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Characterization of Pulmonary T Cell Response to Helper-Dependent Adenoviral Vectors following Intranasal Delivery

Rahul Kushwah,*† Huibi Cao,* and Jim Hu²*†

In spite of the extensive research in the field of gene therapy, host immune responses continue to be the major barrier in translating basic research to clinical practice. Helper-dependent adenoviral (HD-Ad) vectors show great potential for pulmonary gene therapy, but the knowledge of pulmonary immune responses toward these vectors is very limited. In this study, we show that HD-Ad vectors are potent stimulators of dendritic cell (DC) maturation, thus leading to stimulation of T cell proliferation with \(-6\%\) of naive CD4\(^+\) T cells from pulmonary mediastinal lymph node responding to HD-Ad-treated DCs. In contrast to the belief that HD-Ad vectors are unable to prime adaptive immune response, we show for the first time, through in vivo pulmonary studies in mice, that HD-Ad vectors can prime CD4\(^+\) and CD8\(^+\) T cell responses in the lung at high and substantially low doses. This indicates cross-presentation of HD-Ad-derived epitopes by DCs to prime CD8\(^+\) T cell responses. To assess the basis of pulmonary T cell response against HD-Ad vectors, we examined the response of conventional DCs (cDCs) and plasmacytoid DCs (pDCs) in the lung. In response to HD-Ad delivery, there is induction of maturation in both cDC and pDC subsets, but it is the cDCs, not pDCs, that migrate rapidly to draining lymph nodes within the first 2 days after vector delivery to prime adaptive immune response against these vectors. These findings have implications for development of strategies to prevent adaptive immune responses against gene therapy vectors. *The Journal of Immunology, 2008, 180: 4098 – 4108.

The ability of adenoviral-derived vectors to transduce a wide variety of cells, both dividing and nondividing, has led to their development as an efficient system for pulmonary gene transfer (1). However, the problems of host adaptive and innate immune responses have limited the use of adenoviral vectors for gene therapy, because of concern for their safety and efficacy in vivo. Over the last decade, immense research has been conducted on improving the vector design, which has resulted in development of helper-dependent adenoviral (HD-Ad)\(^1\) vectors, which do not encode any viral genes. The only viral sequences present are the terminal repeats along with the packaging signal (2). This has resulted in HD-Ad vectors being selected as the vector of choice for pulmonary gene therapy for diseases such as cystic fibrosis (3–5).

Due to the lack of viral coding sequences, HD-Ad vectors are thought to be less immunogenic than the first generation vectors, and it is widely believed that HD-Ad vectors do not induce an adaptive immune response (6–8). However, other studies have shown that with readministration of HD-Ad vector particles, there is a decrease in transgene expression correlating with an increasing Ab titer against the virus, indicating that there may be an adaptive response being mounted against HD-Ad particles (9). Nevertheless, none of the studies to date have assessed the ability of HD-Ad particles to induce T cell immune response upon airway delivery (9, 10). Among gene therapy vectors, adeno-associated virus (AAV)-derived vectors are thought to be the least immunogenic (11). However, in recent clinical trials using AAV vectors, there has been appearance of cytotoxic CD8\(^+\) T cells upon vector delivery (12). These observations clearly highlight the importance of studying adaptive immune responses to the so-called “less-immunogenic” vectors such as HD-Ad vectors. The ability to develop safe and effective gene therapy therapeutics depends on having a better understanding of the immunological processes in the lung, for targeting/inhibition of these processes will help in increasing the efficacy and persistence of gene therapy vectors.

Dendritic cells (DCs) are potent APCs and lung dendritic cells are ideally positioned in the airway epithelium network to perform surveillance of the inhaled Ags (13–15). Upon uptake of foreign Ags, airway DCs migrate to the T cell zone of the draining lymph nodes, in particular the mediastinal lymph node (MLN), where they interact with naive T cells and prime adaptive immune response (16, 17). Despite the critical role of airway DCs in modulating immune responses to inhaled Ags, our best knowledge, studies have not examined the effects of HD-Ad vectors on pulmonary DCs. Moreover, pulmonary DCs...
include both the conventional (cDC) and plasmacytoid DC (pDC) subsets, and the relative contribution of the two subsets and their response to HD-Ad vectors has not been investigated to date. The understanding of the pulmonary adaptive immune response to HD-Ad can hold the key to development of strategies to prevent immune responses against vectors, and may hold the key to understanding the reason behind sudden appearance of unwanted immune response in many gene therapy clinical trials.

