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Early Interaction of *Yersinia pestis* with APCs in the Lung

Catharine M. Bosio,* Andrew W. Goodyear,* and Steven W. Dow²⁺†¹

Despite the importance of pneumonic plague, little is known of the early pulmonary immune responses that occur following inhalation of *Yersinia pestis*. Therefore, we conducted studies to identify the early target cells for uptake of *Y. pestis* in the lungs following intratracheal or i.v. inoculation. Following intratracheal inoculation, *Y. pestis* was rapidly internalized primarily by a distinctive population of CD11c⁺DEC-205⁺CD11b⁻ cells in the airways, whereas i.v. inoculation resulted in uptake primarily by CD11b⁺CD11c⁺ macrophages and granulocytes in lung tissues. The airway cells internalized and were infected by *Y. pestis*, but did not support active replication of the organism. Intratracheal inoculation of *Y. pestis* resulted in rapid activation of airway CD11c⁺ cells, followed within 24 h by the selective disappearance of these cells from the airways and lungs and the accumulation of apoptotic CD11c⁺ cells in draining lymph nodes. When CD11c⁺ cells in the airways were depleted using liposomal clodronate before infection, this resulted in a significantly increased replication of *Y. pestis* in the lungs and dissemination to the spleen and draining lymph nodes. These findings suggest that CD11c⁺ cells in the airways play an important role in suppressing the initial replication and dissemination of inhaled *Y. pestis*, although these results will also require confirmation using fully virulent strains of *Y. pestis*. Depletion of these airway cells by *Y. pestis* may therefore be one strategy the organism uses to overcome pulmonary defenses following inhalation of the organism. *The Journal of Immunology*, 2005, 175: 6750–6756.

Pneumonic plague is the most deadly form of disease caused by *Yersinia pestis*, but relatively little is known about the early pathogenesis of the disease (1–5). The course of disease is more rapid with a higher mortality rate following inhalation of *Y. pestis* than following cutaneous transmission by flea bite (bubonic plague) (5–7). Thus, it appears that entry via the lungs instead of the skin may provide *Y. pestis* with a selective advantage in terms of early replication and dissemination. Although *Y. pestis* is primarily an extracellular pathogen, in vitro studies indicate that it can infect macrophages under certain conditions (8–10). In addition, histological studies suggest that infection of mononuclear phagocytes in the lungs may occur in the later stages of infection (11, 12). However, the initial target cells for *Y. pestis* in the lungs and the role of innate immunity in controlling pulmonary infection have not been previously determined.

Therefore, we conducted studies to investigate the early immune responses in pneumonic plague. In particular, we were interested in identifying the early target cells in the lungs following inhaled or blood-borne infection. For these studies, we used fluorescently labeled *Y. pestis* organisms to determine how *Y. pestis* interacted with APCs in the airways and lungs. In addition, the effects of infection on early induction of innate immune responses in the lungs were assessed. We found that after intratracheal (IT)³ or intranasal inoculation, the majority of *Y pestis* organisms were taken up by a distinctive population of CD11c⁺ cells that coexpressed DEC-205 and did not express CD11b. Depletion of these CD11c⁺ cells before infection resulted in a significantly increased replication of *Y. pestis* and dissemination to extrapulmonary sites. Thus, CD11c⁺ cells in the airways appear to play a key role in controlling early *Y. pestis* infection following inhalation of the organism.

Materials and Methods

*Mice*

Specific pathogen-free 6- to 8-wk-old female BALB/c and C57Bl/6 mice were purchased from Harlan Sprague Dawley or The Jackson Laboratory. All mice were housed in sterile microisolate cages and provided sterile food and water ad libitum. Protocols for the animal studies described here were approved by the Animal Care and Use Committee at Colorado State University. The *Y. pestis* strain A1122 (an attenuated strain of *Y. pestis* that lacks the pigmentation locus and has also been cured of the low-calcium response (Lcr) plasmid) was provided by M. Chu (Centers for Disease Control, Fort Collins, CO).

