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Restricted Aeroallergen Access to Airway Mucosal Dendritic Cells In Vivo Limits Allergen-Specific CD4^+ T Cell Proliferation during the Induction of Inhalation Tolerance

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Chronic innocuous aeroallergen exposure attenuates CD4^+ T cell-mediated airways hyperresponsiveness in mice; however, the mechanism(s) remain unclear. We examined the role of airway mucosal dendritic cell (AMDC) subsets in this process using a multi-OVA aerosol-induced tolerance model in sensitized BALB/c mice. Aeroallergen capture by both CD11^blo and CD11^bhi AMDC and the delivery of OVA to airway draining lymph nodes by CD8α^- migratory dendritic cells (DC) were decreased in vivo (but not in vitro) when compared with sensitized but nontolerant mice. This was functionally significant, because in vivo proliferation of OVA-specific CD4^+ T cells was suppressed in airway draining lymph nodes of tolerized mice and could be restored by intranasal transfer of OVA-pulsed and activated exogenous DC, indicating a deficiency in Ag presentation by endogenous DC arriving from the airway mucosa. Bone marrow-derived DC Ag-presenting function was suppressed in multi-OVA tolerized mice, and allergen availability to airway APC populations was limited after multi-OVA exposure, as indicated by reduced OVA and dextran uptake by airway interstitial macrophages, with diffusion rather than localization of OVA across the airway mucosal surface. These data indicate that inhalation tolerance limits aeroallergen capture by AMDC subsets through a mechanism of bone marrow suppression of DC precursor function coupled with reduced Ag availability in vivo at the airway mucosa, resulting in limited Ag delivery to lymph nodes and hypoproliferation of allergen-specific CD4^+ T cells. The Journal of Immunology, 2011, 187: 000–000.

Dendritic cells (DC) and their subsets play a central role in coordinating T cell-mediated inflammatory responses to peripheral self-antigens and external Ags, including inhaled aeroallergens that induce allergic inflammatory diseases such as allergic asthma (1–3). In addition to these well-described proinflammatory roles, however, a number of studies have implicated DC in the downregulation of immune responses to peripheral Ags by a variety of mechanisms, including passive mechanisms such as induction of T cell unresponsiveness or anergy through to active induction of CD4^+ T regulatory cells (Treg). In this context, Ag targeted to steady-state DC was shown to induce peripheral T cell unresponsiveness (4, 5), whereas mature, Ag-processing DC can expand Treg that express CD25 and Foxp3 (6, 7). Other potential mechanisms for DC in limiting T cell responsiveness include regulation of tryptophan metabolism by induced expression of IDO in DC (8) or Treg induction through targeting of Ag to subsets of DC including tolerogenic plasmacytoid DC (pDC) (8–10).

Inhalation tolerance is a process whereby exposure to innocuous protein Ags across the respiratory mucosa induces a state of immunological tolerance at either the T or B cell level to subsequent exposure to the Ag. A variety of rodent models have demonstrated this phenomenon, including passive exposure to aeroallergen before sensitization or chronic exposure to aeroallergen after sensitization, with Treg induction and activity implicated in the maintenance of tolerance in both cases (11–16). In contrast, repeated inhalation of complex Ag extracts such as house dust mite leads to persistent inflammation and remodeling in some cases (17), presumably as a consequence of their inherent TLR or proteolytic activity. DC have been implicated in the induction and maintenance of tolerance in the respiratory tract, either as regulators of CD4^+ T cell proliferation in draining lymph nodes (DLN) (18) or inducers of Treg in airway and DLN, or as targets of Treg activity in the airways (12, 14, 19).

In the mouse, a variety of respiratory DC subsets have been described and have been shown to play a role in either the induction or regulation of airways inflammation. Two major populations of CD11^bhi MHC class II^hi myeloid-origin DC that are either CD11^bhi CD103^- or CD11^bhi CD103^+ have been described, as...
well as rarer populations of MHC class II⁺ CD11b⁻ monocyte-like DC and MHC class II⁺ B220⁺ Ly6C⁺ PDC (reviewed in Ref. 3). In the lung, CD11b⁺ CD103⁻ DC have been shown to be major producers of chemokines that contribute to T cell, monocyte, and polymorphonuclear leukocyte recruitment during airways inflammation, whereas CD11b⁺ CD103⁺ lung DC are involved in cross-presentation of exogenous Ags to CD8⁺ T cells and recruitment of TH2 and Tregs (20, 21). In the gut, CD103⁺ DC have been shown to direct the development of Tregs through a TGF-β and retinoic acid-dependent mechanism (22); however, such a role has yet to be directly ascribed to lung-derived CD11b⁻ CD103⁺ lung DC. Lung CD11b⁺ CD103⁺ DC have also been shown to express a range of tight junction proteins, leading to the current concept that these cells can interact with bronchial epithelial cells and directly sample the lumen for inhaled allergens (23, 24). Regulatory roles have also been described for lung pDC (25), and recent studies have also described populations of regulatory myeloid-origin DC that produce IL-10 and can limit TH2-mediated allergic airway responses (26–28).

