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Visualization of Early APC/T Cell Interactions in the Mouse Lung Following Intranasal Challenge

Craig A. Byersdorfer and David D. Chaplin

We have used fluorescent latex beads, with or without covalently conjugated OVA, to facilitate study of Ag trafficking in the mouse lung and draining peribronchial lymph node (LN). At 6 h, and up to 48 h after intranasal administration, beads were observed as intracellular clusters in the tissue parenchyma. Flow cytometry of bead-positive (bead+/H11545) cells from the bronchoalveolar lavage demonstrated that a majority of these cells are CD11c+/H9262, F4/80+/H11011, and CD11b−/H11001. Furthermore, fluorescent microscopy confirmed that a major subset of bead+/H11545 cells in the lung tissue was also CD11c−. In the draining peribronchial LNs, small numbers of beads were present in the subcapsular sinus as early as 6 h after inhalation. By 12 h and beyond, bead+ cells had localized exclusively to the LN T zone. OVA-conjugated latex beads, in addition to stimulating brisk proliferation of naive, OVA-specific DO11.10 transgenic T cells in vitro, could also recruit OVA-specific T cells in vivo. In some cases, bead+ APCs and CD4+ Th1 cells were found adjacent to the lung tissue 6 h after airway challenge. Thus, interactions of bead+/H11545 APCs with Ag-specific CD4+ T cells occurred earlier in the peripheral airways than these same interactions occurred in the draining peribronchial LN. Lastly, after adoptive transfer, in vitro differentiated Th1 cells accumulated at peripheral sites in the lung tissue and airways before Ag challenge and therefore were ideally positioned to influence subsequent immune reactions of the airway.


The immune response of a host to pathogens or exogenous Ags has classically been separated into an initial innate response, beginning immediately after encounter with Ag, and a later adaptive response, which begins hours to days after the initial challenge (1). Situated at the crossroad of these two systems is the peripheral dendritic cell (DC),2 influenced by the microenvironment of innate effectors and yet competent to prime lymphocytes that regulate the adaptive response. DCs sample Ags in the periphery, acting as sentinels at remote sites (2), then travel to lymph nodes (LNs) to prime naive T cells that circulate through these lymphoid organs (3–6).

In the lung, Ag sampling is mediated by an extensive network of Ag-presenting DCs in the airway epithelium, as well as in alveolar septae and around airspaces (7, 8). These cells show high activity for Ag uptake and are capable of presenting Ag to T cells when cultured in vitro (9, 10). In vivo, pulmonary DCs migrate to the regional LNs after encounter with Ag (11–13), up-regulate costimulatory molecules in the process (14), and acquire potent activity for priming naive T cells and initiating a primary immune response (15–18).

In addition to Ag-induced activation of DCs, the overall number of lung DCs also increases in response to a variety of stimuli (19), including proinflammatory cytokines (20, 21), soluble Ag (11), and microbial pathogens (22). Furthermore, DCs in the lung epithelium manifest a short ~2-day half-life, suggesting that they are readily adapted to rapid and continuous sampling of the local environment (23).

Because they present airway Ags so efficiently, lung DCs are thought to be key regulators of T cell-dependent airway diseases such as asthma and allergic airway inflammation (24) (reviewed in Refs. 25 and 26). Indeed, it has been shown that DCs are indispensable for generating chronic eosinophilic airway inflammation in a mouse model of allergic airway disease (27). Furthermore, intratracheal administration of DCs differentiated and incubated with Ag in vitro leads to efficient sensitization of naive animals for a subsequent airway challenge (17). Adoptive transfer of Ag-specific T cells, together with intratracheal instillation of Ag- incubated DCs, permits simultaneous study of T cell activation/proliferation and DC migration (18). In other models, it has been possible to colocalize DC/T cell interactions by labeling DCs ex vivo with one fluorescent dye and T cells with another, permitting their direct visualization in lymphoid tissues (6). However, all of these studies have used DCs differentiated and expanded in vitro. The behavior of in vitro differentiated DCs vs those of naturally occurring in vivo APCs has not been well defined. To study naturally differentiated APCs and track their migration and interactions under in vivo conditions, we developed a system to tag airway DCs with a fluorescent marker that could be easily visualized.

In this study, we used 0.4-μm fluorescent latex beads as a model Ag, because these beads can be detected by both flow cytometry and fluorescence microscopy. We visualized them in the lung after intranasal (i.n.) administration, followed them during subsequent trafficking to the regional LNs, and observed them as they localized to the LN T zone. Bead+/H11545 cells in the bronchoalveolar lavage (BAL) fluid and lung tissue were further characterized by flow cytometry. Lastly, by covalently labeling the widely used immunogen OVA to blue fluorescent beads and using T cells from a DO11.10 transgenic (Tg) mouse (28) tagged with a red fluorophore, we visualized in situ the arrival of APCs in the draining LN.