Preparation of labeled bacteria

*Yersinia pestis* was labeled with CFSE (Molecular Probes) immediately before use. Freshly thawed bacteria were washed twice and resuspended in sterile PBS to remove brain-heart infusion medium. CFSE was added to the bacteria at a final concentration of 10 μM. Bacteria were incubated for 30 min at 37°C in 5%CO₂ centrifuged once, and resuspended in fresh, sterile PBS. Bacteria were incubated for an additional 30 min, washed three times to remove excess CFSE, and resuspended to a final concentration of 1 × 10⁸ or 4 × 10⁷ CFU/ml in sterile PBS. We found that this technique did not result in appreciable CFSE labeling of nonviable bacteria (data not shown).

Preparation of liposomal clodronate

Dichloromethylene bisphosphonate (Sigma-Aldrich) was used to rehydrate phosphatidylcholine liposomes as previously described (13). Control liposomes were prepared using a 10× PBS solution.

Animal infections and administration of liposomes

Mice were anesthetized i.p. with 200 μl of a 2.5% solution of Avertin (Sigma-Aldrich) and injected IT with 50 μl of a 4 × 10⁷ CFU/ml *Y. pestis* A1122 (to deliver 2 × 10⁸ total CFU/mouse) using a steel 22-gauge gavage needle passed blindly into the trachea. In other experiments, mice were infected i.v. with 200 μl of 1 × 10⁸ CFU/ml *Y. pestis* (2 × 10⁷ total CFU/mouse) via the lateral tail vein.

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Collection and flow cytometric analysis of airway and lung cells

Airway cells were collected by bronchoalveolar lavage (BAL) as previously described (14, 15). Single-cell suspensions of lung cells were isolated by enzymatic digestion of lung tissue as previously described (16). Cells in the BAL fluid and lung digests were characterized using 5-7 color flow cytometry. Abs used for these studies included anti CD11c, CD11b, Gr-1, B220, CD8, CD4, CD3, I-A/E, CD86, F4/80, CCR7, anti-CD40 (eBioscience), and anti-DEC-205 (Cedarlane Laboratories) variously conjugated to biotin, allophycocyanin, PE, FITC, PE-Cy5, PE-Cy7, or allophycocyanin-Cy7. For most experiments, 5-6 color staining was done. Data were collected using a Cyan MLE flow cytometer (DakoCytomation). Samples were gated on forward and side scatter characteristics for viable cells and analyzed blinded to the conditions of the experiment. The percentage of positive events was determined after collecting at least 25,000 events (BAL samples) or at least 100,000–150,000 events (lung tissue digest samples).

Enumeration of bacteria in organs

Lungs, spleens, and mediastinal lymph nodes were collected and homogenized in sterile PBS using a stomacher (Teledyne Tekmar). Bacterial colony counts in each organ were determined by plating serial 10-fold dilutions of organ homogenate on blood agar and incubating the plates at 37°C for 24 h.

Brightfield and immunofluorescence microscopy

One hour after IT infection, airway dendritic cells were collected as described above. For light microscopy, BAL cells were stained with modified Wright-Giemsa stain and photographed with a Leica light microscope and deconvolution microscope. Superfrost slides (VWR) for 30 min at 37°C and then stained with anti-CD11c-biotin Ab (eBioscience), followed by incubation with streptavidin biotin-conjugated Alexa-647 (Molecular Probes) and fixation in 1% paraformaldehyde. Fluorescence microscopy indicated that bacteria associated with these cells in the airways of normal mice with a CD11c+CD11b− phenotype. We have also made similar observations in several different strains of mice, including C57BL/6, 129Sv, and ICR mice (C. M. Bosio and S. W. Dow, unpublished data). Thus, Y. pestis appeared to preferentially infect a population of airway cells that phenotypically resembled dendritic cells, although these cells have been previously classified as alveolar macrophages by most investigators.