In this study, we assessed the pulmonary adaptive immune responses to HD-Ad vectors. Because incorporation of a transgene can further elicit/potentiate immune responses, we chose to assess immune responses to an empty HD-Ad vector that did not encode any transgene. We show that in contrast to the commonly held belief, HD-Ad vectors do have the ability to potentiate CD4⁺ and CD8⁺ T cell response upon pulmonary delivery at high and substantially low doses. Furthermore, we also show that HD-Ad vectors are potent stimulators of DC activation in vitro and in vivo with cDCs playing the major role in priming T cells in the draining MLN. Moreover, HD-Ad vector delivery also resulted in maturation of CD8α-DCs within the draining MLN, indicating that this particular subset of DC may play a role in cross-presentation of HD-Ad-derived Ags and inducing CD8⁺ T cell proliferation. In contrast, though pulmonary pDCs do mature upon HD-Ad delivery, they do not migrate to draining MLN, perhaps acting as local source of IFN-α to prime innate and adaptive immune response against HD-Ad vectors.

Materials and Methods

Abs and other reagents

All the Abs were purchased from eBioscience: CD86 PE/Cy5, CD80 PE, MHC II PE, CCR7 PE, BrdU FITC, CD11c PE, mPDCA1 PE, and the following from BD Biosciences: CD11c FITC, CD4 PE/Cy7, CD8 PE/Cy7, CD8α PE/Cy7, and CD3 PE. CD11b Ab was a gift from Dr. Jim Xiang (Saskatoon Cancer Center, Saskatchewan). Isotype control IgGs were obtained from eBiosciences and/or Serotec. CSF was obtained from Molecular Probes and BrdU, DEAE-dextran, heparin and FITC-dextran from Sigma-Aldrich. GM-CSF was obtained from R&D Systems. Cell proliferation ELISA based on BrdU incorporation and chemiluminescent detection and collagenase D was obtained from Roche. Aerrane was obtained from Baxter.

Mice and vector delivery

C57BL/6 mice were purchased from Charles River Laboratories and maintained as per guidelines of SickKids animal facilities. All the animal studies were reviewed and approved by the SickKids Institutional committee for humane use of laboratory animals. Mice that were 8–11 wks of age were lightly anesthetized by Aerrane inhalation, and a highly purified batch of HD-Ad vector particles purified via cesium chloride density gradient centrifugation were delivered intranasally in a volume of 50 μl with 5 x 10^5 or 1 x 10^4 particles in complex with DEAE-dextran for efficient delivery, as described previously (4).

Generation of bone marrow-derived dendritic cells (BMDC) and HD-Ad induced maturation

Bone marrow cells were isolated from the tibia and femur of adult mice and cultured in the presence of GM-CSF as described previously (18). On day 7, weakly adherent cells were isolated, and 85–90% of the cells was confirmed to be CD11c⁺ DCs via FACS analysis. Preparation of “empty” HD-Ad vector particles was performed in our laboratory as described previously (19). CD11c⁺ DCs were treated with HD-Ad vectors at different multiplicities of infection (MOI) and after 24 h marker expression was assessed via FACS analysis.

In vitro T cell proliferation

HD-Ad-treated CD11c⁺ DCs were exposed to single dose of 20 gray of 60Co gamma irradiation and then cultured with CD4⁺/CD25⁻ T cells, isolated from draining MLN via cell sorting for a period of 72 h. BrdU solution was added during the last 12 h to label the cells, after which proliferation was measured using BrdU cell proliferation, chemiluminescent ELISA, according to the manufacturer’s instructions (Roche). To calculate precursor frequencies, HD-Ad-treated DCs were cultured with CFSE-labeled naïve CD4⁺ T cells isolated from draining MLN for a period of 7 days, after which FACS analysis was performed to assess CFSE dilution. T cell precursor frequencies were calculated from CFSE dilution data as described previously (20).

Preparation of tissue lymphocytes

At different time points after HD-Ad vector delivery, mice were sacrificed by i.p. injection of Euthanyl (Bimeda-MTC). To collect bronchoalveolar lavage fluid (BALF), mouse lungs were lavaged as described previously (9). After performing lavage, lungs were perfused with 10 ml of PBS containing 10 U/ml heparin via the right ventricle of the heart to remove blood cells from the lung vasculature. Perfusion was performed until the lungs turned completely white in color. Lungs were dissected out, and after removal of draining MLN, lungs were minced and digested for 25 min at 37°C using 250 U/ml collagenase D solution, with the addition of EDTA (10 mM final) during the last 5 min of incubation. Fragments of digested lungs were passed through a 100-μm cell strainer (BD Biosciences), and hypotonic lysis was used to remove erythrocytes. Cells were then counted and resuspended at appropriate concentrations for different experiments. Similarly, draining MLN were digested, followed by suspension at appropriate concentrations.