The respiratory tract can be divided into two major anatomical and immunological compartments, these being the mucosal barriers of the trachea and conducting airways and the gas exchange barriers of the alveoli and their associated lung parenchymal tissue. We have previously shown that this is functionally important, because the activity of DC is regulated dependent on their anatomical location, and DC residing at airway mucosal surfaces (airway mucosal DC [AMDC]) have a high turnover rate and are involved in the early initiation stages of T cell-mediated allergic airways disease (29–31). Although a number of studies have examined the tolerogenic activity of lung DC as a function of their post-Ag exposure interactions with CD4⁺ T cells, little is known regarding the regulation of aeroallergen movement across the mucosal barrier and the efficiency by which this can be captured by AMDC populations for activation of allergen-specific CD4⁺ T cells in DLN.

In this study, we have examined aeroallergen-capture and CD4⁺ T cell activating activity of mouse AMDC subsets during the induction of inhalation tolerance to inhaled OVA using a multi-OVA aerosol exposure model in sensitized BALB/c mice (13). This model shares several features with other postsensitization models of inhalation tolerance developed in mice (15, 16) and rats (14), including suppressed recruitment of effector CD4⁺ T cells and eosinophils into the airways and attenuated airways hyperresponsiveness through the promotion of Treg activity. We found that both CD11b⁺ CD103⁻ and CD11b⁻ CD103⁺ AMDC captured inhaled OVA in vivo during the early onset of allergic inflammation, and that induction of inhalation tolerance markedly inhibited the capacity of AMDC subsets for aeroallergen capture. The functional consequence of this was a markedly suppressed in vivo proliferation of OVA-specific CD4⁺ T cells in the airway draining lymph nodes (ADLN) of tolerant mice, which could be fully restored by the delivery of OVA-loaded and activated DC into the airways. Reduced activation and in vitro T cell stimulation by AMDC from tolerant mice was observed, as well as delayed delivery of OVA to ADLN by migratory CD8α⁻ DC. Finally, restricted inhaled Ag uptake in tolerant mice was not specific for OVA, as FITC-dextran uptake was also downregulated in OVA-tolerized mice, and not limited to AMDC as OVA and dextran uptake by airway interstitial macrophages (AIM) was also inhibited, potentially via a mechanism of altered allergen deposition and movement across the airway mucosal barrier. These data indicate that aeroallergen movement across airway epithelial barriers is altered during inhalation tolerance induction, limiting allergen capture by AMDC and other APC subsets and their capacity for induction of CD4⁺ T cell proliferation.

Materials and Methods

Animals

Specific pathogen-free 8-wk-old female BALB/c mice were sourced commercially (Animal Resource Centre, Perth, WA, Australia) and housed under standard conditions. BALB/c DO11.10 TCR transgenic mice (The Jackson Laboratory), expressing a TCR recognizing OVA peptide 323–339 (ISQAVHAAHAEINEAGR) (32), were bred under clean conditions at the Telethon Institute for Child Health Research. All animal studies were approved by the Telethon Institute for Child Health Research and Murdoch University Animal Ethics and Experimentation Committee operating under the guidelines of the National Health and Medical Research Council of Australia.

Sensitization and aerosol challenge protocol

Sensitization and aerosol challenge protocol were as previously described (13). In brief, on days 0 and 14, mice received 20 µg OVA (Grade V chicken egg; Sigma-Aldrich, Sydney, NSW, Australia) in 200 µL 2.25 mg/20 µL 0.2% aluminum hydroxide (Serra, Germany) via the i.p. route. Commencing on day 21, mice received either a single 1% OVA aerosol (×1 aero) or a total of 8 × 1% OVA aerosols over a 3-wk period delivered three times a week (multiaero) as described by Burchell et al. (13). Control mice were sensitized to OVA and received endotoxin-free PBS aerosols.