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3 Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; i.n., intranasal; BAL, bronchoalveolar lavage; Tg, transgenic; TAMRA, 5-(and 6)-carboxytetramethylrhodamine; SE, succinimidyl ester; HPF, high power field.

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the time course of T cell recruitment to the lung, and T cell/APC interactions along the way.

This dual-labeling approach provides a flexible system for investigation of not only signals that control the trafficking of Ag, but also the localized interaction between native APCs and Ag-responsive T cells. Data obtained by tracking Ags temporally as well as spatially suggest that, under some circumstances, previously activated T cells first see their Ag in the peripheral tissues of the lung and not in the LN. Furthermore, we make the novel observation that Th1 cells not only accumulate more readily after an airway challenge but are already present at higher numbers in the airway lumen before a primary Ag challenge. Therefore, these cells are positioned to play a specialized role in initiating or modulating inflammatory pathologies of the airway, and fluorescent beads have allowed us to study their rapid interactions with native APCs.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). DO11.10 TCR Tg mice (28) had previously been backcrossed to BALB/c for more than 10 generations and then bred to RAG2−/− mice, also on a BALB/c background. All mice used were female and 6–10 wk of age at the time of Ag challenge. Mice were housed in microisolator cages in a specific pathogen-free facility and provided with food and water ad libitum, according to protocols approved by the Washington University Institutional Animal Care and Use Committee. All mice appeared healthy, and regular monitoring of sentinels showed no serological or histological evidence of respiratory tract infection.

Fluorescent beads

The 0.431-µm carboxylate-modified fluorescent beads were obtained from Sigma-Aldrich (St. Louis, MO) at a concentration of 2.3 × 10^12 particles/ml (catalog nos. L2380 and L4030). Before i.n. administration, beads were washed three times in sterile PBS by centrifugation at 16,000 × g for 12 min and were resuspended in sterile PBS (Life Technologies, Grand Island, NY) at a final concentration of ~7.5 × 10^10 particles/ml. Conjugation of beads to OVA was conducted in 25 mM MES (pH 6.1), 1 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, with beads at 7.5 × 10^10 beads/ml, and 2 mg/ml OVA (all from Sigma-Aldrich). Conjugation reactions were rotated overnight in the dark at room temperature followed by two washes in 25 mM MES. Immediately before use, beads were washed twice in sterile PBS and resuspended at ~7.5 × 10^10 particles/ml. After conjugation, beads were stained with rabbit polyclonal anti chicken egg albumin (Caltag Laboratories, Burlingame, CA), were purchased from BD Pharmingen and include FITC-conjugated anti-CD11c, anti-CD4, and anti-CD11b; PE-conjugated anti-CD11b; and biotin-conjugated anti-CD11c and anti-B220. Biotin conjugates were detected using streptavidin-conjugated APCs (BD Pharmingen). After lysis of red cells, nonspecific Ab staining via FCr was blocked with supernatant from hybridoma 2.4.G2 (American Type Culture Collection, Manassas, VA). Stained cells were stored at 4°C in 1% paraformaldehyde/PBS until just before analysis.

Analysis of cell phenotype by flow cytometry

Flow cytometry was performed using a FACSCalibur and data were analyzed with CellQuest software (BD Biosciences, Mountain View, CA). All Abs, with the exception of the biotin-conjugated monoclonal anti-F4/80 (Caltag Laboratories, Burlingame, CA) and rat anti-NLDC-145 (Bachem, Torrence, CA), were purchased from BD Pharmingen and include FITC-conjugated anti-CD11c, anti-CD4, and anti-CD11b; PE-conjugated anti-CD11b; and biotin-conjugated anti-CD11c and anti-B220. Biotin conjugates were detected using streptavidin-conjugated APCs (BD Pharmingen). After lysis of red cells, nonspecific Ab staining via FCr was blocked with supernatant from hybridoma 2.4.G2 (American Type Culture Collection, Manassas, VA). Stained cells were stored at 4°C in 1% paraformaldehyde/PBS until just before analysis.

