CD11c<sup>+</sup>CD8<sup>α</sup> Dendritic Cells Promote Protective Immunity to Respiratory Infection with <i>Bordetella pertussis</i>

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B. pertussis is a Gram-negative bacterium that causes the severe debilitating disease whooping cough in infants and young children (1). Despite the widespread availability of whole cell and acellular pertussis vaccines, infection still causes significant morbidity and mortality worldwide. Furthermore, adolescents and adults may act as reservoirs for the bacterium and can subsequently infect nonvaccinated infants (2, 3). The bacteria colonize the nasopharynx, trachea, and bronchial tree of the lungs. Clearance of the bacteria is dependent on IFN-γ, produced locally in the lungs, initially by infiltrating NK cells (4) and then by Th1 cells (5, 6). However, infection also induces Ag-specific Th17 cells and IL-10-producing regulatory T (Treg) cells (7, 8). These Treg cells can subvert the development of adaptive immunity and delay clearance of bacteria from the lungs (8).

Immature dendritic cells (DC) are located in almost all tissues of the body, including the various compartments in the lung (9). Following activation through pattern recognition receptors, the DC mature and migrate to the draining lymph nodes where they present Ag to resident naïve CD4+ T cells (10). DC can be subdivided based on the expression of the integrins CD11c, CD8α, and CD45RA (11–16). It has been reported that CD11c+CD8α+ DC can inhibit CD4+ T cell activation and proliferation by causing Fas-induced cell death (13, 14). Based on these studies and others, it has been assumed that CD11c+CD8α+ DC contribute to peripheral tolerance, while CD11c+CD8α− DC initiate immunity to infection. In more recent studies, CD24 and CD45RA have been used to discriminate proinflammatory lymphoid CD11c+CD8α+ DC (CD11c+CD8α−CD24+CD45RA−) from plasmacytoid DC (pDC; CD11c+CD8α+CD24+CD45RA−) (11, 12). Although pDC have a proinflammatory role, particularly against viral infection (17, 18), these cells have been shown to possess immunoregulatory activity in mice (19) and humans (20). CD11c+CD8α− CD24+CD45RA− DC are proinflammatory and are capable of capturing Ags from other DC that migrate from a site of infection, such as the lung (11, 12). Studies in virally infected mice have shown that this transferred Ag is subsequently presented to T cells in draining lymph nodes during the first 3 days of infection (11, 12). Unlike conventional bone marrow-derived (CD8α+) DC, CD11c+CD8α−CD24+CD45RA− DC (referred to as CD11c+CD8α− DC from here on) express the FMS-like tyrosine kinase 3 (Flt3) receptor and are not generated from bone marrow cells by GM-CSF but with Flt3 ligand (FL) in vitro (21).

CD11c+ DC that express the αβε integrin CD103+ have been described in the intestine where they promote inducible regulatory T cells by a retinoic acid and TGF-β-dependent mechanism (22–24). Conversely, proinflammatory CD103+CD11c+CD8α−CD11b−DC have been detected in the mucosal lining of the lung and have been shown to play a role in a murine model of airway hypersensitivity (25, 26). In the present study, we investigated the role of CD11c+CD8α+ and CD11c+CD103+ DC in immunity to B. pertussis infection in mice. We used an established murine model of B. pertussis infection, where vaccine-induced protection correlates with efficacy in children (27). We addressed the hypothesis that CD11c+CD8α+...
DC may either play an immunoregulatory role by activating Treg cells or a protective role by promoting effector Th1 cells. We found significant infiltration of CD11c<sup>+</sup>CD8α<sup>+</sup> DC into the cervical lymph nodes (CLN) and then the lungs 1–7 days after infection. These DC were activated, expressed MHC, costimulatory molecules, as well as CD103, and secreted IFN-γ, IL-4, and IL-10. Depletion of CD8α<sup>+</sup> cells throughout infection and more importantly immediately before and after <i>B. pertussis</i> challenge delayed clearance of the bacteria, suggesting that these DC play a protective role very early in infection. Blocking CD103 function before clearance of the bacteria, suggesting that these DC play a protective role very early in infection. Blocking CD103 function immediately before and after infection also had a dramatic inhibitory effect on bacterial clearance. Finally, transfer of FL-generated CD11c<sup>+</sup>B. pertussis infection also had a dramatic inhibitory effect on bacterial clearance. Our findings demonstrate that CD11c<sup>+</sup>CD8α<sup>+</sup> DC play a significant role in protective immunity to respiratory infection with <i>B. pertussis</i>.

Materials and Methods

Animals

BALB/c mice were purchased from Harlan U.K. Animal experiments and maintenance were approved and regulated by the university ethics committee and the Irish Department of Health.

<i>B. pertussis</i> challenge model

Mice were challenged by aerosol exposure to live <i>B. pertussis</i>. Virulent <i>B. pertussis</i> Wellcome 28 was grown on 36°C in Stainer-Scholte liquid medium. Bacteria from a 48-h culture were resuspended at a concentration of ~2 × 10<sup>10</sup> CFU/ml in physiological saline containing 1% casein. The challenge inoculum was administered to mice over a period of 15 min (1.5 ml/min) with a nebulizer in a sealed container within a class 3 exhaust-protected cabinet. This was followed by a rest period of 10 min. The course of infection was followed by performing <i>B. pertussis</i> CFU on lung homogenates at intervals after infection (8).