Trachea and ADLN preparations

Mouse tracheal and lymph node preparations have been described in detail previously (33). In brief, trachea were collected from pools of five to eight mice and single-cell preparations prepared by type IV collagenase digestion (1.5–2 mg/ml; Worthington Biochemical, Lakewood, NJ). ADLN (upper paratracheal and parathymic) were pooled separately from the same groups of mice, chopped with a scalpel, and digested with type IV collagenase and type I DNase (0.1 mg/ml; Sigma-Aldrich). All digestions and washes were performed in glucose sodium potassium buffer (11 mM NaH₂PO₄, 2 H₂O) with debris and RBCs removed as described previously (33).

Analysis of DC populations and in vivo Ag uptake by flow cytometry

After single-cell suspensions were generated, FcR were routinely blocked using 2.4G2 (BD Biosciences) for 10 min on ice before the addition of phycocyanin-mAb to mouse CD4⁻ CD11c⁺ CD11b⁻ (HL3; BD Biosciences). Migratory DC in ADLN were identified using the above antibodies in combination with CD8⁺ (53-6.7; BD Biosciences) and CD11c (HL3; BD Biosciences), CD11b (M1/70; BD Biosciences), or CD40 (3/23; BD Biosciences). Migratory DC in ADLN were identified using the above markers in combination with CD8α (53-6.7; BD Biosciences) to distinguish CD8α⁺ migratory AMDC from resident lymph node CD8α⁻ DC populations. All Abs were used as direct conjugates to FITC, PE, PE-Cy5, and Alexa Fluor 647 (OVA-A488, OVA-A647; Molecular Probes) in 50 µl saline before use (AffinityPak Detoxi-Gel Column; Pierce, Rockford, IL). In some cases, 40 kDa Dextran-FITC (Molecular Probes, Invitrogen, Life Technologies, Mulgrave, VIC, Australia) was prepared to matching molar concentrations.

For analysis of inhaled Ag uptake in vivo, mice were lightly anesthetized with gasoan isoflurane and received 20 µg OVA-conjugated Alexa Fluor 488 or 647 (OVA-A488, OVA-A647; Molecular Probes) in 50 µl saline delivered per nasally (p.n.) in place of the final OVA aerosol. All Ag solutions were prepared in PBS and detoxified over a polymyxin B column before use (AffinityPak Detoxi-Gel Column; Pierce, Rockford, IL). In some cases, 40 kDa Dextran-FITC (Molecular Probes, Invitrogen, Life Technologies, Mulgrave, VIC, Australia) was prepared to matching molar concentrations and used in place of OVA-A488 or -A647. Mice were sacrificed at indicated time points after fluorescent OVA administration, and trachea and ADLN tissue were harvested.

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In vitro CD4+ T cell stimulation by AMDC and bone marrow-derived DC

Pooled trachea collected 24 h after the final aerosol were digested to single-cell suspensions as described earlier, labeled with fluorochrome-conjugated mAbs to mouse CD11c, I-A/I-E, and CD11b, and passed through a sterile nylon mesh before cell sorting. CD11c+I-A/I-E+ AMDC were digested to CD11bhi and CD11blo expressing cells after light scatter gating for use in vitro as APCs (BD FACs Aria Cell Sorter: BD, North Ryde, NSW, Australia). In some cases, bone marrow-derived DC (BMDC; see later) were cultured from bone marrow cells isolated 24 h after final OVA aerosol for use as APCs in separate assays. Responder (BMDC; see later) were cultured from bone marrow cells isolated 24 h after final OVA aerosol for use in vitro as APCs (BD FACS Aria Cell Sorter: BD, in OPTILUX dishes (BD Falcon) in IMDM with 10% FCS (Trace Biosciences, Australia). Cells were prepared in RPMI 1640 with glutamine (Invitrogen Life Technologies, Australia) supplemented with 10% FCS, 20 μg/ml gentamicin, and 20 μM 2-ME, and 1.25 × 10^5 APCs cultured with 1 × 10^5 T cells in round-bottom 96-well plates (Nunclon, Nunc, Nordenmark) together with OVA peptide 323–339 (ISOQVHAAAHEINEAGR, LPS free, ProSci, Castle Hill, NSW, Australia) at 20 μg/ml/gentamicin, 20 μM 2-ME, and 10 μg/ml polyoxynin B (Sigma-Aldrich, Australia), and 10% GM-CSF (generated as tissue culture supernatant from the X68 GM-CSF transfected cell line; kind gift of Dr. B. Stockinger). At day 8 of culture, 2 × 10^5 cells/ml were transferred to 25-cm² tissue culture flasks (BD Biosciences, Australia), pulsed with 1 mg/ml OVA for 2 h, then stimulated for 24 h with either 1 μg/ml LPS or 10 μg/ml anti-CD40 mAb (FGK45, Alexis Biochemicals, San Diego, CA), Isolated BMDC were then washed and transferred at 1 × 10^6 cells in 200 μl PBS i.v. or 5 × 10^5 cells in 50 μl PBS i.p.