Assessment of apoptosis

Apoptosis of airway cells, lung cells, and lymph node cells were assessed using annexin V and propidium iodide staining and flow cytometry according to the manufacturer’s directions (TACS Annexin V kit; R&D Systems). For detection of apoptosis of specific cell types, surface immunostaining was done first, followed by incubation with annexin V reagent. Immediately before analysis by flow cytometry, propidium iodide was added. Early apoptotic cells were those that were positive for annexin V alone, and late apoptotic cells were those that were positive for both annexin V binding and propidium iodide uptake.

Statistical analysis

Statistical differences between two groups were determined using an unpaired t test, with significance set at p < 0.05. For comparisons among three or more groups, analysis was done by one-way ANOVA, followed by Tukey’s multiple comparisons test, with significance determined for p < 0.05.

Results

Inhaled Y. pestis infects a phenotypically distinctive population of airways cells after IT inoculation

To identify the initial target cells for Y. pestis in the lungs following inhalation of the organism, BALB/c mice were challenged IT with 2 × 10⁶ CFSE-labeled Y. pestis organisms. Airway cells were harvested by BAL 1 h later and analyzed by flow cytometry. We observed that the large majority (~80%) of cell-associated CFSE+ bacteria in the airways were contained within a distinctive population of CD11c+CD11b− cells (Fig. 1). These CD11c+ cells in the airways also uniformly coexpressed DEC-205 (data not shown). In contrast, only 7–8% of labeled bacteria in the airways were associated with CD11b+ cells (Fig. 1A). The remaining 10–12% of cell-associated bacteria were associated with cells that did not stain with the panel of Abs used for these experiments. When the number of CD11c+ and CD11b+ cells containing labeled Y. pestis organisms in BAL samples following IT inoculation was determined, this analysis also confirmed that the majority of organisms were taken up by CD11c+ cells (Fig. 1C). Similar results in terms of cellular distribution of labeled bacteria were obtained when bacteria were inoculated intranasally, although the numbers of bacteria were lower (data not shown).

Lung digests were also analyzed to track the uptake of CFSE-labeled Y. pestis following IT inoculation. Again, labeled Y. pestis was found primarily in the population of CD11c+CD11b− cells (Fig. 1B). These results suggested that shortly after inhalation, Y. pestis in the airways was primarily associated with a distinctive population of CD11c+DEC-205+ cells that also did not express CD11b. Expression of CD11c by alveolar macrophages has been described previously, although in previous reports many alveolar macrophages coexpressed CD11b, and expression of DEC-205 was not examined (15, 18). We are not aware of previous reports demonstrating a large population of cells in the airways of normal mice with a CD11c+CD11b−DEC-205− phenotype. We have also made similar observations in several different strains of mice, including C57BL/6, 129Sv, and ICR mice (M. Bosio and S. W. Dow, unpublished data). Thus, Y. pestis appeared to preferentially infect a population of airway cells that phenotypically resembled dendritic cells, although these cells have been previously classified as alveolar macrophages by most investigators.

Experiments were done next to determine whether the route of inoculation influenced uptake of Y. pestis by different populations of APCs in the lung. Mice were inoculated i.v. with CFSE-labeled Y. pestis and cellular uptake in lung tissues was assessed 1 h later. In contrast to the results obtained following IT inoculation, i.v. inoculation resulted in uptake almost exclusively by CD11b+ cells (Fig. 1, A and B). In the example shown, ~60% of all CD11b+ cells present in lung tissues contained CFSE-labeled Y. pestis after i.v. inoculation, which accounted for nearly 98% of all cell-associated bacteria present in the lungs. In contrast, <1% of CD11c+ cells contained labeled Y. pestis after i.v. inoculation (Fig. 1B).