Depleting and blocking Abs

Hybridoma cells were obtained from the American Type Culture Collection and secreted IgG that was purified from hybridoma supernatants using a MiniTrap affinity chromatography kit (GE Healthcare). The optimal concentration of Ab required to deplete >95% of the relevant target cells was determined by titration. Briefly, mice were injected i.p. with increasing concentrations of depleting Ab. The spleens of these mice were subsequently investigated for the target cell 24, 48, and 72 h after injection by flow cytometric analysis. The hybridoma cell lines, clones 2.43 and N418, were used to generate rat anti-mouse (IgG1) CD8<sup>+</sup> hybridoma supernatants were removed and analyzed for IL-10, IL-12p70, IFN-γ, and IL-4 by ELISA (R&D systems). Proliferation was assessed 72 h after activation by tritiated thymidine incorporation.

Generation of CD11c<sup>+</sup>CD24<sup>-</sup>CD45RA<sup>-</sup> cells with <i>FL</i> in vitro

Bone marrow cells were isolated from the femurs of BALB/c mice and cultured at a concentration of 2 × 10<sup>5</sup> cells/well in Stemline dendritic cell maturation medium (Sigma-Aldrich) supplemented with 0.3 g/l t-glutamine and 300 mg/ml recombinant FL (ImmunoTools). Cells were incubated in humidified air for 8 days at 37°C and in 10% CO2. Additional FL (300 ng/ml) was added on days 3 and 6. These cells were subsequently washed and stained with Abs against CD24, CD45RA, and CD11c. CD11c<sup>+</sup>CD24<sup>-</sup>CD45RA<sup>-</sup> cells (FL-DC) were purified using a MoFlo cell sorter and counted. In brief, 0.5 × 10<sup>6</sup> CD11c<sup>+</sup>CD45RA<sup>-</sup> cells were injected i.p. into mice 30 min after infection.

Uptake of green fluorescent latex bead (FLB) particles by DC

Plain yellow-green fluorescent 0.5-μm latex particles (Polysciences) were diluted 1/25 in PBS for a final administration of ~3.64 × 10<sup>4</sup> particles/mouse. Twenty microliters of this solution was administered intranasally (i.n.) 15 min before infection. Control mice received latex particles in the absence of infection.

Statistical analysis

Results, expressed as mean ± SEM were compared using the Student t test or by ANOVA. Values of p < 0.05 were considered statistically significant.

Results

<i>CD11c</i><sup>+</sup><i>CD8α</i> DC infiltrate the lungs and draining lymph nodes during acute phase of infection with <i>B. pertussis</i>

We investigated the infiltration and function of CD11c<sup>+</sup>CD8α<sup>+</sup> lymphoid DC in the lungs and draining lymph nodes of mice through the course of infection with <i>B. pertussis</i>. Respiratory challenge of naïve BALB/c mice by exposure to an aerosol of live <i>B. pertussis</i> resulted in a reproducible nonlethal infection with peak bacterial counts on day 14 and approached complete clearance by 28 days (Fig. 1A). The relative percentage of CD11c<sup>+</sup>CD8α<sup>+</sup> DC was determined by gating on viable lymphocytes from forward vs side scatter plots in conjunction with the appropriate isotype and compensation controls. The total number of cells expressing various surface Ags was calculated based on the total number of viable cells and the percentage of CD11c<sup>+</sup>CD8α<sup>+</sup> cells within the viable lymphocyte gate. The numbers of CD11c<sup>+</sup> and CD11c<sup>+</sup>CD8α<sup>+</sup> DC were low in the CLN, thoracic lymph nodes (TLN), and lungs of noninfected control mice (Fig. 1, B–D). However, during <i>B. pertussis</i> infection, we consistently found an early influx of CD11c<sup>+</sup> and CD11c<sup>+</sup>CD8α<sup>+</sup> DC into the CLN and a later
Representative FACS profiles for CD11c

FIGURE 1. Recruitment of CD11c⁺CD8α⁺ DC to the lungs and draining lymph nodes soon after respiratory challenge with B. pertussis. Mice were infected by exposure to an aerosol of live virulent B. pertussis. The course of infection was followed by performing CFU counts on lung homogenates at intervals after B. pertussis challenge-infected mice. B–D, The CLN, TLN, and lungs were removed and digested with Collagenase D at various time points after challenge. Cells were counted and stained for CD11c and CD8α. The relative percentage of CD11c⁺CD8α⁺ DC was determined by gating on viable lymphocytes in conjunction with the appropriate isotype and compensation controls. The total number of CD11c⁺ (B) or CD11c⁺CD8α⁺ (C) was calculated from the total number of viable cells and the percentage of CD11c⁺ or CD11c⁺CD8α⁺ cells within the viable lymphocyte gate. D, Representative FACS profiles for CD11c⁺CD8α⁺ cells in either CLN or lung tissue from naive control mice or B. pertussis-infected mice 1 and 4 days after challenge. These results are representative of four separate experiments. *, p < 0.05 and **, p < 0.01 vs noninfected control mice.

