{low} DC. Interestingly, neither adjuvant had an effect on the number of OVA⁺CD8 α ^{high} DC in the DLN, suggesting that the increase in OVA uptake could not be attributed to an increase in the permeability of pulmonary tissue. Because there was little difference in the number of OVA⁺pDC and B cells in the DLN at 4 h, it appears that CT and LPS stimulated or increased the migration of these cell types to the DLN. Whether pDC and B cells are normally involved in the delivery of p.n. Ag to the DLN cannot be determined at this time, though de Heer et al. (27) have previously reported Ag delivery by pDC. However, we only observed very low levels of Ag processing (as detected by DQ OVA) in pDC, CD8 α ^{high} DC, and B cells in the DLN, prompting us to question whether these cells are able to participate as APCs in the immune response against airborne allergens. Previous adoptive transfer studies with purified CD8 α ⁺ DC (26) or pDC (27) have shown that Ag presentation by these populations can have a profound impact on sensitization of the airways, though in these experiments the cells were loaded with Ag *in vitro*. A detailed study of Ag delivery and presentation by CD8 α ^{high} DC, pDC and B cells in the DLN will be necessary to clarify their role in initiating or perpetuating the immune response in the DLN.

LPS and CT stimulated up-regulation of CD80 and CD86 on CD8 α ^{low} DC 24 h after p.n. immunization. This effect was largely confined to CD8 α ^{low} DC that were OVA⁺, suggesting both adjuvants acted directly on cells exposed to OVA in the airways and lungs. Up-regulation of CD80 and CD86 is typically associated with an increase in the stimulatory capacity of DC, and indeed, we detected increases in T cell activation, clonal expansion, and perhaps cytokine priming after administering OVA with CT and LPS. Thus, LPS and CT could act in two ways to improve Ag presentation: first, by increasing the amount of Ag delivered to the DLN, which was most obvious when LPS was used for immunization; and second, by stimulating up-regulation of CD80 and CD86, two crucial costimulatory molecules known to enhance T cell activation. Curiously, LPS had no effect on CD40 expression, while CT consistently induced down-regulation of CD40 expression. The functional significance of this effect cannot be deduced from our results, but it is clear that reduced levels of CD40 did not hamper T cell activation in the DLN. Ligation of CD40 has been shown to be important for IL-12 production by DC (46), so lower CD40 expression may favor the induction of a Th2 cells by DC exposed to CT.

In this report, we have relied on the level of class II MHC and CD11c expression to differentiate between CD8 α ^{low} and CD8 α ^{high} DC. class II MHC is prone to modulation on DC, especially after exposure to LPS, so our study of OVA uptake by CD8 α ^{high} DC may have been confounded by a increase in class II MHC expression in mice administered LPS. In this instance, OVA uptake attributed to CD8 α ^{low} DC may have included a proportion of CD8 α ^{low} DC that had up-regulated their class II MHC expression. We do not believe that this is the case for two reasons: first, there was no corresponding decline in the number of OVA⁺CD8 α ^{high} DC in the DLN; and second, subsequent experiments using CD205, CD11c, and CD8 α to identify DC in the DLN revealed there was no change in class II MHC expression on CD8 α ^{low} or CD8 α ^{high} DC 24 h after p.n. administration of LPS (M. E. Wikstrom and P. A. Stumbles, unpublished observations). It is important to note that the two flow cytometry analysis gates used to

define CD8 α ^{low} and CD8 α ^{high} DC also contained other DC populations. For example, the CD8 α ^{low} DC gate contains a small proportion of DC that do not express CD205 and that can be divided into further subpopulations on the basis of CD8 α and CD11b expression (M. E. Wikstrom and P. A. Stumbles, unpublished observations). Henri et al. (47) and Itano and Jenkins (37) have previously commented on the complexities associated with identifying and/or classifying DC populations in the lymph nodes. In the future, we intend to develop a six-color protocol where we can include CD205, CD8 α , and CD11b in our OVA tracking studies to further refine our description of the DC populations that deliver airborne Ag to the DLN.

The primary T cell response to Ag administered via the airways has previously been described in some detail. Tsitoura et al. (7) found that three consecutive doses of 100 μ g of OVA induced significant T cell activation and proliferation, while Lambrecht et al. (48) reported that the T cell response elicited by intratracheal delivery of peptide-pulsed DC was similar to that elicited by peptide alone. Our results compare well with these earlier studies, as well as studies on other routes of Ag administration (49–54) or pulmonary infection (1, 2). One notable exception, however, is related to the kinetics of cytokine-priming by p.n. OVA: previous studies have shown that cytokine priming appears relatively late after T cell activation in cells that have divided at least three times (2, 54–56). In our model, IL-4, IL-5, and IFN- γ production were detected in the DLN as early as 24 h after p.n. immunization (data not shown) with a peak around 36 h when the bulk of the CD4⁺TgTCR⁺ cells had yet to divide. By 72 h, when the CD4⁺TgTCR⁺ cells had undergone multiple rounds of cell division, cytokine production was barely detectable. CT or LPS did not alter the kinetics of cytokine priming, even though both adjuvants were able to increase cytokine production. Laouar and Crispe (57) have shown that cytokine production can be uncoupled from cell division *in vivo*, so we have no reason to doubt the validity of our results. However, we cannot provide any insight on the mechanism that might be driving the rapid induction of cytokine production, though clearly, this is an intriguing feature of the T cell response that warrants further investigation.