Immunohistochemical staining and confocal microscopy

Mice were lightly anesthetized using inhaled isoflurane and a single 50-μg dose of OVA-A488 instilled p.n. after the last aerosol as described earlier. Two hours after instillation, animals were sacrificed and trachea were dissected. The trachea was fixed in 70% ethanol immediately after dissection and kept at 4°C overnight, then permeabilized with 0.2% Triton X-100 for 30 min at room temperature. After rinsing with 0.1% BSA in PBS, the tissue was incubated with a biotinylated I-A/I-E mAb (BD, Australia) at 1:100 in 0.1% PBS/BSA on a shaker at 4°C overnight. Samples were washed in 0.1% PBS/BSA and were incubated with streptavidin Alexa 547 (Invitrogen Life Technologies, Australia; dilution 1:100 in PBS + 0.1% BSA) on a shaker at 4°C overnight. After staining, whole tracheas were snap frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA) and cross sections were made using a cryomicrotome (Leica Microsystems, North Ryde, NSW, Australia). Sections were mounted on microscope slides and dried at room temperature for a maximum of 30 min. Samples were embedded using Vectashield mounting medium (Vector Laboratories, CA) and analyzed using an Olympus BX51 microscope (Leica Microsystems). All sections were analyzed by unpaired, two-tailed Student t test with 95% confidence intervals using Prism analysis software (GraphPad Software).

Results

In vivo Aeroallergen capture by AMDC subsets and delivery to ADLN is attenuated in multi-OVA tolerized mice