The population of CD11b+ cells that contained CFSE-labeled Y. pestis was further subdivided into Gr-1+ cells (granulocytes) and Gr-1− cells (macrophages). This analysis revealed that after i.v. inoculation ~70% of labeled Y. pestis organisms were contained in CD11b+Gr-1− granulocytes, whereas 30% were contained within CD11b+Gr-1+CD11c− macrophages. The CD11b+Gr-1+ population was also F4/80+, consistent with their identification as granulocytes (data not shown). Thus, blood-borne infection of the lungs with Y. pestis resulted in infection of CD11b+CD11c− lung macrophages and granulocytes, whereas inhalation led to infection of a distinctive population of CD11c+CD11b− cells.

The cellular localization of Y. pestis within infected cells in the lungs was also assessed using light microscopy and deconvolution immunofluorescence microscopy. By light microscopy, large numbers of bacteria were found associated with alveolar macrophages obtained by BAL (Fig. 1D). Analysis by deconvolution fluorescence microscopy indicated that bacteria associated with these CD11c+ cells were internalized and not present on the cell surface (Fig. 1D and our unpublished data). In addition, addition of trypan blue failed to quench the fluorescence of CFSE+ bacteria in lungs.
cells, again consistent with an intracellular location (data not shown).

When airway cells obtained by BAL were infected in vitro, live intracellular *Y. pestis* could be recovered 24 h after eradication of all extracellular bacteria by gentamicin treatment (data not shown). In addition, the numbers of intracellular bacteria did not increase over time in culture over a 72-h period (data not shown). Fully virulent *Y. pestis* strain CO92 also infected airway cells, but again without evidence of intracellular replication (data not shown). These results are therefore most consistent with phagocytosis and internalization of *Y. pestis* by airway cells following in vivo infection, rather than simply binding of the organism to the cell surface.

*Y. pestis* infection induces rapid activation of CD11c<sup>+</sup> airway cells

Previous reports suggested that certain *Y. pestis* proteins may interfere with activation of APCs in vitro (19, 20). Therefore, we investigated directly in vivo the effects that infection with the entire *Y. pestis* organism had on CD11c<sup>+</sup> airway cells. As early as 1 h after IT inoculation with *Y. pestis*, significant up-regulation of CD86 expression (10-fold increase over mock-infected controls) by CD11c<sup>+</sup> airway cells was observed (Fig. 2). In addition, significant and rapid up-regulation of expression of 4-1BBL and MHC class II expression was also observed. In contrast, i.v. inoculation with *Y. pestis* did not result in significant up-regulation of CD86, MHC class II, or 4-1BBL expression by CD11c<sup>+</sup> cells (data not shown). These results indicate that direct exposure to *Y. pestis* in the airways, rather than indirect exposure as occurred in the case of i.v. inoculation, was required to induce activation of CD11c<sup>+</sup> airway cells.

Cytokine responses to infection were assessed using BAL cells that were collected 1 h after IT inoculation with *Y. pestis* and processed as described in *Materials and Methods* for detection of intracellular TNF-α production. CD11c<sup>+</sup> airway cells rapidly produced TNF-α in response to *Y. pestis* infection (Fig. 2). TNF-α was also released into supernatants following overnight culture (Fig. 2) and significant increases in secretion of IL-6 and MCP-1 were also observed, whereas IL-10 was not produced (our unpublished data). Thus, CD11c<sup>+</sup> airway cells were rapidly activated to up-regulate costimulatory molecules and produce proinflammatory cytokines following infection with *Y. pestis*.

Direct infection with *Y. pestis* required for up-regulation of CCR7 expression by CD11c<sup>+</sup> airway cells

Up-regulation of CCR7 is necessary for dendritic cell migration to draining lymph nodes (21, 22). Therefore, we assessed the effects