influx into the lungs (Fig. 1, B–D). The numbers of both DC subsets in the CLN decreased dramatically between 4 and 24 h of aerosol challenge with live B. pertussis and steadily diminished thereafter to baseline levels (Fig. 1, B and C). Conversely, the number of CD11c⁺ and CD11c⁺CD8α⁺ DC increased in the lung from day 1 and peaked on days 5–7 of infection. The number of DC subsets in the draining lymph nodes and lungs declined to that in control mice after 14–21 days (Fig. 1, B and C). There was a small, but not statistically significant, increase in the frequency of CD11c⁺CD8α⁺ DC in the TLN 3 days after B. pertussis aerosol challenge (Fig. 1C). However, the number had returned to baseline values by day 5 (Fig. 1C) and remained there when examined 7, 14, and 21 days after challenge (data not shown). Our findings suggest that following B. pertussis aerosol challenge, DC are recruited to the CLN and then may migrate to the lungs.

CD11c⁺CD8α⁺ DC in the CLN acquire particulate Ag from the respiratory tract within 4 h of respiratory challenge with B. pertussis

We have shown that CD11c⁺CD8α⁺ DC with a mature phenotype are present in the CLN of mice within 24 h of B. pertussis challenge. However, it is unclear whether CD11c⁺CD8α⁺ DC migrate from the lung or reside in the CLN and acquire Ag from other migrating cells types. A recent report suggested that CD11c⁺CD8α⁺ DC are resident in the draining lymph nodes and rarely migrate (28). Therefore, we hypothesized that these cells acquire particulate Ag from dying APC that migrate from the lung and in turn present Ag via MHC to T cells within the lymph node. To test this hypothesis, green FLB were administered i.n. to mice 15 min before infection with B. pertussis and the number of FLB⁺ DC were determined in the CLN and lung at different time points after infection. FLB were found in the total leukocyte population and in CD11c⁺CD8α⁺ DC isolated from the CLN 4 h after infection (Fig. 2A). On the other hand, very few total FLB⁺ leukocytes or FLB⁺CD11c⁺CD8α⁺ DC were detected in the CLN or lungs of
noninfected mice up to day 3 after infection (Fig. 2B and data not shown). Furthermore, very few FLB \(^+\) cells were detected in the lungs from either infected or noninfected control mice up to day 7 after challenge (data not shown). These findings suggest that CD11c\(^+\) CD8\(\alpha\)^+ DC in the CLN can acquire particulate Ag from the upper respiratory tract within hours of infection. It remains unclear however whether or not the CD11c\(^+\) CD8\(\alpha\)^+ DC activated in the CLN are the same as those observed in the lung some days later. Based on this data, it would seem that they are not the same cell.

**CD11c\(^+\) CD8\(\alpha\)^+ DC cells from the CLN and lungs of infected mice are mature and secrete proinflammatory cytokines**

We next examined the activation status of CD11c\(^+\) CD8\(\alpha\)^+ DC in the CLN and lungs. Infiltrating CD11c\(^+\) CD8\(\alpha\)^+ DC expressed surface markers indicative of maturation, but at different stages of infection in the CLN and lungs. Expression of MHC class I and CD40 was enhanced on CD11c\(^+\) CD8\(\alpha\)^+ DC in the CLN, with peak expression 1 day after challenge (Fig. 3, A and B). MHC class II expression was also enhanced with peak expression on day 3. A similar pattern was observed on CD11c\(^+\) CD8\(\alpha\)^+ DC in the lung, except that peak expression of these surface Ags was delayed by at least 24 h when compared with the CLN (Fig. 3, A and B). We also investigated whether CD11c\(^+\) CD8\(\alpha\)^+ DC expressed surface CD205, a marker of Ag uptake, during acute infection with *B. pertussis*. CD205 expression was significantly enhanced in the CLN after 24 h and in lungs 36–48 h after *B. pertussis* challenge and gradually declined during the first 7 days of infection (Fig. 3, A and B). The number of CD11c\(^+\) CD8\(\alpha\)^+ cells expressing either CD80 or CD86 DC peaked by days 3 and 5 in the CLN and lungs, respectively, in mice after challenge with *B. pertussis* (Fig. 3A). Finally, the majority of CD11c\(^+\) CD8\(\alpha\)^+ DC from CLN and lungs early in infection expressed CD24 (data not shown).