We have previously shown that a multi-OVA aerosol exposure regimen of LPS-free OVA into sensitized BALB/c mice induced airways hyperresponsiveness tolerance by a mechanism involving, in part, low levels of CD4+ CD69+ effector T cell recruitment into the airways and expansion of regulatory Foxp3+ CD25+ CD4+ T cells (13). In this study, we aimed to determine whether the mechanism underlying both of these events could be related to alterations in the aeroallergen capture and presenting capacity of AMDC, because these cells have been implicated in the early priming of aeroallergen-specific CD4+ Th2 cells in airways and ADLN (33, 36). To test this, we modified our multi-OVA aerosol exposure protocol to include a p.n. delivery of LPS-free OVA conjugated to Alexa 647 (OVA-A647) in place of the last OVA aerosol to track the Ag-capture activity of AMDC in vivo. Conducting airways were isolated 2 h after OVA-A647 exposure, and single-cell suspensions were analyzed for OVA-A647 uptake by FACS. AMDC were identified in saline aerosol control, ×1 OVA, and multi-OVA aerosol mice by a combination of high expression of CD11c and I-A/I-E and multi-OV A aerosol mice by a combination of high expression of CD11c and I-A/I-E (Fig. 1A, left panel) and further classified as CD11bhi (CD103<sup>hi</sup>) and CD11blo (CD103<sup>lo</sup>) AMDC subsets by multiparameter flow cytometry (Fig. 1A, right panel). Analysis of OVA-647 uptake by AMDC subsets in single OVA-aerosol exposed mice demonstrated a high capacity for OVA-647 capture by both CD11bhi (Fig. 1B, upper panels) and CD11blo (Fig. 1B, lower panels) AMDC subsets (72.2 and 54.7% of cells OVA<sup>+</sup>, respectively) when compared with control mice (Fig. 1B, left panels). However, CD11bhi AMDC from ×1 OVA mice captured OVA most efficiently, both in terms of numbers of OVA<sup>+</sup> cells and intensity of OVA uptake. In contrast, OVA-A647 capture by both CD11bhi and CD11blo AMDC subsets (28.8 and 9.0% of cells OVA<sup>+</sup>, respectively) was markedly attenuated in tolerized, multi-OVA aerosol mice when compared with nontolerized ×1 OVA aerosol mice (Fig. 1B, right panels), with an almost complete abolition of OVA uptake by the CD11blo subset (Fig. 1B, lower panel). The number of OVA<sup>+</sup> AMDC in the airways declined over the next 24 h after OVA-A647 inhalation for both the CD11bhi and CD11blo AMDC subsets of single-OVA aerosol mice, consistent with the migration of these cells out of the airways to local DLN (Fig. 1C, black bars). This was confirmed by a corresponding increase in the number of OVA<sup>+</sup> migratory CD8<sup>+</sup>CD11bhi and CD8<sup>+</sup>CD11blo DC in the ADLN (but not nondraininginguinal nodes; data not shown) of single-aerosol mice over the same 24-h period (Fig. 1D, black bars), consistent with the rapid emigration of OVA-bearing AMDC to the DLN rather than degradation of the OVA signal in the airways. Similarly, the number of OVA-647<sup>+</sup> AMDC in the airways of tolerized multi-OVA exposed mice declined over a 24-h period for both the CD11bhi and CD11blo AMDC subsets (Fig. 1C, gray bars). However, as expected, the percentage of OVA<sup>+</sup> cells was markedly lower in multi-OVA aerosol mice than that observed for ×1 OVA aerosol mice, and the rate of decline was faster such that few OVA<sup>+</sup> AMDC were observed in the airways of multi-OVA exposed mice at 24 h after OVA-A647 inhalation (Fig. 1C). In the ADLN of multi-OVA-exposed mice, the percentage of OVA<sup>+</sup> CD8<sup>+</sup>CD11bhi migratory DC remained reduced at 24 h after OVA inhalation when compared with single-aerosol control mice (Fig. 1D, left panel). Although this reduction was not statistically significant for...
ADLN were harvested from the same mice as in of inhaled OVA in ADLN DC subsets of OVA aerosol-exposed mice. Fluorescence determined within the CD11bhi (CD11c, I-A/I-E, CD11b, and CD103 as described in Materials and Methods. AMDC were identified as CD11chi I-A/I-Ehi cells after standard forward scatter and side scatter gating (left panel) and then gated as CD11bhi (CD103hi and CD11bhi CD103hi) for subsequent analysis (right panel). B. Uptake of inhaled OVA-A647 by AMDC subsets in vivo. Tracheal tissue was isolated from sensitized BALB/c mice 2 h after control saline aerosol (left panels), single-OVA (middle panels), or multi-OVA (right panels) aerosol exposure, using p.n. OVA-A647 as the single OVA exposure or in place of the last multi-OVA aerosol exposure. The cells were labeled for FACS analysis as described earlier, and OVA-A647 fluorescence determined within the CD11bhi (upper panels) and CD11bhi (lower panels) AMDC subsets. Data are from pooled tissue of five animals for each treatment group, and one representative of four separate experiments using pooled tissue of five animals for each treatment group at each time point. Results are shown as mean values ± SEM. *p < 0.05. D. Persistence of inhaled OVA in ADLN DC subsets of OVA aerosol-exposed mice. ADLN were harvested from the same mice as in C and labeled for FACS as the percentage of cells positive for OVA (p = 0.06), it did reach significance for absolute numbers of cells recovered (×1 OVA = 1085 ± 55 cells; multi-OVA = 529 ± 6 cells; p = 0.009). In contrast, the percentage of OVA+ CD8α+ CD11bhi DC in ADLN were equivalent in both ×1- and multi-OVA aerosol-exposed mice at the 24-h time point (Fig. 1D, right panel).

In vivo aeroallergen-specific CD4+ T cell proliferation is suppressed in multi-OVA tolerized mice that can be restored by activated OVA-loaded DC