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**FIGURE 1.** Infection of CD11c<sup>+</sup> airway cells by inhaled *Y. pestis*. BALB/c mice (four animals per treatment group) were inoculated IT or i.v. with 2 × 10<sup>6</sup> CFSE-labeled *Y. pestis*. One hour later, the mice were sacrificed and airway cells were collected by BAL. Total lung cells were also prepared by enzymatic digestion of lung tissues. Airway and lung cells that contained CFSE<sup>+</sup> *Y. pestis* were identified using multicolor flow cytometry as described in *Materials and Methods*. A, Flow cytometric analysis of BAL cells obtained after IT inoculation with CFSE-labeled *Y. pestis*. Labeled *Y. pestis* was found primarily to be associated with CD11c<sup>+</sup>CD11b<sup>+</sup> alveolar macrophages. These data are representative of results obtained in at least four independent experiments. B, Flow cytometric analysis of total lung cells obtained by enzymatic digestion 1 h after IT or i.v. inoculation with CFSE-labeled *Y. pestis*. Intravenous inoculation resulted in uptake primarily by CD11b<sup>+</sup>CD11c<sup>+</sup> cells, whereas IT inoculation resulted in uptake primarily by CD11c<sup>+</sup>CD11b<sup>+</sup> cells. C, The mean number (±SD) of CD11c<sup>+</sup> or CD11b<sup>+</sup> cells taking up CFSE-labeled *Y. pestis* following IT or i.v. inoculation of mice was determined (five animals per group). Similar results were obtained in two independent experiments. D, Left panel, Wright-Giemsa stain of alveolar macrophages obtained 1 h after IT inoculation with *Y. pestis*. Right panel, Immunofluorescent micrograph of CD11c<sup>+</sup> airway cells (orange) containing internalized CFSE-labeled *Y. pestis* (green). Scale bar, 2 μm. Data are representative of three independent experiments.
of *Y. pestis* infection on CCR7 expression by CD11c+ airway cells. Within 24 h of IT inoculation with CFSE-labeled *Y. pestis*, there was significant up-regulation of CCR7 expression by airway CD11c+ cells (Fig. 3). Of note, the up-regulation of CCR7 expression was restricted primarily to airway cells that contained CFSE-labeled *Y. pestis*, whereas airway cells from the same infected mice that did not contain *Y. pestis* failed to exhibit significant up-regulation of CCR7 expression. These results indicate that direct infection with *Y. pestis* served as the primary stimulus for up-regulation of CCR7 expression, rather than up-regulation being mediated indirectly by cytokine release.

**Y. pestis** infection induces depletion of CD11c+ cells in the lungs and airways

The effects of *Y. pestis* infection on numbers of CD11c+ cells in the lungs and airways was assessed. Between 1 and 4 h after IT inoculation, the numbers of CD11c+ airway cells in BAL samples or lung digests did not change significantly (data not shown). However, by 24 h after infection, the total number of CD11c+ cells in both BAL and lung digest samples declined significantly (Fig. 4). For example, the percentage of CD11c+ cells in the airways declined from an average of 80 to 4% at 24 h after infection. The decline in the numbers of CD11c+ cells in BAL specimens was not however due to retention in alveolar spaces, as the number of CD11c+ cells present in lung digests was also decreased significantly (Fig. 4). Along with the decrease in CD11c+ cells, there was a large increase in the number of CD11b+Gr-1+ neutrophils in lung tissues and airways (data not shown). Thus, pulmonary infection following inhalation of *Y. pestis* induced selective depletion of CD11c+CD11b+ cells in the lungs and airways.

**Depletion of CD11c+ cells before infection results in increased *Y. pestis* replication and dissemination to lymph nodes and spleen**

Experiments were done next to determine the effect of depletion of CD11c+ airway cells before infection with *Y. pestis*. IT administration of liposomal clodronate has been used previously to deplete alveolar macrophages and has also been used to deplete dendritic

**FIGURE 2.** *Y. pestis* infection induces rapid activation and release of proinflammatory cytokines by CD11c+ airway cells. Mice (four per group) were infected IT with 2 x 10^6 *Y. pestis* organisms and lung, and BAL cells were obtained for analysis of activation or cytokine release. A, One hour after IT inoculation, lung cells were prepared by enzymatic digestion of lung tissues and immunostained for cell surface and activation markers (CD86, 4-1BBL, and MHC class II) and analyzed by flow cytometry. Data were representative of three independent experiments (n = 5 mice per experiment). B, Cell surface expression of CD86, 4-1BBL, or MHC class II was determined. IT inoculation with *Y. pestis* induced statistically significant (p < 0.01) increases in the number of CD11c+ cells expressing CD86, 4-1BBL, and MHC class II, compared with control animals. Data are representative of three independent experiments (n = 5 mice per experiment). C, Lung tissues and airways (data not shown). Thus, pulmonary infection following inhalation of *Y. pestis* induced selective depletion of CD11c+CD11b+ cells in the lungs and airways.