Assessment of lymphocyte proliferation in vitro

CD4+ lymphocytes were purified from the spleens of RAG2−/−/DO11.10 TCR Tg mice (kindly provided by O. Kanagawa, Washington University, St. Louis, MO) using anti-mouse CD4 Dynabeads and the mouse CD4 DETACHaBEAD system (Dynal BioTech, Lake Success, NY), according to the manufacturer’s instructions. CD11c+ cells were purified from the Liberase-digested lungs of naive mice using a FITC-conjugated anti-CD11c mAb (BD Pharmingen), anti-FITC magnetic beads, and a magnetic column (Miltenyi Biotec, Auburn, CA). Purity of CD11c+ cells after magnetic sorting, as assessed by flow cytometry, was >94%. Purified T cells (3 × 10^5) were cultured in 200 µl of Iscove’s medium supplemented with 10% FCS (HyClone Laboratories), 0.1 mM nonessential amino acids, 2 mM sodium glutamate, 1 mM sodium pyruvate, 10 U/ml penicillin, 100 µg/ml streptomycin, and 5.5 µM 2-ME (all from Life Technologies) with 2 × 10^7 CD11c+ cells and 20 µg of soluble OVA, 10 µl of fluorescent beads only, or 10 µl of fluorescent beads conjugated to OVA. Cells were cultured for 3 days at 37°C under 5% CO2. One microcurie of [3H]thymidine (Amersham, Arlington Heights, IL) was then added to each well, and the culture was continued for an additional 24 h. Cells were harvested on a PHD cell harvester (Cambridge Technology, Watertown, MA), and incorporation was measured on a scintillation counter (LS8301; Beckman Coulter, Fullerton, CA).

Adoptive transfer of T cells

OVA-specific T cells were differentiated in vitro into Th1 or Th2 cells as previously described (29–31). On day 7 of culture, cells were centrifuged over Histopaque 1119 (Sigma-Aldrich) to remove dead cells, and they were washed twice in PBS. Before labeling, a sample of Th1 and Th2 cells were stained for expression of CD25 and CD69. Levels of these activation markers were the same between the two groups, suggesting that there was no general difference in the activation status of the two populations. Cells were then fluorescein labeled by incubation for 15 min at 37°C with 6 µg/ml TAMRA-succinimidyl ester (SE) (Molecular Probes) in Ca2+ - and Mg2+-free PBS. Fluorescently labeled cells were washed twice in sterile PBS and transferred to anesthetized, naive animals i.v. via retro-orbital injection. Naive CD4+ T cells were recovered from spleens and LNs of RAG2−/−/DO11.10 Tg mice, and sorted using anti-mouse CD4 Dynabeads and mouse CD4 DETACHaBEAD (Dynal BioTech). Then they were incubated with TAMRA-SE and injected as described above. Purity of naive CD4+ cells was >95% as determined by flow cytometry.
Results
Fluorescent beads are taken up by cells of the lung

To investigate factors that control Ag uptake, trafficking, and presentation in the mouse lung, we have used fluorescent latex beads as a model inhaled Ag. The beads are well suited for this purpose because they are readily taken up by phagocytic cells, visualized by fluorescence microscopy, and quantitated by flow cytometry. Furthermore, they can also be modified by covalent coupling to immunogens and followed in parallel to immunogen-specific T cell responses. To test their behavior as particulate Ags, we administered $3 \times 10^8$ 0.4-$\mu$m fluorescent latex beads in 40 $\mu$l of sterile PBS to lightly anesthetized C57BL/6 mice. At various times later, the mice were sacrificed, their lungs were harvested, and frozen sections of lung were analyzed by fluorescence microscopy to determine the locations of the beads.

Beads were detected in lung cells as early as 2 h after i.n. instillation and remained present until at least 46 h later. This observation is congruent with other studies demonstrating that lung DCs harvested after Ag challenge retain the ability to present Ag in vitro up to 7 days after initial administration of the Ag (11). The majority of beads detected in this fashion resided within cells of the lung, and not free in the airways, based upon their localization adjacent to 4',6'-diamidino-2-phenylindole-positive nuclei (Fig. 1, A and B). Beads were also found in large quantities in cells recovered by BAL (Fig. 1, C and D). Fluorescence microscopy in conjunction with Wright-Giemsa staining demonstrates that beads could be found in $>50\%$ of BAL cells and that the majority of these cells were of the monocyte/myeloid phenotype.

**Bead**$^+$ lung cells are CD11c$^+$ and bead$^+$ BAL cells are CD11c$^+$F4/80$^+$CD11b$^+$

To identify phenotypic characteristics of bead$^+$ lung cells, we performed Ab staining of frozen lung sections followed by dual fluorescence microscopy. Using an anti-CD11c Ab, we determined...
that >30% of the cells in the lung that had taken up fluorescent beads were also CD11c− (Fig. 1, E and F). Analysis by confocal microscopy confirmed that beads were present within the CD11c− cells (data not shown). To permit broader characterization of bead− cells, we performed flow cytometry on cells positive for bead uptake. Bead+ cells are easily differentiated from those that have not taken up beads (Fig. 2). By gating on bead+ cells, we were able to then test for expression of CD11c and CD11b, F4/80, or NLDC-145 on these same cells (Fig. 2 and Tables I and II). The markers CD11b, F4/80, and NLDC-145 were chosen because of their suggested differential expression among the myeloid and lymphoid DC subsets (32–34).