The reduced aeroallergen capture capacity of AMDC subsets suggested that multi-OVA aerosols induced tolerance-generated populations of CD11bhi AMDC with suboptimal naive CD4+ T cell-stimulating capacity. To test this in vivo, we injected CFSE-labeled, BALB/c DO11.10 TCR transgenic T cells i.v. into multi-OVA aerosol tolerized or nontolerized (×1 OVA) mice 24 h after final OVA aerosol. Transgenic T cell proliferation was then assessed 3 d later by preparing single-cell suspensions of ADLN, labeling for CD4 and the transgenic TCR (KJ-126), and assessing CFSE profiles of CD4+ KJ-126+ cells by FACS. As expected, the transgenic CD4+ T cells proliferated vigorously in the ADLN of mice receiving an ×1 OVA aerosol, with ~70% of the cells entering into one or more divisions, as indicated by only 30.3% of cells remaining in the undivided peak (Fig. 2A, left panel). In contrast, transgenic T cells transferred into multi-OVA–tolerized mice proliferated poorly in the ADLN, with 59.4% of cells remaining in the undivided peak and with dividing cells entering into only one or two division cycles (Fig. 2A, right panel). To test whether the lack of T cell division was truly related to a migrating DC defect and not a secondary inhibition of T cell division by Tregs in the DLN, we used GM-CSF to expand CD11bhi DC from the bone marrow of normal BALB/c mice (BMDC). BMDC were pulsed with OVA, stimulated with LPS overnight, then washed and delivered p.n., or in some cases, i.v., at 2 h after final OVA into multi-OVA–tolerized mice. Twenty-four hours later, CFSE-labeled DO11.10 T cells where then transferred i.v. and proliferation assessed as described earlier. OVA-loaded and LPS-stimulated BMDC (OVA-LPS BMDC) effectively restored T cell division (5.5% of cells remained undivided) in the ADLN of multi-OVA mice (Fig. 2B, right panel) when compared with control multi-OVA–exposed mice receiving p.n. saline (54.3% of cells remaining undivided; Fig. 2B, left panel). In addition, OVA-LPS BMDC restored CD4+ T cell division in the trachea to a similar extent to ADLN (Fig. 2C), although this may, in part, have also represented cells that have divided in the ADLN and trafficked back to the airways. Finally, restoration of CD4+ T cell proliferation by OVA-LPS BMDC was not an artifact of residual LPS contamination, because anti-CD40 mAb-induced maturation of OVA-loaded BMDC in place of LPS was also effective (data not shown).

Multi-OVA–induced tolerance alters the frequency and function of AMDC subsets

To further investigate the function of AMDC subsets after multi-OVA–induced tolerance, we used FACS to analyze the frequency and activation status of ex vivo-derived AMDC from multi-OVA–tolerized mice. The frequency of total CD11c+ MHC II+ AMDC described, including CD8a as an additional marker to identify CD8α− airway migratory DC. Subsets of migratory DC were identified by high expression of CD11c and I-A/I-E, and further gated as CD8a− and either CD11bhi (left panel) or CD11bhi (right panel). Data are from three experiments using pooled tissue from five animals for each treatment group at each time point. Results are shown as mean values ± SEM.
Inhibition of OVA-specific CD4+ T cell proliferation in the ADLN was significantly increased 2 h after aerosol exposure in both ×1- and multi-OVA aerosol-exposed mice compared with control mice (Fig. 3A, 3B, left panels). In addition, a small but significant decrease in the relative proportion of CD11bhi AMDC and increase in CD11blo AMDC (as a proportion of total AMDC) was observed in single-aerosol-treated mice but had normalized in multi-OVA–tolerized mice when compared with saline aerosol-exposed controls (Fig. 3B, middle and right panels). To investigate the functional activity of AMDC after multi-OVA exposure, we purified CD11bhi and CD11blo AMDC subsets from the trachea of single- or multi-OVA–exposed mice by cell sorting and used them in an ex vivo Ag-presenting assay to D011.10 CD4+ T cells after pulsing with the H-2d restricted OVA peptide 323–339. Both the CD11bhi (Fig. 3C, left panel) and CD11bhi (Fig. 3C, right panel) subsets of AMDC from single-OVA aerosol-exposed mice showed higher levels of peptide-presenting capacity to OVA-specific CD4+ T cells than saline aerosol-exposed control mice, consistent with

FIGURE 2. OVA-specific CD4+ T cell proliferation in the ADLN of mice exposed to inhaled OVA. A, Inhibition of OVA-specific CD4+ T cell proliferation in the ADLN of multi-OVA–exposed mice. Enriched CD4+ T cells from the lymph nodes of naïve D011.10 OVA-TCR transgenic donor mice were labeled with CFSE and 3 x 10^6 cells transferred i.v. into ×1-OVA (left panel) or multi-OVA (right panel) aerosol mice 24 h after the final OVA exposure. Forty-eight hours later, ADLN cells were isolated and stained for CD4 and the KJ-126 transgenic TCR, and CFSE intensity was determined by FACS as shown in (left panel) or multi-OVA (right panel) aerosol mice 24 h after the final OVA exposure. Forty-eight hours later, ADLN cells were isolated and stained for CD4 and the KJ-126 transgenic TCR, and CFSE intensity of CD4+ KJ-126+ transgenic cells was determined by FACS as shown in A and B. Each data point indicates results obtained from a pool of tissue from five mice, and each treatment was repeated as indicated. **p < 0.01.