**FIGURE 3.** Infection of airway CD11c+ cells with *Y. pestis* directly induces up-regulation of CCR7 expression. Mice (three to four per group) were infected IT with CFSE-labeled *Y. pestis*, then airway cells were collected 24 h later. CCR7 expression was assessed by flow cytometry as described in Materials and Methods. In infected mice, there was a significant overall increase in CCR7 expression compared with uninfected control mice. However, only CD11c+ cells that contained labeled *Y. pestis* had significantly increased expression (p < 0.001) of CCR7, whereas CCR7 expression was not significantly increased on uninfected CD11c+ cells from infected mice. Similar results were obtained in one additional experiment.

**FIGURE 4.** Inhalation of *Y. pestis* induces the disappearance of CD11c+ airway cells. Mice (four per group) were inoculated IT with 2 x 10^6 CFU of *Y. pestis*. At 24 and 48 h after inoculation, BAL and lung digest samples were collected and prepared for immunostaining and flow cytometry. A, The percentages of CD11c+ airway cells present in the airways (BAL) and lung digests (Lung) were determined by flow cytometry in uninfected and *Y. pestis*-infected mice 24 h after infection. B, The mean number (±SD) of CD11c+ cells present in BAL and lungs of infected mice at 24 and 48 h after IT inoculation was calculated and plotted. Similar results were obtained in two additional experiments. *p < 0.05.
cells (23–25). We observed that IT instillation of liposomal clodronate eliminated >90% of CD11c+ airway cells (Fig. 5). Therefore, mice were pretreated with liposomal clodronate and inoculated IT with *Y. pestis* 18 h later. We found that 24 h after infection, mice depleted of CD11c+ airway cells had significant increases in bacterial counts in the lung (*p < 0.01*) compared with untreated mice or to mice treated with control (PBS) liposomes (Fig. 5). By day 4 of infection (1 day before the mice were euthanized due to progressive bacterial replication), liposomal clodronate-treated mice had significant increases in bacterial numbers in both the lung (*p < 0.01*) and spleen (*p < 0.01*), compared with untreated mice or mice treated with control liposomes. In addition, the number of bacteria in the mediastinal lymph nodes of liposomal clodronate-treated mice was also significantly increased (*p < 0.05*) compared with control mice or mice treated with PBS liposomes. These results indicate that CD11c+ airway cells are critical to controlling the initial replication of *Y. pestis* in the lungs, as well as the dissemination of *Y. pestis* to draining lymph nodes and extrapulmonary organs such as the spleen.

**Y. pestis infection induces increased trafficking and apoptosis of CD11c+ cells in draining lymph nodes**

The preceding experiments indicated that *Y. pestis* infection induced significant depletion of CD11c+ cells in the airways and lungs. Therefore, experiments were conducted to assess the site and mechanism of CD11c+ cell depletion. Airway cells, lung cells, and mediastinal lymph node (MLN) cells were collected at 4 and 24 h after IT infection with *Y. pestis* and induction of apoptosis was assessed using a combination of CD11c staining and staining with annexin V and propidium iodide (Fig. 6). A significant increase in the number or percentage of apoptotic CD11c+ cells in the airways or lungs was not observed at either time point following infection with *Y. pestis* (data not shown). However, when the MLN were examined, we observed a significant increase in the number of CD11c+ cells 24 h after *Y. pestis* infection (Fig. 6A). In addition, there was also a significant increase in the number of apoptotic CD11c+ cells in the MLN (Fig. 6C), along with an overall increase in the number of apoptotic cells in the MLN of *Y. pestis*-infected mice (Fig. 6B). These results indicated therefore that the loss of CD11c+ airway cells following *Y. pestis* infection was most likely due to trafficking of these cells to draining MLN, followed by apoptosis in situ.