CD11c\(^+\) DC have been shown to secrete IFN-\(\gamma\), IL-12p70, IL-4, and IL-10 in a variety of murine models of infection and inflammation (29–34). Therefore, we performed intracellular cytokine staining on CD11c\(^+\) CD8\(\alpha\)^+ DC during the acute stage of infection.
with *B. pertussis*. Cells isolated from the CLN or lungs of infected mice were permeabilized and stained directly with anti-cytokine Abs, without reactivation or incubation with BFA in vitro. A significant number of CD11c<sup>+</sup>CD8α<sup>+</sup> in the CLN secreted IL-4 and IL-10 and this peaked 4 days after infection. A smaller number of CD11c<sup>+</sup>CD8α<sup>+</sup> DC in the CLN of *B. pertussis*-infected mice secreted IFN-γ (Fig. 4). In contrast, a high frequency of IFN-γ-secreting CD11c<sup>+</sup>CD8α<sup>+</sup> DC were detected in the lungs of infected mice, especially 3–5 days after *B. pertussis* challenge (Fig. 4). IL-4- and IL-10-secreting CD11c<sup>+</sup>CD8α<sup>+</sup> DC were also detected in the lungs, but at a lower frequency than the IFN-γ-secreting CD11c<sup>+</sup>CD8α<sup>+</sup> DC (Fig. 4). Although a number of CD11c<sup>+</sup>CD8α<sup>+</sup> DC expressed both IL-4 and IFN-γ or IL-10 and IFN-γ in the CLN, very few coproducers were detected at any stage during acute infection in the lung (Fig. 4). The percentage of CD11c<sup>+</sup>CD8α<sup>+</sup>IFN-γ<sup>+</sup> DC was lower than CD11c<sup>+</sup>CD8α<sup>+</sup>IL-4<sup>+</sup> or CD11c<sup>+</sup>CD8α<sup>+</sup>IL-10<sup>+</sup> DC in the CLN on day 4 of infection when compared with noninfected control mice (Fig. 4, B and C). In contrast, the percentage of CD11c<sup>+</sup>CD8α<sup>+</sup>IFN-γ<sup>+</sup> DC was increased in the lungs of infected animals on day 7 (Fig. 4D). These findings demonstrate that activated CD11c<sup>+</sup>CD8α<sup>+</sup> DC found in the CLN soon after respiratory challenge with *B. pertussis* predominantly express IL-4 and IL-10 with a lower frequency expressing IFN-γ, whereas the CD11c<sup>+</sup>CD8α<sup>+</sup> DC infiltrating the lungs a few days later expresses predominantly IFN-γ.

### CD11c<sup>+</sup>CD8α<sup>+</sup> DC from infected mice can induce proliferation and proinflammatory cytokine secretion by CD4<sup>+</sup> T cells in vitro

We next investigated whether CD11c<sup>+</sup>CD8α<sup>+</sup> DC were as effective as total CD11c<sup>+</sup> DC at stimulating CD4<sup>+</sup> T cells and if the activated T cells had a similar cytokine profile. Total CD11c<sup>+</sup> DC or CD11c<sup>+</sup>CD8α<sup>+</sup> DC were sorted from the lungs of mice at the peak of infection 7 days after challenge with *B. pertussis* (Fig. 5A) and cocultured in vitro with CD4<sup>+</sup> T cells purified from the spleens of infected mice. CD11c<sup>+</sup>CD8α<sup>+</sup> DC were as effective as total CD11c<sup>+</sup> DC in inducing proliferation of CD4<sup>+</sup> T cells in response to anti-CD3 (Fig. 5B). IFN-γ (Fig. 5C) and IL-4 (Fig. 5D) were also detected in supernatants of CD4<sup>+</sup> T cells stimulated with CD11c<sup>+</sup>CD8α<sup>+</sup> DC as well as CD11c<sup>+</sup> DC, although the concentrations were higher with the latter (Fig. 5C). IL-10 and IL-12p70 production were undetectable in supernatants from CD4<sup>+</sup> T cells stimulated with either population of DC (data not shown).

The results show that CD11c<sup>+</sup>CD8α<sup>+</sup> DC from the lungs of mice during the acute stage of infection with *B. pertussis* can activate CD4<sup>+</sup> T cells in vitro.

### Depletion of CD8α<sup>+</sup> cells impairs bacterial clearance

Having shown that functionally active, mature CD11c<sup>+</sup>CD8α<sup>+</sup> DC infiltrate into the lungs during acute infection with *B. pertussis*, we examined the hypothesis that they may play a positive role in antibacterial immunity and thereby function in bacterial clearance. To examine the function of CD11c<sup>+</sup>CD8α<sup>+</sup> DC rather than total CD11c<sup>+</sup> DC, we depleted CD8α<sup>+</sup> cells in vivo using an anti-CD8α-depleting Ab. Although this approach will also deplete CD8<sup>+</sup> T cells, unlike other approaches (35), it will not deplete other DC subsets. CD8α<sup>+</sup> cells were depleted from mice infected with *B. pertussis* by injecting anti-CD8α Abs i.p. on days −1, +1, +3, +6, +13, and +19 after *B. pertussis* challenge. Depletion of CD8α was confirmed by flow cytometric analysis on spleen cells (Fig. 6A). Depleting CD8α<sup>+</sup> cells throughout infection had a marginal impact on the numbers of total CD11c<sup>+</sup> cells in the lung (Fig. 6B); however, CD11c<sup>+</sup>CD8α<sup>+</sup> DC were almost completely absent from the lungs of infected mice treated with anti-CD8α Ab (Fig. 6C).