At 6 and 12 h after instillation of beads, most bead+ cells from the BAL were also positive for the DC marker CD11c (>80%). In turn, a great majority of these CD11c+ cells were negative for the myeloid-specific marker CD11b. For example, at 6 h compare 85.9% CD11clow/CD11b− vs 2.7% CD11clow/CD11b+ (Table I). By 24 and 48 h, a greater portion of bead+ BAL cells were low or negative for expression of CD11c compared with the populations at 6 or 12 h, although they remained negative for CD11b expression. This CD11clow/CD11b+ population of cells also showed lower side light scatter and forward light scatter values than the bead-positive population at 6 h. It remains unclear whether this increase in the CD11clow/CD11b+ subset at 24 and 48 h represents a newly emigrated population of cells entering into the airway lumen or merely indicates the down-regulation of surface markers on cells, which phagocytosed beads at some earlier time. To continue characterization, staining with F4/80 revealed that >85% of bead+ BAL cells were also F4/80+ at all times examined (Table II). Furthermore, of all bead+ cells at 6 and 12 h, ~65% of these were F4/80+ CD11clow−.

In the lung tissue itself, regardless of the time after challenge, ~25% of bead+ cells are CD11c−CD11b+, with a majority (50–60%) being CD11clow−CD11b−. As in the BAL, <10% of bead+ lung leukocytes are either CD11c−CD11b+ or CD11c+CD11b+. Flow cytometry data regarding CD11c positivity correlate closely with those obtained using tissue staining and then dual fluorescence microscopy (~30%). For bead+ cells recovered from the lung, nearly equal numbers of CD11c−F4/80+, CD11clow−/F4/80−, and CD11clow−/F4/80− populations were observed (Table II). Lastly, at 6 and 12 h, ~20% of bead+ cells from the BAL or lung were also NLDC-145+.

OVA covalently conjugated to fluorescent latex beads can stimulate OVA-specific CD4+ T cells in vitro

To enhance the utility of fluorescent beads as a model Ag, we tested whether beads would be recognized by Ag-specific CD4+ Tg T cells if covalently conjugated to the Ag recognized by the transgenic TCR. For this purpose, we tested whether CD11c− lung cells given OVA-conjugated fluorescent beads could stimulate proliferation of OVA-specific DO11.10 Tg T cells. CD11c− lung cells were recovered from naive, wild-type animals; incubated with OVA-conjugated fluorescent beads, beads alone, or unconjugated OVA (soluble OVA); and then cultured for 96 h with CD4+ DO11.10 Tg T cells. When CD4+ DO11.10 T cells were stimulated with OVA-conjugated beads using CD11c+ cells from the lung as APCs, robust proliferation was observed at 96 h (Fig. 3). Beads alone stimulated no proliferation over background.

i.n. administered fluorescent beads traffic to the T cell zone of peribronchial LNs

Other studies have demonstrated that in vitro differentiated DCs traffic from the skin to the draining LN within 24 h (6). To determine whether fluorescent beads administered i.n. exhibit a similar temporal progression, we analyzed the accumulation of beads in peribronchial LNs harvested at varying times after challenge. Small numbers of beads (2–3 bead− cells/section) were detected in peribronchial LN sections as early as 6 h after i.n. administration. These bead− clusters initially localized exclusively in the subcapsular sinus (Fig. 4A). In contrast, 12 h after challenge, peribronchial LNs showed large numbers of bead− clusters (>10 per section) in nearly each section examined (20 sections from eight mice). Furthermore, by this time the majority of fluorescent bead+ cells were localized within the T zone of the LN (Fig. 4B). By 24 and 48 h, there was a further increase in the number of bead+ cells per LN section, showing similar exclusive localization to the T zone (Fig. 4C).

Table I. Characterization of CD11c and CD11b expression on bead+ cells

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>CD11c&lt;sup&gt;high&lt;/sup&gt;</th>
<th>CD11c&lt;sup&gt;low&lt;/sup&gt;</th>
<th>CD11b&lt;sup&gt;high&lt;/sup&gt;</th>
<th>CD11b&lt;sup&gt;low&lt;/sup&gt;</th>
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<tr>
<td>BAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>85.9 ± 5.3</td>
<td>2.7 ± 1.0</td>
<td>10.6 ± 5.4</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>12</td>
<td>78.7 ± 8.1</td>
<td>3.4 ± 0.4</td>
<td>16.6 ± 7.3</td>
<td>1.4 ± 1.1</td>
</tr>
<tr>
<td>24</td>
<td>44.5 ± 14.3</td>
<td>2.4 ± 0.1</td>
<td>51.6 ± 13.8</td>
<td>1.8 ± 1.2</td>
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<tr>
<td>48</td>
<td>51.9 ± 8.2</td>
<td>1.6 ± 0.4</td>
<td>46.0 ± 8.5</td>
<td>0.5 ± 0.3</td>
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<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>22.0 ± 8.2</td>
<td>9.9 ± 3.5</td>
<td>53.3 ± 16.0</td>
<td>14.7 ± 15.8</td>
</tr>
<tr>
<td>12</td>
<td>23.9 ± 2.9</td>
<td>8.4 ± 0.4</td>
<td>63.0 ± 3.1</td>
<td>4.7 ± 0.9</td>
</tr>
<tr>
<td>24</td>
<td>24.1 ± 12.2</td>
<td>13.1 ± 3.2</td>
<td>56.0 ± 13.3</td>
<td>6.9 ± 2.0</td>
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<td>48</td>
<td>30.6 ± 1.9</td>
<td>12.4 ± 4.6</td>
<td>52.5 ± 4.2</td>
<td>4.5 ± 0.4</td>
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* Data are expressed as percentage of total bead-positive cells and represent quadruplicate determinations ± SEM.
Th1 cells preferentially accumulate in the lung and BAL of mice after i.n. challenge with OVA-conjugated beads