BrdU labeling

At different time points after vector delivery, mice were anesthetized using Aerrane inhalation and then BrdU was administered intranasally in a volume of 50 μl at 16 mg/ml concentration. Mice were killed 24 h after BrdU delivery, and lungs/draining MLN were isolated.

Ex vivo assessment of T cell specificity upon HD-Ad delivery

Six days after delivery of HD-Ad vector particles, mice were sacrificed and MLN were isolated. T cells isolated from MLN by nylon-wool enrichment were cocultured with saline/HD-Ad-treated irradiated BMDCs for a period of 72 h. BrdU solution was added during the last 12 h to label the cells, after which proliferation was measured using BrdU cell proliferation ELISA according to the manufacturer’s instructions.

In vivo labeling of DCs

Mice were anesthetized using Aerrane inhalation and 50 μl of 1 mg/ml FITC-dextran or 50 μl of 5 mM CFSE was delivered intranasally, 2 h before viral delivery. At indicated time points, mice were killed and draining MLN were isolated.

Measurement of absolute DC counts

Lung or draining MLN were isolated and single cell suspension was prepared as described above. Cell suspensions were stained for DC-specific markers, and absolute counts were assessed in the lung or draining MLN for each mouse separately.

Ab labeling and flow cytometry

For in vivo BMDC experiments, DCs were labeled at 4°C with CD11c and maturation markers. To assess pulmonary T cell proliferation, cells from BALF/lung or MLN single cell suspensions were stained for CD3 and CD4/CD8 with/without BrdU staining as described previously (21). Migration of FITC-dextran-labeled DCs was assessed by labeling MLN single cell suspensions with CD11c. DCs in the airways were identified by staining single cell lung suspensions with CD11c and CD11b Abs for cDCs and with mPDCA1 Ab for pDCs. Maturation of DCs in vivo was assessed by staining with CD86 Ab as marker for DC maturation. Maturation of CD8α DCs was assessed by gating on CD11c⁺CD8α⁺ cells from the draining lymph nodes and staining with CD86 Ab as a maturation marker. Cells were gated according to their forward scatter vs side scatter characteristics to discriminate highly autofluorescent macrophages from DCs as described previously (22). Flow cytometry data was acquired for each of the experiments using a BD FACSCalibur (BD Biosciences) at the SickKids-University Health Network Flow Cytometry Facility and was analyzed using FlowJo flow cytometry analysis software (Tree Star).

Statistical analysis

Student’s t test was used to assess statistical significance between means. Significance was set at p < 0.05. All the data is presented as mean ± SD.
Results

HD-Ad vectors induce maturation of BMDCs

BMDCs generated in presence of GM-CSF showed classical characteristics of immature myeloid DCs, characterized as CD11c⁺ with low expression of MHC II, CD80, CCR7, and CD86 (Fig. 1A). The effect of HD-Ad vector on the surface phenotype of BMDCs (day 7) was assessed 24 h after treatment with HD-Ad vector particles. Incubation with HD-Ad caused an increase in the intensity of expression along with the percentage of DCs positive for CD86, CD80, and MHC II expression. Using flow cytometry, expression of different markers was assessed on CD11c⁺ DCs. Isotype matched IgGs were used as isotype controls and all values represent mean of 4–5 independent experiments; *, p < 0.05 compared with the DCs treated with saline (-Ad).