Depletion of CD8α<sup>+</sup> cells from mice throughout infection with *B. pertussis* delayed bacterial clearance; the CFU counts were significantly higher (*p < 0.01*) in depleted animals when compared with *B. pertussis*-infected control mice 21 days after challenge (Fig. 6D). This was a consistent finding in six experiments. Furthermore, deaths were observed among the group of mice depleted of CD8α<sup>+</sup> cells, a feature previously observed in mice lacking functional IFN-γ (6). We next investigated the impact of depleting CD8α<sup>+</sup> cells (throughout infection) on CD4<sup>+</sup> T cell cytokine production. Cells were isolated from the lungs of infected animals 30 days after challenge and intracellular cytokine staining was performed. IFN-γ-secreting and IL-10-secreting CD4<sup>+</sup> T cells were detected in the lungs of mice infected with *B. pertussis* (Fig. 6E). In contrast, IL-17-producing T cells were almost undetectable. The
frequency of CD4+IFN-γ+ T cells infiltrating the lung 30 days after infection was reduced in CD8α-depleted compared with non-infected control and nondepleted B. pertussis-infected control mice (Fig. 6E). Conversely, the percentages of CD4+IL-10− T cells and CD4+IL-17− T cells were enhanced in CD8α-depleted mice (Fig. 6E). These findings are consistent with the data in Fig. 5 showing that CD8α DC could activate IFN-γ-producing CD4+ T cells.

To determine the role of CD8α+ cells at different stages of infection, infected mice were injected with anti-CD8α-depleting Ab either on day −1 and +1, from day −1 through to +19, or from +6 through to +19. Depleting CD8α+ cells either early in infection or throughout the course of infection resulted in a significant enhancement of bacterial burden in the lungs (p < 0.05) when compared with infected control mice (Fig. 7A). In addition, the number of cells in the perivascular spaces of lung tissue (isolated 30 days after infection) was greater in mice depleted of CD8α+ cells either during the early phase or throughout infection when compared with B. pertussis-infected undepleted control animals (Fig. 7B).

Since B. pertussis causes a predominant extracellular infection, it was assumed that CD8+ T cells would not play a protective role in immunity to B. pertussis, in particular during the acute phase of infection. Indeed, we have previously demonstrated that transfer of CD8+ T cells delays bacterial clearance in infected mice. Mice were infected by exposure to an aerosol of live virulent B. pertussis. Mice received either 200 µg/mouse anti-CD8α-depleting Ab (anti-CD8α) or irrelevant IgG2b isotype control Ab (200 µg/mouse) i.p. 24 h before infection and on days 1, 3, 7, 14, and 19 thereafter. A. FACS analysis on spleen cells isolated from CD8α-depleted mice 24 h after infection showing absence of CD8α+ cells. The number of CD11c+B and CD11c+CD8α+ DC infiltrating the lung in B. pertussis-infected mice, control mice, and mice treated with anti-CD8α 24 h before challenge with B. pertussis and on days 1, 3, 7, 14, and 19 (−1 to +19). D. The course of infection in B. pertussis-infected control mice and in CD8α-depleted infected animals was followed by perfuming CFU counts on lung homogenates at intervals after the initial challenge. One of five animals died from the CD8α-depleted group after challenge with B. pertussis, representative dot plots, gated on viable CD3+CD4+ T cells, showing intracellular cytokine expression in cells isolated from the lungs of mice from control and experimental groups on day 30 after B. pertussis challenge. These results are representative of four separate experiments.

FIGURE 5. CD11c+CD8α+ cells from the lungs of B. pertussis-infected mice can stimulate CD4+ T cells in vitro. A, Infiltration of CD11c+ or CD11c+CD8α+ DC into lungs at intervals after B. pertussis challenge. B, CD11c+ or CD11c+CD8α+ DC were sorted from lung homogenates of mice on day 7 of respiratory infection with B. pertussis. Each cell subset was activated with 2 µg/ml immobilized anti-CD3 in the absence or presence of conventional CD4+ T cells (purified from the spleens of infected animals). Proliferation was determined by [3H]thymidine incorporation 72 h later. *, p < 0.05 and **, p < 0.01 vs noninfected control mice. The concentrations of IFN-γ (C) and IL-4 (D) in the supernatants were quantified by ELISA 48 h after activation. These results are representative of two separate experiments. Results were compared using Student’s t test. *, p < 0.05 and **, p < 0.01 vs DC or T cells alone.

FIGURE 6. Depleting CD8α+ cells delays bacterial clearance in infected mice. Mice were infected by exposure to an aerosol of live virulent B. pertussis. Mice received either 200 µg/mouse anti-CD8α-depleting Ab (anti-CD8α) or irrelevant IgG2b isotype control Ab (200 µg/mouse) i.p. 24 h before infection and on days 1, 3, 7, 14, and 19 thereafter. A. FACS analysis on spleen cells isolated from CD8α-depleted mice 24 h after infection showing absence of CD8α+ cells. The number of CD11c+B and CD11c+CD8α+ DC infiltrating the lung in B. pertussis-infected mice, control mice, and mice treated with anti-CD8α 24 h before challenge with B. pertussis and on days 1, 3, 7, 14, and 19 (−1 to +19). D. The course of infection in B. pertussis-infected control mice and in CD8α-depleted infected animals was followed by perfuming CFU counts on lung homogenates at intervals after the initial challenge. One of five animals died from the CD8α-depleted group after challenge with B. pertussis (+). **, p < 0.01 vs nondepleted B. pertussis-infected mice. E. Representative dot plots, gated on viable CD3+CD4+ T cells, showing intracellular cytokine expression in cells isolated from the lungs of mice from control and experimental groups on day 30 after B. pertussis challenge. These results are representative of four separate experiments.
enhanced bacterial clearance when compared with nondepleted infected control mice (Fig. 7D). This observation points to a possible regulatory role for CD8α+ T cells in immunity to *B. pertussis*. In contrast, the enhancement of infection observed following depletion of CD8α+ cells early in infection along with the early appearance of CD8α+ DC suggest a protective role for this DC population in immunity to *B. pertussis*.