Other groups have shown that naive T cells first encounter their Ag in the regional LN. To test whether previously activated cells follow the same paradigm, we combined analysis of OVA-conjugated fluorescent beads and migration of OVA-specific Tg T cells. In vitro differentiated DO11.10 Th1, Th2, or naive CD4^+ cells were labeled with the fluorescent dye TAMRA-SE and transferred i.v. to naive BALB/c mice. Three days later, these mice were challenged i.n. with one administration of OVA-conjugated beads. At 12 h, some bead^+ cells were found in the T zone of the LN between baseline and 48 h after airway challenge (data not shown). This indicates that the accumulation of Th1 cells in the airway was specifically localized to the region of Ag challenge.

Percentages of TAMRA^+ cells in the lung parenchyma were also analyzed. Th1 cells at baseline were represented at a higher frequency in lung tissue than either Th2 cells or naive, undifferentiated cells (Fig. 5B). There were modest increases in the percentage of lung Th1 cells over the 48 h after OVA challenge and smaller transient changes in Th2 cells. The failure to detect a substantial increase in naive or Th2 cells in the BAL or lung was not due to a gross loss or death of these populations, because both groups could be found at other anatomical locations, including the peribronchial LN, nondraining inguinal LN, and spleen (Fig. 6D and data not shown).

T cells are positioned to encounter Ags in the regional LNs early after Ag challenge

As shown in Fig. 4D, naive TAMRA^+ T cells are present in the T zone of the peribronchial LN before an airway challenge. This is also true, to a lesser extent, for mice given Th1 cells (data not shown). Conjugated fluorescent beads administered to naive, wild-type mice, as shown earlier, traffic to the T zone of the LN between 6 and 12 h. Transfer of Ag-specific Th1, Th2, or naive cells did not affect this time course, and bead^+ cells do not reach the T zone 6 h after i.n. administration of beads. At 12 h, some bead^+ cells are found in the T zone and can be seen localized adjacent to fluorescently labeled T cells (Fig. 4E). Higher magnification verifies the proximity of this localization (Fig. 4F). This type of APC/T cell interaction in the LN continues to occur at higher frequency 24 and 48 h after Ag challenge.

Adjacent localization of Th1 cells and APCs in the lung 6 h after Ag challenge

Because large amounts of the Ag remained in the lung over the first several days, we reasoned that APC/T cell interactions might be occurring in the peripheral lung tissue at time points before, or directly concurrent with, initial presentation of Ag to T cells in the draining LN. To test this, we investigated localization of TAMRA-labeled T cells and bead^+ APCs in the lung 6 h after challenge with OVA-conjugated beads.

As demonstrated in Fig. 6, at 6 h after i.n. Ag challenge, OVA-specific Th1 cells were found in much higher numbers per high power field (HPF) than either Th2 or naive cells (Fig. 6A). In contrast, the numbers of bead^+ APCs/HPF were relatively constant between groups (Fig. 6B). Significantly, Th1 cells were found colocalizing with bead^+ APCs 4-fold more frequently than either Th2 or naive cells (Fig. 6C). T cell-APC interactions characterized as colocalization are depicted microscopically in Fig. 4, G and H. The frequency of these interactions appeared to be dependent upon the subset of helper cells transferred, because the average number of interactions per T cell was 0.072 interactions/Th1 cell (7.2%), but only 0.018 interactions/Th2 cell (1.8%). T cells and bead^+ APCs were also frequently found in close proximity, but without

### Table II. Characterization of CD11c and F4/80 expression on bead^+ cells^a^

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>BAL</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD11c^high</td>
<td>CD11c^low</td>
</tr>
<tr>
<td></td>
<td>F4/80 (%)</td>
<td>F4/80 (%)</td>
</tr>
<tr>
<td>6</td>
<td>1.3 ± 0.6</td>
<td>64.1 ± 9.0</td>
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<td>1.2 ± 0.2</td>
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</tr>
<tr>
<td>48</td>
<td>0.4 ± 0.2</td>
<td>47.5 ± 6.05</td>
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</table>