HD-Ad vector-treated DCs are potent inducers of T cell proliferation in vitro

Because HD-Ad-treated DCs had the surface phenotype of mature myeloid DCs, we next assessed induction of T cell proliferation by HD-Ad-treated DCs in vitro. Lymphoid drainage from the lung is predominantly into MLN; thereby, we isolated naive CD4⁻ T cells from MLN as representative of T cells that encounter HD-Ad-presenting DCs in lymph node upon pulmonary delivery of the vector. To determine the potency of adenoviral matured DCs to induce T cell proliferation, BMDCs were matured overnight in the presence of different MOI of HD-Ad vectors, ranging from 40 to 400 MOI. HD-Ad-matured DCs were irradiated and cocultured with naive CD4⁺ T cells from draining MLN for 3 days to assess T cell proliferation via BrdU incorporation. Increasing MOI of
HD-Ad used to treat DCs led to a parallel increase in levels of T cell proliferation, which plateaued around 80–200 MOI, indicating that this particular MOI was inducing maximal T cell proliferation (Fig. 2A). Therefore, MOI of 100 was used for all the experiments, and T cell proliferation in response to HD-Ad vectors was also confirmed using CFSE dilution analysis (Fig. 2B). Furthermore, we wanted to estimate the frequency of naive T cells from draining MLN, responding to adenosivector. To do that, we assessed T cell proliferation in a coculture of DCs with CFSE-labeled naive CD4+ T cells. As a CFSE-labeled cell undergoes division, the CFSE is halved and every round of division gives a different peak due to the varying CFSE intensity upon FACS analysis. Eventually the frequency of responsive T cells can be calculated. Therefore, naive CD4+ T cells from draining MLN labeled with CFSE were cultured with HD-Ad-matured DCs for a period of 7 days, after which CFSE dye dilution was assessed via flow cytometry (Fig. 2B). The results indicated ~9% of naive CD4+ T cells from draining MLN to be responding to HD-Ad epitopes presented by DCs (Fig. 2C). As a parallel control, we used coculture of CFSE-labeled naive T cells with DCs without addition of any HD-Ad vectors for maturation, because this would indicate the percentage of nonspecific T cell proliferation. Nonspecific proliferation was determined to be around 3% by CFSE dilution analysis (Fig. 2C). Therefore, upon accounting for nonspecific proliferation, results indicate that ~6% of naive T cells underwent proliferation in response to HD-Ad-derived epitopes presented by HD-Ad-treated DCs.

**FIGURE 2.** Induction of CD4+ T cell proliferation in vitro by HD-Ad-treated BMDCs. A, Assessment of CD4+ T cell proliferation using BrdU incorporation 3 days after a coculture of naive CD4+ T cells isolated from draining MLN with DCs treated with HD-Ad at different MOI (RLU, relative light units, was a chemiluminescent measurement of BrdU incorporation by proliferating cells). B, Representative histogram depicting CFSE dilution profile of CFSE+ naive CD4+ T cells which were cultured with HD-Ad-treated DCs or medium-treated DCs for a period of 7 days. C, Frequency of T cells proliferating in coculture of naive CD4+ T cells (CFSE+) with HD-Ad/media-treated DCs, calculated from CFSE dilution analysis. *p < 0.05, compared with precursor frequency of T cells cultured in presence of media-treated DCs. Results for panel A were normalized to control measuring T cell proliferation in naive CD4+ T cell and DC coculture, where DCs were not treated with HD-Ad vectors results are representative of three independent experiments.

HC-FT knockout mice (19). The second dose was a low dose of 5 × 10^9 vector particles/mouse and is 3-fold lower than the low dose normally used for HD-Ad pulmonary delivery (9, 19). At first, we looked at the infiltration of T cells in the BALF of mice at different time points after vector delivery. Under basal conditions in the absence of any inflammatory response, and before delivery of HD-Ad vectors, BALF is primarily composed of macrophages, and only 2–3% of the cells can be identified as T lymphocytes (Fig. 3A). However, upon delivery of a high dose of HD-Ad vector (1 × 10^11 vector particles), there was a gradual recruitment of T lymphocytes in BALF, which peaked around day 7, when approximately half of the cells in BALF were T lymphocytes. The infiltration gradually receded and was close to basal levels by day 14 (Fig. 3A). Further assessment of the phenotype of T cells indicated that there was infiltration of both CD4+ and CD8+ T cells, which followed a similar trend as overall T cell responses, peaking around day 7 with ~10–15% of the cells in BALF being CD8+ T cells and 30–40% being CD4+ T cells (Fig. 3B). In contrast to high dose, delivery of a low dose of HD-Ad vector (5 × 10^9 particles), which is even lower than the low dose used in gene therapy experiments, did not result in any significant changes to the composition of the BALF (Fig. 3A).