Transfer of in vitro-expanded FL-DC into infected mice enhances cellular infiltrate and promotes bacterial clearance from the lung

The results of the depletion experiments suggested that CD11c+ CD8α+ DC contributed to antibacterial immunity in the lungs; we sought to confirm these findings using a cell transfer approach. We used FL to expand CD11c+CD24+CD45RA− (FL-DC) from freshly isolated bone marrow cells and removed CD11c+CD24+CD45RA+ pDC by cell sorting (36). CD8α is not expressed on these cells in vitro; however, CD8α expression is up-regulated when they are transferred in vivo (37). The CD11c+CD24+CD45RA− (FL-DC), which were routinely 95% pure (Fig. 8A), were transferred 30 min after *B. pertussis* challenge. Transfer of FL-DC significantly enhanced bacterial clearance when compared with *B. pertussis*-infected controls. Mice that received the DC transfer had significantly lower (*p* < 0.05) CFU counts, particularly on day 14 after challenge (Fig. 8B). To exclude the possibility that transfer of any DC subset could enhance bacterial clearance in *B. pertussis*-infected animals, experiments were performed where mice received either DC expanded in culture with GM-CSF and IL-4 (GM-DC) or FL-DC 30 min after infection. Only FL-DC transfer significantly accelerated bacterial clearance (*p* < 0.05) in infected animals (Fig. 8C).

Transfer of FL-DC was associated with an enhancement in the number of CD4+ T cells and B cells but not neutrophils infiltrating the lungs (Fig. 8D). Although not statistically significant, this was a consistent finding over five separate experiments (data not shown). Finally, the numbers of CD11c+ or CD11c+CD8α+ DC in the lungs of infected mice were not significantly altered by transfer of FL-DC when examined 7 days after challenge (Fig. 8E).

**Blocking CD103 function in vivo impairs bacterial clearance**

A number of studies have reported that DC, particularly in the gut and lung, express the αEβ7 integrin CD103 whose ligand, E-cadherin, is expressed by epithelial cells on mucosal surfaces (23, 25). Therefore, we examined expression of CD103 on DC in the lungs of infected mice were not statistically significant over five separate experiments (data not shown). Additionally, significant numbers of CD11c+CD8α+CD103+ DC in the CLN on day 4 after infection when compared with noninfected control mice (Fig. 9, A and B). In contrast, significant numbers of CD11c+CD8α+CD103+ DC were detected in lung by day 6 after challenge (Fig. 7, A and B). It is possible that CD11c+CD8α+CD103+ DC from the CLN require surface CD103 to track into the lung by interacting with E-cadherin on epithelial cells in the mucosal surface. We next investigated the possible role of CD103 in protective immunity to *B. pertussis* infection. Mice were injected once with an anti-CD103-blocking Ab 24 h before infection. It was not possible to specifically eliminate CD11c+CD8α+CD103+ cells, as only 40% of the CD103+ cells in either CLN or lung, during the acute phase of infection, express CD8α (data not shown). Nevertheless, blocking CD103 function with a single injection of anti-CD103 Ab had a dramatic impact on bacterial clearance, with a significant (*p* < 0.01) enhancement in CFU counts 21 days after *B. pertussis* challenge (Fig. 9C). Inhibition

CD8α− T cells exacerbated infection with *B. pertussis* (5). Nevertheless, we examined the possibility that the enhanced infection seen in CD8-depleted mice resulted from depletion of CD8α− T cells. We first examined the infiltration of CD8α− T cells and found that they do infiltrate into the lungs of *B. pertussis*-infected mice, especially late in infection (Fig. 7C). Depletion of CD8α− cells from day 6 after *B. pertussis* challenge significantly (*p* < 0.05)
in infected animals that received either 0.5 × 10⁸ live, virulent *B. pertussis*. Infected lungs and CLN were removed and digested at various time points after infection. The percentage (A), based on the viable lymphocyte gate and total (B) number of CD11c⁺CD8α⁺ CD103⁺ DC in CLN from noninfected control mice and in CLN and lungs from infected animals, was determined by flow cytometric analysis daily, up to day 7 after challenge with *B. pertussis*. *, p < 0.05 and **, p < 0.01 vs uninfected control mice. C and D. Mice were injected with 50 μg of either anti-CD103 or rat anti-HRP IgG1 Ab (control) i.p. 24 h before infection to block CD103 function in vivo. Bacterial clearance (C) and cellular infiltration into the lung (D) was examined on day 21 after *B. pertussis* infection. Results are representative of two separate experiments. **, p < 0.01 vs nondepleted *B. pertussis*-infected control mice.