**Discussion**

There are several major findings to emerge from these studies. First, we observed that *Y. pestis* readily infected a population of CD11c+CD11b− cells in the airways of mice shortly after inhalation of the organism. Second, infection of these cells led to their rapid activation, as reflected by cytokine secretion and up-regulation of costimulatory molecule. This was followed by the rapid disappearance of significant numbers of these cells from the airways and lungs within 24 h of infection, most likely due to increased expression of CCR7 followed by apoptosis in draining lymph nodes. Finally, we observed that depletion of these airway cells before IT inoculation resulted in significantly enhanced replication of *Y. pestis* in the lungs and dissemination to draining lymph nodes and spleen. These results therefore point to a critical role for these CD11c+ airway cells in the initial control of pneumatic plague. However, it should also be noted that these results will also need to be confirmed using a fully virulent strain of *Y. pestis*, rather than an attenuated strain as was used in these studies.

We found that the airway CD11c+ cells were readily infected with *Y. pestis* in vivo, rather than simply binding the organism on the cell surface. Previous in vitro experiments have suggested that the F1 glycoprotein, found in virulent strains of *Y. pestis* and in the attenuated strain used in these studies, functions to prevent phagocytosis of the bacterium (8–10, 26). Our data countered these observations and demonstrated that F1 may have a limited role in the

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**FIGURE 5.** Depletion of CD11c+ cells before infection results in increased replication of *Y. pestis* in lungs and dissemination to lymph nodes and spleen. The effects of prior depletion of CD11c+ airway cells on replication and dissemination of *Y. pestis* were assessed in mice subjected to IT challenge. Mice were depleted of CD11c+ cells by administration of liposomal clodronate 18 h before *Y. pestis* challenge by IT inoculation as described in Materials and Methods. Mice were euthanized at 24 and 96 h after challenge and bacterial numbers in lung, spleen, and MLN tissues were determined as described in Materials and Methods. A, IT instillation of liposomal clodronate induced significant depletion of CD11c+ airway cells (*, *p < 0.01*). Similar results were obtained in two additional experiments. B, Bacterial counts (CFU/ml) in the lung, MLN, and spleen were determined 24 or 96 h after IT inoculation with 2 × 10^6 *Y. pestis*. The mean number of bacteria (± SE) in each organ was determined in untreated mice, mice pretreated 18 h before with clodronate liposomes (Clodronate), or mice pretreated with PBS control liposomes (Liposome). ***, *p < 0.01* for clodronate-treated mice compared with untreated mice or mice treated with control liposomes. ***, *p < 0.01* for bacterial counts in lung and spleen tissues of clodronate-treated mice vs untreated mice or control liposome-treated mice; *p < 0.05* for MLN in clodronate-treated compared with untreated or liposome-only treated mice. Data are representative of three independent experiments.
inhibition of *Y. pestis* phagocytosis by resident airway APCs and neutrophils. The ability of *Y. pestis* to rapidly infect these airway APCs suggests the presence of specific receptors for uptake of the organism. Although the strain of *Y. pestis* used in these studies (strain A1122) is considered attenuated due to the absence of the pigment locus and the Lcr plasmid, this strain is still lethal at the doses used in these studies (data not shown). The Lcr plasmid encodes for several proteins important for survival of the bacteria in the mammalian host, including the *Yersinia* outer surface proteins (Yops) and the V Ag (1, 27, 28). The direct effects of these virulence factors on alveolar macrophages have not been previously investigated, but it is likely that infection with fully virulent *Y. pestis* would lead to even greater depletion of alveolar macrophages in vivo. Furthermore, our data also indicate that the fully virulent *Y. pestis* strain CO92 (which expresses both Yops and V Ag) readily infects alveolar macrophages (our unpublished data).