Because the presence of T cells in BALF is indicative of extensive inflammation, and the absence of T cells in BALF does not indicate absence of T cell proliferation in response to HD-Ad, we also assessed T cell proliferation within the airways along with the draining MLN. To directly estimate the frequency of proliferating T cells responding to intranasal delivery, we used the strategy of in vivo uptake of the thymidine analog BrdU with a flow cytometry-based analysis to identify BrdU+ proliferating cells within the airways and draining lymph nodes. BrdU was delivered 24 h before sacrificing mice, and single cell suspensions of lung and lymph node cells were analyzed via flow cytometry to look at BrdU+ T cells. Fig. 4A shows representative histograms indicating BrdU staining on
Among proliferating BrdU+ T cells in the lung, ~20% of proliferating T cells were CD8+ T cells and 80% were CD4+ T cells (Fig. 4C). Though the absolute frequencies of proliferating T cells were lower at the low dose than those observed at a high dose, the overall trend stayed the same, with frequencies returning to basal levels by day 14. To confirm the specificity of T cell proliferation being observed in the draining MLN, T cells isolated from draining MLN of HD-Ad-treated animals on day 6 were cultured with HD-Ad-treated BMDCs and T cell proliferation was assessed by BrdU incorporation. As a control, T cells isolated from MLN of mice treated with saline were cocultured with saline-treated BMDCs, which resulted in basal levels of proliferation, perhaps indicating some nonspecific proliferation. In contrast, coculture of T cells from animals treated with saline with HD-Ad-treated BMDCs resulted in higher levels of proliferation, perhaps indicating the proliferation of naive T cells in response to HD-Ad (Fig. 4E). However, coculture of T cells isolated from MLN of mice treated with a high or low dose of HD-Ad resulted in significantly higher levels of T cell proliferation, indicating that HD-Ad-treated animals had significantly higher levels of proliferating T cells responding to HD-Ad-derived epitopes (Fig. 4E). Therefore, the results clearly indicate that HD-Ad vectors also induce T cell proliferation in vivo even at a dose below the so-called low dose used in gene therapy experiments (9).

**HD-Ad vectors induce maturation of cDC and alter their levels within the lung**

To assess the response of DCs to HD-Ad in vivo, we delivered HD-Ad intranasally and looked at effects on cDCs at different time-points. cDCs are usually identified via high expression of CD11c along with CD11b (23). To clearly identify lung cDCs, we first performed bronchoalveolar lavage to remove most of alveolar macrophages before isolation of cells from the lung. Moreover, gating characteristics were further used to clearly identify lung cDC subset.

To assess maturation of cDCs, we looked at the expression of CD86 costimulatory marker on lung cDCs at different time-points after HD-Ad delivery. Fig. 5A shows a representative histogram depicting that in response to HD-Ad, there is an increase in CD86 expression on day 1, which returns to basal levels by day 5. The percent increase in mature lung cDCs was calculated relative to the levels in control mice receiving saline instead of HD-Ad vectors. Maximum increase in DC maturation was observed 1 day after HD-Ad delivery, which decreased on day 2 and eventually returned to basal levels by day 5 (Fig. 5B). At the same time, we also looked at the absolute numbers of lung cDCs in response to HD-Ad delivery and found that around day 2 after HD-Ad delivery, there was a marked decrease in absolute numbers of lung cDCs which eventually returned to normal levels by day 5 (Fig. 5C). The decrease in absolute numbers of cDCs mirrored the increase in maturation of cDCs in response to HD-Ad vectors, with increased maturation correlating with reduced numbers of cDCs in the lung.