^a Data are expressed as percentage of total bead-positive cells and represent quadruplicate determinations ± SEM.
apparent cell-cell contact (data not shown). The latter were consistent with cell-cell interactions in the process of development and were found to occur around vessels or in collections of cells at branching points of alveolar walls. In total, these studies substantiate the hypothesis that in vitro differentiated Th1 cells are capable of localizing adjacent to APCs in the peripheral lung tissue on a time course that precedes detection of the same Ag or APC/T cell interaction in the draining LN and that the frequency of this early

**FIGURE 4.** Beads traffic to the T zone of the LN by 12 h, but colocalize with Th1 cells in the lung earlier. A, Six hours after administration, beads can be seen in the subcapsular sinus of the peribronchial LN (†, lower right), whereas by 12 h they reach the T zone (B), where their accumulation peaks at 24 h (C). A–C, Fluorescent beads are yellow-red, green represents staining with FITC-conjugated anti-CD4, and blue is B220-biotin followed by Alexa-350-conjugated neutravidin. In adoptive transfer experiments, TAMRA+ cells (red) can be found in the draining LN before challenge in mice receiving naïve cells (D) and to a lesser extent Th1 cells (not shown), but beads (blue) do not arrive at the T zone until 12 h post-challenge (E). E, B220+ cells are stained with B220-biotin and then streptavidin-FITC. F, Higher magnification highlights the Th1/APC interaction seen in E. In contrast, TAMRA+ Th1 cells interact with bead+ APCs in the lung parenchyma as early as 6 h after Ag challenge (G and H; higher magnification).
Th1, or naive cells. All numbers are the average of at least three experiments. The difference at baseline between mice given Th1, Th2, or naive CD4+CD45R0+ cells, and Ag-specific antigen-presenting cells (APCs) present in the BAL, is significant. This study was designed to investigate the behavior of Ag-presenting cells that recruit T cells in mice after airway administration of Ag via the airway. As a particulate Ag we have used fluorescent latex beads, with or without covalently conjugated OVA, to facilitate the study of native APC trafficking in the mouse lung. Beads, when given alone, are rapidly engulfed by cells of the lung and can be seen gathered together in “bead” clusters” as early as 2 h after administration. How long they remain in the lung remains undefined, but we have observed their continued persistence for at least 48 h after the initial i.n. administration.

We have used both histological and flow cytometric approaches to define the phenotype of cells responsible for bead uptake. In both BAL and lung samples, a large fraction of bead+ cells express the cell surface marker CD11c. CD11c has been described by Steinman and colleagues (35) as a specific marker for splenic DCs and is present on at least a subset of DCs from other lymphoid organs (33, 34). In this study, bead+ cells recovered by BAL, a majority of which are CD11c+ by flow cytometry, appeared by Wright Giemsa staining to be myeloid in origin. Their high expression of CD11c argued against an exclusive macrophage designation (36), and so we postulated that these bead+ cells belonged to the myeloid DC subset. However, particularly within the first 12 h, bead+ cells are predominantly CD11c+CD11b-, with CD11b being a marker commonly positive on myeloid DCs. This suggests that bead+ CD11c+CD11b- cells represent a unique subset of airway DCs. Alternatively, they may simply represent incompletely committed precursor cells of monocytic origin. It is of interest that the macrophage marker F4/80 is highly expressed on this same bead+ cell subset. High expression of F4/80 does not preclude the classification as DCs because other groups have shown that some subsets of DCs, before culturing and when freshly isolated from tissues, also express the macrophage marker F4/80 (34). We speculate then, based upon bead+ cells being CD11c+ F4/80+CD11b-, that this subset may represent a cell type similar to the CD11c+ F4/80+ epidermal Langerhans cell (37) and may perform a similar function in trafficking Ag from the periphery to the draining LN.

Another report published recently used FITC-conjugated OVA to track DCs in the mouse lung and LNs (38). The phenotypic markers expressed on APCs in that study and on those reported in this study differ substantially. These differences might be explained in several ways. First, our model evaluated the phenotype of all cells that took up the fluorescent Ag. Our only inclusion

Discussion

This study was designed to investigate the behavior of Ag, Ag-transporting cells, and Ag-specific CD4+ T cells in mice after administration of Ag via the airway. As a particulate Ag we have used fluorescent latex beads, with or without covalently conjugated OVA, to facilitate the study of native APC trafficking in the mouse lung. Beads, when given alone, are rapidly engulfed by cells of the lung and can be seen gathered together in “bead” clusters” as early as 2 h after administration. How long they remain in the lung remains undefined, but we have observed their continued persistence for at least 48 h after the initial i.n. administration.