Our studies also provide new information regarding the phenotype of the predominant APCs present in the airways of mice. The majority of cells present in the airways of normal mice are classified as alveolar macrophages based primarily on cytologic features (29–32). However, alveolar macrophages have also been reported to express some surface markers more often associated with dendritic cells, including expression of CD11c (15, 18). Our results concur with the earlier reports in that the majority of airway cells classified as alveolar macrophages expressed CD11c, but differ in the fact that we found that the large majority of these airway cells did not express CD11b. In addition, we found that virtually all CD11c+ airway cells coexpressed DEC-205, a molecule typically expressed by dendritic cells (33, 34). Therefore, the airway APCs that most would classify as alveolar macrophages appear to actually resemble cells with a phenotype intermediate between macrophages and immature dendritic cells. For this reason, we have chosen in this article to refer to these cells as “CD11c+ airway cells” to emphasize their unique phenotype relative to classical macrophages or dendritic cells.

When *Y. pestis* was inoculated i.v., we found that an entirely different population of APCs was infected. Following i.v. inoculation (Fig. 1), the target cells for *Y. pestis* uptake were either typical CD11b+CD11c− macrophages or neutrophils (Gr-1+CD11b−). Thus, there appears to be tight compartmentalization of the different APC populations within the lungs and *Y. pestis* is restricted to infecting these separate populations of APCs depending on the route of inoculation. The initial restriction to different pulmonary APC populations may therefore have important consequences for the outcome following infection of the lungs by inhalation (pneumonic plague) or via the bloodstream (bubonic plague). For example, our studies suggest that the CD11c+ cells in the airways are protective, inasmuch as depleting these cells before infection resulted in significantly enhanced replication in the lungs, as well as increased dissemination of the organism (Fig. 4).

The ability of *Y. pestis* to induce the depletion of CD11c+ airway cells may represent a survival strategy for the organism, since these cells suppressed the replication and dissemination of *Y. pestis* in the lungs. The rapid disappearance of CD11c+ cells following inhalation of *Y. pestis* (Figs. 3 and 6) was most likely due to first migration from the site of infection followed by death in the draining lymph nodes. This conclusion is based on two primary observations. First, CD11c+ cells in the airways increased expression of CCR7. The majority of these CD11c+CCR7+ cells were infected with *Y. pestis* (Fig. 3) indicating that an increase in CCR7 is a direct effect of infection. Up-regulation of CCR7 is typically attributed to dendritic cells undergoing maturation and enables their movement to the draining lymph nodes (21, 22). Second, we observed a significant increase in apoptosis of CD11c+ cells in the lymph node draining the lung (MLN) after *Y. pestis* infection (Fig. 6C). We did not observe evidence of increased apoptosis of CD11c+ cells in the airways or lungs following *Y. pestis* infection. Therefore, although we cannot exclude the possibility that apoptotic cells in the airways were removed too rapidly to detect an increase in apoptosis, it is more likely that they exited the airways, traveled to the MLN where they underwent apoptosis, and successfully seeded the MLN with *Y. pestis*.

A final conclusion to emerge from our studies is the idea that inflammatory cells such as neutrophils recruited to the lungs following inhalation of *Y. pestis* are relatively ineffective in controlling the overall pathogenesis of *Y. pestis* infection compared with classical APCs. This is apparent in mice treated with liposomal clodronate to deplete APCs. Although liposomal clodronate-treated mice retained the ability to recruit granulocytes after infection (data not shown), they had significantly more bacteria in the lung and spleen after infection (Fig. 5). Thus, CD11c+ airway cells are the initial target cell for infection by inhaled *Y. pestis* and also appear to be uniquely suited to controlling early *Y. pestis* replication in the lungs and preventing systemic dissemination. Destruction of the CD11c+ cells therefore appears to represent one
strategy by which Y. pestis is able to escape innate immune defenses in the lungs.

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Disclosures

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References