**HD-Ad vectors induce migration of pulmonary DCs to draining MLN**

To better understand the basis for the decrease in lung cDC levels after HD-Ad delivery, we next used FITC-dextran to track the location of lung cDCs by assessing their migration. FITC-dextran is a carbohydrate with a very high molecular mass, which is easily uptaken by phagocytic cells including DCs, and hence allows for tracking the location of phagocytic cells. To assess DC migration after HD-Ad delivery, we delivered FITC-Dextran 2 h before HD-Ad delivery to label phagocytic cells, including DCs within...
FIGURE 4. Assessment of T cell proliferation in the lung and the draining mediastinal lymph node in response to pulmonary HD-Ad delivery. A, Representative histogram showing BrdU incorporation among CD3$^+$ T cells in the mouse lung upon HD-Ad delivery (low dose - $5 \times 10^9$ particles) on days 0 (i) and 6 (ii) compared with mice receiving saline. B, BrdU incorporation by CD3$^+$ T cells in the lung at different times after delivery of high ($1 \times 10^{11}$ particles) or low dose ($5 \times 10^9$ particles) of HD-Ad. C, Proportion of CD4$^+$ and CD8$^+$ T cells among proliferating BrdU$^+$ T cells on day 6, upon delivery of high ($1 \times 10^{11}$ particles) or low dose ($5 \times 10^9$ particles) of HD-Ad. D, BrdU incorporation by CD3$^+$ T cells in the draining MLN at different time points after delivery of high ($1 \times 10^{11}$ particles) or low dose ($5 \times 10^9$ particles) of HD-Ad. E, Proliferation of draining MLN-derived T cells from HD-Ad- or saline-treated mice upon coculture with HD-Ad-treated DCs (control refers to coculture of T cells derived from saline-treated animals with saline-treated DCs). RLU, relative light units, was a chemiluminescent measurement of BrdU incorporation by proliferating cells. *, $p < 0.05$ compared with control and saline groups. The percentage of BrdU expression ($B$ and $D$) is shown for CD3$^+$ gated T cells at the indicated number of days after HD-Ad delivery. All values represent $n = 3–4$ mice per time point and $n = 8$ per group ($E$).
the airways. The efficacy of using FITC-Dextran is demonstrated in Fig. 6A, which clearly shows that 1 day after FITC-dextran and HD-Ad delivery, FITC-dextran+/CD11c+ cells can be observed within the draining MLN. In general, cDCs rapidly migrate from peripheral tissues to the draining MLN in response to infection. Using FITC-dextran, we were able to identify the subset of DCs that migrated from the lung to the draining lymph node in response to HD-Ad vectors. In response to HD-Ad, there was a dramatic increase in the percentage of FITC+ DCs (CD11c+ DCs) in the lymph node 24 h after HD-Ad delivery (Fig. 6B). In contrast, control receiving only saline did not show this dramatic increase; instead, a small increase on day 1 was observed, indicating basal level of DC migration (Fig. 6B). The dramatic increase seen in response to HD-Ad delivery compared with delivery of saline was thus an event specific to HD-Ad delivery. FITC+ DCs observed in MLN were CD11c+ and mPDCA1+, indicating that they were cDCs and not pDCs (data not shown). FITC+ DCs observed in MLN demonstrated the phenotype of mature DCs, indicated by CD11c+CD86+CD80+CD86+CCR7high, consistent with the notion of migration of mature DCs to the draining LN (data not shown). At different times we also measured the absolute count of DCs in the draining MLN by looking at total DC count along with the count of FITC+ and FITC- DCs. The trend observed was similar to that seen with percent of FITC+ DCs, for both the FITC+ and FITC- DCs increased in levels after day 0 and peaked around day 2, after which they receded to basal levels by day 5 (Fig. 6C). This is consistent with the knowledge that mature DCs have a very short lifespan, so probably mature DCs are entering LN, where they are priming T cell responses and then dying within 24–48 h.

The peak in DC count within the MLN occurred around day 2 (Fig. 6B), which correlates with the observed reduction in absolute number of mature cDCs within the lung on day 2 (Fig. 5C). Similarly, the percentage of mature DCs was also reduced on day 2 (Fig. 5B), indicating that probably migration of mature cDCs from the lung to the draining MLN resulted in decrease of cDCs within the lung and a dramatic increase of FITC+ mature DCs within the MLN.

**HDAd vectors induce maturation of plasmacytoid DCs but do not affect their levels in the lung**

Since pDCs may play a key role in priming immune response to viral particles, we also looked at the effects of HD-Ad vectors on
pDCs within the airways (24). First, we assessed the effects of HD-Ad on maturation of pDC. Fig. 7A shows representative histograms showing CD86 expression on lung pDCs at day 0 and day 1 after HD-Ad vector delivery. We observed that upon HD-Ad delivery pDCs matured rapidly, with maximum maturation being observed around day 1 and eventually receding by day 2 and returning to basal levels by day 5 (Fig. 7A and B). Thus, the trend was similar to that observed with cDCs. Since the trend observed with cDCs was associated with migration to the draining LN, we next assessed the migration of pDCs from the lung to draining LN in response to HD-Ad. It has been reported that FITC-Dextran cannot be used for identifying pDCs, because pDCs do not readily uptake FITC-Dextran. CFSE has been used before to track pDCs, and therefore we used intranasal delivery of CFSE to track movement of pDCs from the lung to the draining LN (25). CFSE was delivered 2 h before viral delivery to label all the cells in the airways. At different times after HD-Ad delivery, we assessed the presence of CFSE+ DCs within the lymph nodes and found results similar to that observed with FITC-Dextran, with percentage of CFSE+ DCs peaking around day 2 and then receding to basal levels by day 5 (data not shown). However, we did not detect presence of mPDCA1+ cells among CFSE+ population within the lymph nodes, indicating that there was no migration of pDCs induced in response to HD-Ad vectors (Fig. 7C). At the same time, we also measured the absolute numbers of pDC in the lung stayed very consistent, further confirming the lack of pDC migration from the lung (Fig. 7D).