Naive T cells were not activated in the lungs during the first 2 days following p.n. immunization. However, a population of activated and divided T cells appeared in the lungs on day 3, presumably after migrating out of the DLN, and a small proportion of these cells were primed to produce IL-4, IL-5, and IFN- γ for a short period of time. Previous studies on the primary T cell response to pulmonary infection have reported very similar results, though activated T cells were not observed in the lungs until day 5 or 6 (1, 2). CT and LPS both increased the number of T cells entering the lungs on day 3 but they did not induce any bias in cytokine priming. The number of activated T cells declined in the lungs after day 3 to leave a small population of memory T cells. The size of this population appeared to correlate well with the number of T cells entering the lungs on day 3, which in turn, appeared to depend on the amount of clonal expansion in the DLN. Thus, memory T cells are generated against airborne Ags or allergens as a consequence of T cell activation and proliferation in the DLN. This process does not depend on the presence of an adjuvant, though they will act to increase the number of memory T cells generated.

The persistence of memory T cells in the lungs is crucial for viral protection (58) and appears to be important for the development of allergic disease in the airways (11, 59, 60). We found that CT increased the number of memory T cells ~2-fold compared with immunization with OVA alone, and this was associated with

an increase in the incidence and extent of eosinophilia upon secondary OVA challenge. However, LPS generated a larger number of memory T cells in the lungs, yet failed to sensitize the airways. Thus, a population of resident memory T cells may be necessary, but not sufficient, to sensitize the airways.

The differential effects of LPS and CT on the development allergic disease in our model might be best explained by preferential induction of Th1 and Th2 immunity, respectively. However, there was no indication that LPS and CT preferentially induced Th1 and Th2 cytokines in CD4⁺TgTCR⁺ cells in the DLN or the lungs during the primary response. It is possible that CT and LPS had differential effects on the endogenous T cell population of the recipient mice used in our adoptive transfer studies. However, we found no evidence of an increase in Th2 priming among the endogenous CD4⁺ T cell population in the DLN or lungs of mice administered CT, though LPS may have increased IFN- γ priming in the lungs on day 3 (M. E. Wikstrom and P. A. Stumbles, unpublished results). Previous studies in leishmaniasis have found that while the preferential induction of Th1 or Th2 immunity influenced the course of infection, no bias in cytokine production was evident until 4 wk after infection (61). These findings strengthen the notion that the immune response commences in a relatively undifferentiated manner and is refined over time according to the influence of the host and/or the local environment (62, 63). Clearly, it will be important to examine the cytokine repertoire of the memory T cells that persist in the DLN and lungs to determine whether preferential induction of Th1 and Th2 memory can account for the differential effects of LPS and CT in our model.

Previous studies have suggested that differential Th priming can be attributed to specific DC subsets. Thus, Th1 priming has been attributed to lymphoid DC (CD8 α ⁺ CD11b⁻), and Th2 priming to myeloid DC (CD8 α ⁻ CD11b⁺) (24, 25). Consistent with these findings, Hammad et al. (26) found that CD8 α ⁻ DC, but not CD8 α ⁺ DC, induced allergic disease in the airways after intratracheal delivery. Our results do not support this association, because CD8 α ^{low} DC were the major OVA presenting population in the DLN whether mice were immunized with CT or LPS. We believe that Th1 and Th2 priming can be modulated by exogenous factors (e.g., a mucosal adjuvant) acting on the APC and/or the host environment. Accordingly, our laboratory has previously shown that purified pulmonary DC preferentially induce Th2 immunity in vivo unless they are treated with GM-CSF, and then they induce Th1 immunity (20). This thesis allows for the influence of the tissue environment on the context in which airborne allergens are collected and presented to T cells, which is known to have a profound impact on the development of allergic sensitization (reviewed by Ref. 64). Our data suggests such mechanisms may operate beyond the DLN to influence the population of T cells (and other proallergic cell types) attracted to, retained in, or otherwise maintained in the airways and lungs during the development of immunological memory and secondary immunity.

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Disclosures

The authors have no financial conflict of interest.

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