FIGURE 3. Frequency and function of AMDC subsets after OVA aerosol exposure. A and B, AMDC frequency increased in tracheal tissue after ×1- and multi-OVA aerosol exposure. Tracheal tissue was isolated from sensitized and saline aerosol-exposed control mice (control), ×1-OVA, and multi-OVA aerosol-exposed mice 2 h after final aerosol; stained for CD11c, I-A/I-E, and CD11b; and assessed by FACS. A, AMDC were identified as CD11c+ I-A/I-Ehi cells, and B (the frequency of total (left panel), CD11bhi (middle panel), and CD11bhi (right panel) AMDC were assessed. The mean ± SEM percentage frequency of each subset is shown for each treatment group of pools of five animals per experiment for three independent experiments. C, In vitro OVA-specific CD4+ T cell stimulation by AMDC subsets after OVA-aerosol exposure. Tracheal cells were isolated 2 h after final aerosol for each exposure group and sorted for AMDC subsets as described in Materials and Methods. CD11bhi AMDC (left panel) and CD11bhi AMDC (right panel) were plated at 1.25 x 10^3 cells/well of 96-well microtiter plates and pulsed with 5 μg/well of the H-2d restricted OVA 323–339 peptide. Purified CD4+ T cells from pooled lymph nodes of three D011.10 OVA-TCR transgenic mice were then added at 1 x 10^5 cells/well, incubated for 48 h, and then pulsed with [3H]thymidine for 20 h. Cell proliferation was assessed by [3H]thymidine deoxyribosyl incorporation and expressed as mean cpm ± SEM of triplicate cultures using AMDC isolated from pooled tracheal tissue from five mice, showing one representative experiment of three repeat experiments with similar results. **p < 0.01, ***p < 0.001, p versus control cultures, ’p versus ×1 OVA cultures.
the enhanced in vivo Ag-presenting activity of these cells. In contrast, CD11b\(^+\) AMDC from multi-OVA-tolerized mice stimulated OVA-specific CD4\(^+\) T cell proliferation to a significantly lower extent than those from x1 OVA-aerosol mice, with no increase above CD11b\(^+\) AMDC isolated from saline aerosol-exposed control mice (Fig. 3C, left panel). Similarly, CD11b\(^+\) AMDC from multi-OVA-tolerized mice showed a reduced T cell stimulatory capacity compared with x1 OVA-aerosol mice, with the stimulatory capacity of these cells now similar to, or slightly below, that of CD11b\(^-\) AMDC from saline aerosol-exposed control mice (Fig. 3C, right panel).

Inhalation tolerance inhibits DC maturation from bone marrow precursors

Populations of AMDC residing within the airway mucosa have a rapid rate of turnover, with replacement of resident cells from bone marrow myeloid progenitors occurring, on average, every 12 h (30). Thus, the reduced functional capacity of AMDC observed in multi-OVA aerosol-tolerized mice may have reflected a decreased functional activity of influxing precursor cells from the bone marrow. To test this, we isolated bone marrow cells from saline control, x1 OVA, or multi-OVA-aerosol-exposed mice and examined their capacity to generate functional BMDC in vitro after culture in GM-CSF. Bone marrow cell cultures from control mice generated a high proportion of mature CD11b\(^+\) BMDC (85% of total DC generated), with 45% of these cells expressing high levels of MHC class II (Table I). Although the total MHC class II expression on BMDC derived from x1 OVA aerosol mice was slightly reduced when compared with control cultures, there was no difference in the proportion of CD11b\(^+\) or CD11b\(^-\) MHC class II\(^+\) BMDC generated from these mice (Table I, Supplemental Fig. 1). In contrast, bone marrow cell cultures from multi-OVA-exposed mice generated BMDC with significantly lower total levels of MHC class II, and also a significantly lower proportion of CD11b\(^+\) MHC class II\(^+\) cells, when compared with bone marrow cultures of both single-aerosol–exposed and control mice (Table I, Supplemental Fig. 1). Although the in vitro uptake of OVA, and CD40 upregulation after LPS exposure, by BMDC was similar for all groups (data not shown), the ability to present OVA 323–339 CD40 upregulation after LPS exposure, by BMDC was similar for CD11b\(^+\) MHC class II\(^+\) cells, when compared with bone marrow cultures of both single-aerosol–exposed and control mice (Fig. 3). In contrast, bone marrow cell cultures from multi-OVA-exposed mice generated BMDC with significantly lower total levels of MHC class II, and also a significantly lower proportion of CD11b\(^+\) MHC class II\(^+\) cells, when compared with bone marrow cultures of both single-aerosol–exposed and control mice (Table I, Supplemental Fig. 1).