We have used both histological and flow cytometric approaches to define the phenotype of cells responsible for bead uptake. In both BAL and lung samples, a large fraction of bead+ cells express the cell surface marker CD11c. CD11c has been described by Steinman and colleagues (35) as a specific marker for splenic DCs and is present on at least a subset of DCs from other lymphoid organs (33, 34). In this study, bead+ cells recovered by BAL, a majority of which are CD11c+ by flow cytometry, appeared by Wright Giemsa staining to be myeloid in origin. Their high expression of CD11c argued against an exclusive macrophage designation (36), and so we postulated that these bead+ cells belonged to the myeloid DC subset. However, particularly within the first 12 h, bead+ cells are predominantly CD11c+CD11b-, with CD11b being a marker commonly positive on myeloid DCs. This suggests then that bead+ CD11c+CD11b- cells represent a unique subset of airway DCs. Alternatively, they may simply represent incompletely committed precursor cells of monocytic origin. It is of interest that the macrophage marker F4/80 is highly expressed on this same bead+ cell subset. High expression of F4/80 does not preclude the classification as DCs because other groups have shown that some subsets of DCs, before culturing and when freshly isolated from tissues, also express the macrophage marker F4/80 (34). We speculate then, based upon bead+ cells being CD11c+ F4/80+CD11b-, that this subset may represent a cell type similar to the CD11c+ F4/80+ epidermal Langerhans cell (37) and may perform a similar function in trafficking Ag from the periphery to the draining LN.

Another report published recently used FITC-conjugated OVA to track DCs in the mouse lung and LNs (38). The phenotypic markers expressed on APCs in that study and on those reported in this study differ substantially. These differences might be explained in several ways. First, our model evaluated the phenotype of all cells that took up the fluorescent Ag. Our only inclusion

![FIGURE 5.](image1.png) **A** Th1 cells preferentially accumulate in response to challenge with OVA-conjugated beads. A total of 5 x 10^6 in vitro differentiated DO11.10 Th1 or Th2 cells or 6 x 10^6 DO11.10 naive cells were transferred to naive BALB/c recipients. Total number of TAMRA-SE+ cells recruited to the BAL (A) and the percent of TAMRA-SE+ cells present in enzymatically digested lung samples (B) at various times after challenge with OVA-conjugated fluorescent beads. C, Number of TAMRA-SE+ BAL cells before challenge, highlighting the difference at baseline between mice given Th1, Th2, or naive cells. All numbers are the average ± SE.

![FIGURE 6.](image2.png) **A** Th1 cells interact more frequently with bead+ APCs 6 h after airway Ag challenge than do either Th2 or naive cells. A total of 7 x 10^6 in vitro differentiated OVA-specific DO11.10 Th1 cells, Th2 cells, or naive CD4+ T cells were transferred i.v. to naive BALB/c recipients. One day later, mice were challenged with OVA-conjugated fluorescent beads. At 6 h, lungs were harvested and analyzed for localization of labeled T cells and bead+ APCs. A, TAMRA DO11.10 Th1 cells are found in higher numbers/HPF than either Th2 cells or naive cells 6 h after Ag challenge (p < 0.001 and p < 0.0001, respectively), whereas at the same time the number of APCs/HPF is equivalent (B). C, Number of interactions (scored as colocalization of T cells and bead+ APCs) between Th1 cells and bead+ APCs was significantly higher than between either Th2 or naive cells and APCs (p < 0.004 vs either Th2 or naive cells).
criterion was that cells be positive for bead uptake. Secondly, our system uses a particulate rather than a soluble Ag. Although it is unclear what difference particulate vs soluble nature of the Ag makes in terms of Ag delivery or cellular uptake, this difference may drive the selection of different phagocytic subsets. Finally, our model highlights the identity and surface marker characterization of Ag-positive BAL cells. The study by Vermaelen et al. (38) focuses on tissue phagocytes and not BAL cells.

After uptake from the airway, beads trafficked efficiently to the peribronchial LN on a time course that closely approximates that seen in other systems. Ingulli et al. (6) demonstrated the appearance of dye-labeled DCs in the draining lymph 24 h after s.c. injection, whereas other studies have observed the appearance of intratracheally administered DCs in the peribronchial LNs 36 h post-administration (16). Vermaelen et al. (38) detected initial accumulation of FITC-conjugated OVA in the peribronchial node 6 h after administration. However, in this latter case, the Ag was soluble and early arrival may represent rapid draining via lymph channels and sinuses, as has been demonstrated to occur in other situations (39). In our model, the appearance of cell-associated particulate Ag in the peribronchial node began ~6 h after challenge. However, at this early time, beads were localized exclusively to the area of the subcapsular sinus. Based upon this observation, we speculate that the sinus represents a location where bead-bearing cells first enter the LN, trafficking only later to the T cell zone. Twelve hours after i.n. instillation, bead+ clusters were seen in the T zone of the LN, with peak accumulation in this area by 24 h. Using these observations as a foundation, we envision true T cell/APC interactions in the LN to begin in earnest only 6–12 h after administration of the particulate Ag.