**HD-Ad vectors induce maturation of CD8α DCs in the draining MLN**

CD8α DCs are thought to play a key role in cross-presentation of Ags, and because we observed induction of a CD8+ T cell proliferation even without viral transcription upon HD-Ad delivery, we assessed the effects of HD-Ad delivery on CD8α DCs to identify whether CD8α DCs can play a potential role in induction of CD8+ T cell proliferation in response to intranasal delivery of HD-Ad vectors. Because CD8α DCs are known to be lymphoid resident, we did not observe recruitment of CD8α DCs in the lungs. However, upon assessing for maturation of draining MLN resident
CD8α DCs by assessing for expression levels of CD86, we observed a dramatic increase in the levels of CD86 expression by CD8α DCs in response to HD-Ad delivery, indicating that intranasal delivery of HD-Ad was resulting in maturation of CD8α DCs within the draining MLN (Fig. 8).

**Discussion**

Several clinical trials with first generation adenoviral vectors were initiated and ultimately discontinued due to an induction of immune response, attributed to leaky expression of viral genes from this vector in airway epithelium (5). In contrast, development of HD-Ad vectors with no viral coding sequences led to the assumption that these vectors will not initiate adaptive immune responses because there is no leaky expression of any viral genes. Due to this assumption, adaptive immune responses to HD-Ad vectors were never extensively studied and hence the knowledge of pulmonary immune responses to these vectors is practically absent. To the best of our knowledge, our study is the first to demonstrate induction of pulmonary adaptive immune response to intranasal delivery of HD-Ad vectors, and clearly illustrate the ability of HD-Ad vectors to induce both CD4+ as well as CD8+ T cell response at high and substantially low dose. CD4+ T cells responses can possibly be induced via uptake of exogenous HD-Ad vector particles by DCs and presentation via MHC II pathway. However, induction of CD8+ T cell response in absence of any viral transcription was

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**FIGURE 7.** Effect of HDAd delivery on pulmonary plasmacytoid DC levels and maturation within the lung. A, Representative histograms showing expression of CD86 on mPDCA1+ pDCs before HDAd delivery (day 0) (i) and 1 day after delivery (ii). B, Increase in mPDCA1+CD86+ DC population in the lungs of mice at different time points after HDAd delivery, relative to mPDCA1+CD86+ pDC population in the lungs of mice receiving saline. C, Representative histogram showing lack of mPDCA1 expression on CFSE+ cells from the draining MLN, 24 h after delivery of CFSE and HD-Ad vector particles. D, Absolute numbers of pulmonary pDCs in the lung at different time points after HD-Ad delivery to mice. All values represent n = 3–4 mice per time point.

**FIGURE 8.** Effects of HD-Ad delivery on CD8α DCs in the draining MLN. Flow cytometric analysis of CD86 expression was performed on CD8α DCs, 24 h after HD-Ad delivery. Isotype matched IgG was used as isotype control and results are representative of n = 8 animals.
unexpected, because viral gene expression is usually needed for Ags to be shuttled through the MHC I pathway.

In two recent gene therapy clinical trials using AAV serotype 2 vectors, which are thought to be the least immunogenic, there were reports of apparent immune responses with appearance of CD8+ effector T cells (26). This gave rise to the so-called capsid T cell hypothesis, which postulates that exogenous vector proteins may be shuttled into the MHC class I pathway for T cell priming (12). Our results demonstrate similar phenomena occurring upon pulmonary delivery of HD-Ad vectors, whereby we are seeing proliferation of CD8+ T cells along with CD4+ T cells. This observation indicates that HD-Ad-derived peptides are being shuttled to MHC I pathway and cross-presented in context with MHC class I to induce CD8+ T cell response along with presentation with MHC class II to induce CD4+ T cell response. Five dominant CD8+ T cell epitopes on the capsid hexon protein of adenovirus have been identified and because HD-Ad vector does indeed has the capsid proteins, it is possible that perhaps cross-presentation of these epitopes may play a role in inducing CD8+ T cell responses (27). Our results indicate that perhaps CD8α DCs may play a role in cross-presenting HD-Ad-derived epitopes, because this subset of DC undergoes maturation within the draining MLN upon HD-Ad delivery. We did not observe recruitment of CD8α DCs in the lungs as expected, because these DCs are thought to be nonmigratory. However, maturation of CD8α DCs indicates that perhaps cDCs are migrating to the draining MLN and transferring the Ags to CD8α DCs, which results in maturation of CD8α DCs. Mature CD8α DCs may then go on to induce CD8+ T cell response, as has been observed with lung and s.c. infection using HSV (28, 29). Moreover, CD103+ DCs with the ability to cross-present inhaled Ags and migrate to draining MLN have also been identified in the mouse lung (30). Therefore, there may be a specialized population of DCs that may be initiating CD8+ T cell responses upon HD-Ad delivery via cross-presentation, which may include CD103+ DCs and CD8α DCs, which we have shown to mature in draining MLN in response to HD-Ad. MHC:peptide complexes of immunodominant epitopes can have a half-life of >7 days (31, 32). Therefore, HD-Ad transduced epithelial cells may present vector-derived epitopes for substantial amounts of time to allow for responding T cells to migrate to the airways and gradually target/eliminate some transduced cells. Furthermore, upon readministration, memory T cells are probably activated which are recruited to the site within the first 3 days and mediate gradual clearing of transduced cells, and hence may account for observed loss in transgene expression upon vector readministration (9, 33). Moreover, the extent of both CD4+ and CD8+ proliferation in the lung and draining MLN peaked around day 6–7, which is similar to the timeline of pulmonary T cell responses associated with other pulmonary infections such as influenza (34, 35).