**FIGURE 8.** Transfer of in vitro-expanded FL-DC before *B. pertussis* challenge enhances bacterial clearance. CD11c⁺CD24⁻CD45RA⁻ DC (FL-DC) were generated in vitro from freshly isolated bone marrow cells cultured for 8 days in DC medium supplemented with 300 ng/ml FL. A. Representative dot plots showing CD11c⁺ cells, which were sorted before transfer in vivo. B. The course of infection in *B. pertussis*-infected control mice and in infected animals that received 0.5 × 10⁸ FL-DC (30 min before challenge) was followed by performing CFU counts on lung homogenates at fixed time points after the initial challenge. *, p < 0.05 vs control *B. pertussis*-infected mice. C. The bacterial load in *B. pertussis*-infected control mice and in infected animals that received either 0.5 × 10⁸ FL-DC or GM-DC before infection was assessed by performing CFU counts on lung homogenates on day 21 after infection. *, p < 0.05 vs control *B. pertussis*-infected mice. D. The number of CD3⁺CD4⁺, CD3⁺CD8⁺, B220⁺, and GR1⁺ cells in lung homogenates from *B. pertussis*-infected mice and infected animals that received FL-DC was determined 7, 14, and 21 days after infection. E. The number of CD11c⁺ and CD11c⁺CD8α⁺ DC infiltrating the lungs 7 days after challenge in control-infected mice and mice that received sorted FL-DC. Results are representative of three separate experiments. *, p < 0.05 vs control *B. pertussis*-infected mice.

**FIGURE 9.** Blocking CD103 function in vivo impairs bacterial clearance. Mice were infected with 2 × 10⁹ live, virulent *B. pertussis*. Infected lungs and CLN were removed and digested at various time points after infection. The percentage (A), based on the viable lymphocyte gate and total (B) number of CD11c⁺CD8α⁺ CD103⁺ DC in CLN from noninfected control mice and in CLN and lungs from infected animals, was determined by flow cytometric analysis daily, up to day 7 after challenge with *B. pertussis*. *, p < 0.05 and **, p < 0.01 vs uninfected control mice. C and D. Mice were injected with 50 μg of either anti-CD103 or rat anti-HRP IgG1 Ab (control) i.p. 24 h before infection to block CD103 function in vivo. Bacterial clearance (C) and cellular infiltration into the lung (D) was examined on day 21 after *B. pertussis* infection. Results are representative of two separate experiments. **, p < 0.01 vs nondepleted *B. pertussis*-infected control mice.

**Discussion**

CD11c⁺CD8α⁺ DC of lymphoid origin have been considered as primarily immunoregulatory (34, 38 – 40); however, it has been suggested that CD11c⁺CD8α⁺ may also have proinflammatory functions (29, 30, 33, 41–44). In this study, we have demonstrated that CD11c⁺CD8α⁺ DC infiltrate the CLN and then the lungs of early stages of infection with *B. pertussis* where they promote cellular immune responses that mediate clearance of the bacteria from the respiratory tract.

**of CD103 constrained cellular infiltration into the lungs 21 days after challenge (Fig. 9D). These findings suggest that CD11c⁺ CD103⁺ DC infiltrate the CLN and then the lungs in the early stages of infection with *B. pertussis* where they promote cellular immune responses that mediate clearance of the bacteria from the respiratory tract.**
It is widely accepted that CD11c+CD8α+ cells are the principle DC subset involved with cross-presentation of cell-associated Ags by MHC class II (41, 45, 46). We found that CD11c+ CD8α+ DC that infiltrate the draining lymph nodes very soon after infection with *B. pertussis* had enhanced expression of a number of activation markers, including CD40, CD80, CD86, and MHC class I and II. This profile changes in CD11c+CD8α+ DC that appear in the lungs a few days later, with higher expression of MHC class II and lower expression of CD40. Furthermore, we found that CD11c+CD8α+ DC in the CLN acquire i.n. delivered fluorescent particles within hours of infection. Since these cells are very efficient at acquiring Ag from apoptotic and necrotic cells (47), it is possible that they are capable of acquiring Ag from newly arrived apoptotic APCs in the CLN of mice infected with *B. pertussis* and thereby activating MHC class II-restricted CD4+ T cells, which play a critical role in protection against *B. pertussis*.

Interestingly, expression of CD205, a marker of endocytic activity and Ag internalization (48), was also enhanced on CD11c+ CD8α+ DC early in infection and this inversely correlated with CD103 in particular in the lung. It has been suggested that CD205 on the surface of DC might interfere with CD103 integrin binding to its ligand E-cadherin on endothelial cells (49). Therefore, it is possible that during the initial phase of Ag uptake and processing, the DC is prevented from migrating from the CLN to the lung, but on maturation, CD205 expression diminishes and CD103 increases, which would permit the DC to migrate to the mucosal surface. However, we have yet to elucidate whether CD11c+CD8α+ DC observed in the draining lymph nodes are the same cells seen in the lung some days later.