Because of this time course and the fact that large amounts of Ag remain in the peripheral tissue, we hypothesized that productive Ag/APC/T cell interactions might occur in the lung periphery before the Ag reached the T zone of the draining LN. Given the fact that nearly one-third of all bead+ cells in the lung (>75% in the BAL) are positive for CD11c and that CD11c+ cells are capable of presenting beads and their conjugated Ags to OVA-specific T cells in vitro, we reasoned that CD11c+ cells responsible for taking up Ag in the lung might also be capable, in vivo, of presenting this same Ag to T cells residing in the surrounding lung tissue.

Our data demonstrating colocalization of bead+ APCs and OVA-specific T cells are consistent with this hypothesis, namely that some T cells first encounter their Ag at a peripheral site outside the LN. Our ability to observe Th1/APC interactions in distal airspaces 6 h after Ag challenge provides evidence that previously activated T cells may encounter Ag in extralymphoid compartments quite early in a secondary immune reaction. This is in contrast to what we and others have observed using naive or undifferentiated cells (40). Furthermore, because beads are already gathered together in clusters 2 h after administration, we believe that T cell/APC interactions are capable of occurring even earlier than 6 h. Whether or not this early interaction occurs at a higher frequency appears to be dependent on the subset of the T cell involved.

In addition to early APC/Th1 cell interactions in the tissue, it was interesting to observe that Th1 cells were also recovered in much higher numbers than Th2 or naive cells from the BAL fluid of animals in the absence of challenge by Ag. As shown in Fig. 5C, Th1 cells number in the thousands in the BAL of naive mice before challenge, whereas Th2 or naive cells are observed at the very limits of detection. This is despite the fact that in vitro differentiated Th1 and Th2 cells have similar levels of the activation markers CD25 and CD69 before transfer and have been stimulated for the same duration in vitro. This suggests that the different behavior of Th1 and Th2 cells is not due to differences in their state of activation. Furthermore, Th1 cell numbers do not increase over time in either the spleen or the nondraining inguinal LN, indicating that there is specific accumulation of these cells in the lung and airways after airway challenge. The precise reason Th1 cells accumulate in the BAL, particularly in the absence of overt inflammation, remains undefined. It is certainly possible that differential expression of chemokine receptors (41) or ligands (42), differential thresholds of stimulation (43), or expression of other currently unidentified signaling molecules leads to preferential recruitment or retention of Th1 cells in the distal airspaces, even in the absence of inflammation. In this regard, Th1 localization to peripheral lung sites is reminiscent of specific Th1 recruitment to the peritoneal cavity under Ag-dependent and Ag-independent inflammatory scenarios (44).

The relatively rapid accumulation over time of TAMRA+ Th1 cells in the BAL of mice after airway Ag challenge is not, in fact, unexpected. Earlier adoptive transfer studies in our lab demonstrated the accumulation of Th1 cells in the BAL of challenged animals with only minimal recruitment of Th2 cells (31). We predict that predominance of Th1s in the BAL 24 h after a primary airway challenge localizes this subset in an environment where they are capable of responding almost immediately to a second inhalation of Ag. Furthermore, coupled with early Th1/APC interactions in an airway response, peripheral localization would allow Th1 cells to exert a profound influence over the outcome of not only the ongoing immune response, but over subsequent immune responses as well. Th1 modulation of an immune response might occur in a multiplicity of ways, including the release of specific inflammatory mediators at the site of Ag encounter or the recruitment, directly or otherwise, of cells types known to be pathogenic (30).

It is clear from our own experience and that of others (38), that fluorescent Ags provide a powerful way of tracking Ags and APCs in vivo. When combined with fluorescent labeling of other cells, visualization in this fashion helps clarify issues of migration and cell contact. Together with data tracking fluorescent Ag to the peribronchial LN, we conclude that, after airway challenge, a significant portion of previously activated Th1 cells first encounter inhaled Ag in the periphery of the lung. The presence of the cells at these peripheral sites facilitates a T cell-mediated response immediately upon subsequent inhalation of immunogen or Ag. Predominance of specific T helper subsets at sites of primary Ag contact (e.g., the skin, the lung, the gut) is important for understanding the regulation of subsequent allergic and nonallergic immune reactions. Additional studies using fluorescently labeled cells and beads will permit better characterization of cell interactions immediately upon Ag uptake, help elucidate the role of inflammatory proteins in bead/Ag handling using genetic knockout animals, and begin to define the differences in the handling and immunogenicity of soluble vs particulate Ags.

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References
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