We assessed the response of pulmonary DCs to HD-Ad to gain an insight behind priming of T cell response to HD-Ad vectors upon pulmonary delivery. Our results demonstrated that early on after HD-Ad delivery, there is rapid maturation of both cDC along with pDC, which peaks around day 1. As DCs mature, there is migration of only cDCs to the lymph nodes, resulting in reduction of percent of mature cells, as well as reduction in absolute count of cDC in the lung by day 2 after HD-Ad vector delivery. During the first 48 h after delivery, there is massive increase in the numbers of lung-derived DCs in the draining MLN, which eventually recedes to basal levels by day 5. In contrast to cDCs, pDCs do not migrate and probably act as local sites of IFN-α secretion to prime innate and adaptive immune responses. Ex vivo studies have further demonstrated that adenovirus can induce IFN-α secretion by pDCs via TLR-MyD88 pathway (36). The observation of priming of DC maturation and migration within the first 48 h is consistent with the previous findings involving other respiratory viruses such as paramyxoviruses (22). This may in part explain the role of clinically used mucolytic agent Nacystelyn, which can inhibit DC maturation, in enhancing HD-Ad mediated gene transfer to mouse airways (4, 37). Thus, cDCs seem to play the central role in priming pulmonary immune responses against HD-Ad vectors, and hence intervention within the first 24 h of vector delivery to prevent DC maturation/migration may help in mitigating the adaptive immune responses induced by HD-Ad vectors. In contrast to our findings that HD-Ad vectors act as potent stimulators of pulmonary DC maturation and pulmonary T cell response, it was recently reported that infection with adenovirus can suppress lung DC function, and such suppression was related to adenovirus transcription (38). We reason that the report’s conclusion may be instead attributed to gene products encoded by E3 region, which function to modulate host-immune response (39). In particular, E3gp19K protein encoded by E3 region of the adenoviruses genome has been shown to sequester MHC I complexes in endoplasmic reticulum, thereby suppressing CD8+ T cell responses (40). Since HD-Ad particles do not encode any viral genes including E3 region, these vectors cannot suppress pulmonary DC responses and are rather initiators of adaptive immune response.

In conclusion, our data demonstrate that pulmonary delivery of HD-Ad results in induction of CD4+ as well as CD8+ T cell responses that peak around day 5–7. These responses are primarily orchestrated by cDCs that mature and migrate to draining MLN within the first 48 h after HD-Ad delivery. At the same time, pDCs also mature but do not migrate to MLN, and hence may act as local source of IFN-α to prime innate and adaptive immune response. Therefore, pulmonary delivery of known DC maturation inhibitors such as prostaglandin E2, Bortezomib, FK778, a derivative of the active leflunomide-metabolite, within the first 24 h after HD-Ad vector delivery, may inhibit priming of pulmonary adaptive immune responses by preventing maturation and thereby migration of cDCs (41–43). Moreover, because T cell proliferation peaked around a week after HD-Ad delivery, transient immunosuppression within a week after HD-Ad vector delivery using cyclosporine A or CTLA4Ig to inhibit CD28/B7 pathway or inhibition of CD40/CD40L pathway via CD40LIg administration will prevent T cell proliferation, and hence suppress adaptive immune response to HD-Ad vectors (44–46). Strategies to inhibit adaptive immune responses will inhibit induction of memory T cells. This may go on to prevent the observed loss of transgene expression and anti-HD-Ad Ab titer, observed in mice upon subsequent re-administrations. Identification of adaptive immune responses initiated upon pulmonary delivery of HD-Ad vectors indicates that strategies targeted to improve vector delivery and persistence need to target both adaptive and innate immune responses. Moreover, identification of the time-line of these responses may help in identifying the time-point for intervention to prevent subsequent unwanted immunological responses.

Disclosures
The authors have no financial conflict of interest.

References