Although the precise mechanism by which CD8α+ DC mediate protection against *B. pertussis* infection is not clear, the findings suggest that they promote a protective Th1 response in the CLN and lungs, which is mediated by innate IFN-γ production. CD11c+CD8α+ DC from the CLN of *B. pertussis*-infected mice expressed significant IL-4 and IL-10 and a lower frequency expressed IFN-γ. This is consistent with the regulatory phenotype attributed to these cells (34, 38). However, we found that CD11c+CD8α- DC that infiltrated the lungs a little later in the bacterial infection produced predominantly IFN-γ, with a lower frequency expressing IL-4 and IL-10. CD11c+CD8α-IFN-γ+ DC primed with bacterial Ag might become the dominant subset in the lung as a result of activation through TLR ligands or other bacterial-derived pathogen-associated molecular patterns or through interaction with other innate cells and CD4+ T cells within the lung. Interestingly, Th1 responses were reduced and IL-10-producing T cells enhanced in mice depleted of CD8α+ cells. Thus, CD11c+CD8α+IFN-γ- DC may help to promote Th1 responses, which help to clear the infection. The simultaneous presence of IL-10-secreting DC in the lungs and CLN is consistent with the coinduction of Th1 and Tr1-type cells and the role of the latter cells in suppressing effector responses early in infection and in controlling immunopathology in the lungs (8, 50).

Cell transfer and depletion experiments demonstrated that CD11c+CD8α+ DC made a positive contribution to protective immunity in the respiratory tract. Depleting CD8α- cells during the first 7 days of infection with *B. pertussis* delayed bacterial clearance, implying that CD11c+CD8α- DC play a significant role in initiating protective immunity to infection with this organism. It has previously been suggested that CD11c+CD8α- DC are required for the generation of protective virus-specific CTL responses in mice respiratory infected with influenza virus (32). However, that conclusion was based on depletion of total CD11c+ cells, which included myeloid and lymphoid DC (32). In contrast to the results described here, Leef et al. (51) have found that administering the same CD8α- depleting Ab as that used in our study had little or no effect on the course of bacterial colonization with *B. pertussis* in mice. However, in that study, mice were immunized with formalin-fixed bacteria or microencapsulated pertussis Ags before *B. pertussis* challenge (51). Therefore, CD11c+ CD8α- DC might have a significant role in primary infection in the lung, but perhaps a lesser role during secondary immune responses. We also demonstrated that transfer of CD11c+CD24-CD45RA-DC in vitro with FL, and which acquire CD8α when transferred in vivo (37), consistently caused a small but significant enhancement in bacterial clearance in infected mice. This was not observed with DC expanded with GM-CSF and IL-4. It has been shown that repeated injections of mice with high concentrations of recombinant FL greatly expands CD11c+CD8α+ in vivo (16). Indeed, FL has been used in phase I and II clinical trials as an adjuvant in a vaccine against hepatitis B infection (44, 52–54) and has been used to treat a number of different cancers in mice and humans, including metastatic lung cancer (55, 56). Our results indicate that FL represents an exciting therapeutic option against lung infections. However, it has been shown recently that transfer of CD11c+CD8α- DC enhanced HSV type I latency in mice (39).

We also found evidence that CD103 has a positive role in driving cellular immunity against *B. pertussis* infection. We found significant infiltration of CD11c+CD8α+CD103+ DC into the CLN and lungs 4–6 days after reparation challenge with *B. pertussis*. Furthermore, injection of a blocking Ab to CD103 before infection caused a significant delay in clearance and in infiltrating lymphocytes in the lungs. Since CD103 is also expressed on other cell types, we cannot exclude their role in protection, but our data do suggest that CD11c+CD8α-CD103+ DC may have a protective function in the lungs. This conclusion is consistent with recent studies describing proinflammatory CD103+CD11chighCD11blow DC that reside in the lung mucosa and vascular wall (25, 26). In short, DC that express CD103 can have proinflammatory function. These DC have been shown to migrate across the airway epithelia for Ag transport and presentation and contribute to the pathogenesis of airway hypersensitivity in mice (25). Furthermore, they express CCR7, which allows them to migrate to the bronchial nodes during viral infection (26). Therefore, it is possible that blocking CD103 function during *B. pertussis* infection inhibited CD11c+CD8α+CD103+ in the lung and in the CLN, causing a delay in Ag transfer from the lung to the draining lymph nodes. A protective proinflammatory role for CD11c+CD103+ DC in the lungs is at variance with the proposed regulatory function of these cells in the gut, where they have been shown to generate inducible Treg cells from effector CD4+ T cells, by a retinoic acid and TGF-β-dependent mechanism (22–24). However, proinflammatory CD11c+CD103+ DC have also been described in murine models of airway hypersensitivity and viral infection (25, 26). Therefore, it is possible that CD103 might not be a marker of proinflammatory or regulatory DC per se but an integrin, which is involved with DC homing between mucosal surfaces and draining lymph nodes.

In conclusion, we hypothesize that lymph node resident CD11c+CD8α+ DC can acquire *B. pertussis* Ag from dying macrophages or other DC subtypes. These cells could traffic to the site of infection, perhaps with the aid of surface CD103, within the lung where they become polarized to predominantly CD4+Th1 cells.
secrete IFN-γ and subsequently promote a Th1 response. Thus, CD11c+CD8α+ dendritic cells represent a subset that is crucial for establishing immunity against Bordetella pertussis infection of the respiratory tract.

Disclosures

The authors have no financial conflict of interest.

References