Inhalation tolerance alters Ag localization and uptake at airway mucosal surfaces

Although the preceding experiments confirmed that the decreased capacity for OVA-specific CD4\(^+\) T cell stimulation by AMDC observed in vivo in tolerized mice could be reproduced using in vitro cocultures, at no point could we replicate the reduced in situ capacity for OVA capture by AMDC subsets that we observed in tolerized mice using in vitro OVA uptake systems with purified AMDC. In this respect, AMDC subsets from all groups of mice showed an equivalent capacity to capture OVA-647 in vitro when assessed by FACS (data not shown), suggesting that microenvironmental factors may be influencing inhaled Ag availability to resident APC populations. Therefore, we next investigated the possibility that inhalational exposures to AECs in the airway mucosa was reduced under conditions of inhalation-induced tolerance, thereby limiting the amount of Ag available for capture and transport by AMDC subsets and other cell types with endocytic capacity. To test this, we examined inhaled Ag capture by resident AIM that can be identified in airway tissue by FACS among a population of cells expressing intermediate levels of CD11c and high levels of MHC class II (Fig. 5A, left panel, left box), and that we have previously shown to express high levels of CD11b and to be F4/80\(^+\) and be highly endocytic (30, 37). We examined inhaled OVA uptake and also the Ag specificity of inhaled allergen capture by replacing OVA with inhaled 40 kDa FITC-labeled dextran (Dextran-FITC) and examining uptake of this marker by CD11b\(^+\) and CD11b\(^-\) AMDC (Fig. 5A, right upper panel) and CD11b\(^+\) AIM (Fig. 5A, right lower panel). Dextran-FITC was effectively captured in vivo by CD11b\(^+\) (but not CD11b\(^-\)) AMDC (Fig. 5B, middle left panel) and AIM in x1-OVA aerosol-exposed mice. In contrast, dextran-FITC capture was downregulated after multi-OVA exposure in AMDC and AIM (Fig. 5B, right panels). Similar to AMDC shown earlier, inhaled OVA-647 uptake by AIM was high in x1 OVA aerosol-exposed mice (Fig. 5C, left panel), but significantly reduced in multi-OVA–tolerized mice (Fig. 5C, right panel), mimicking the downregulation of OVA-A647 uptake shown earlier for AMDC after multi-OVA aerosol exposure.

In parallel studies, we used confocal microscopy of mouse tracheal tissue to visualize the distribution of inhaled OVA-A647 in x1 OVA or multi-OVA mice, in conjunction with staining for actin to visualize epithelial cells and MHC class II to visualize intraepithelial and submucosal DC. In x1 OVA aerosol-exposed mice, OVA was distributed mainly on the apical surface of airway epithelial cells, in close association with MHC class II\(^+\) intraepithelial AMDC (Fig. 6, left panel, arrows). In contrast, in multi-OVA aerosol-tolerized mice, OVA was rarely seen on the epithelial surface, but rather was now scattered throughout the epithelial layer and into the submucosa (Fig. 6, right panel, arrows).

Discussion

In this study, we show that induction of inhalation tolerance to innocuous aeroallergen induces a state of allergen-specific CD4\(^+\)
T cell hyporesponsiveness in DLN and the airway mucosa that is mediated by a marked downregulation of allergen capture by AMDC subsets. These data are consistent with our and other studies showing induction of physiological and immunological tolerance after repeated exposure to innocuous allergens (11, 13, 16, 38) and with a previous report that this process is dependent on the CCR7-guided migration of allergen-bearing DC to the DLN (18). We observed that inhaled aeroallergen is effectively captured by both CD11bhi and CD11blo subsets of AMDC, equating to the CD103− DC subset that have recently been described in both the lung and gut (3). Recent studies have shown that CD11bhi CD103− lung DC express the tight junction proteins Claudin-1, Claudin-7, and ZO-2, leading to the suggestion that this subset is dominant over CD11blo CD103− DC in their capacity to sample the airway lumen and capture inhaled allergens (23, 24). Our data are not entirely consistent with this functional separation, but suggest that both CD11bhi and CD11blo AMDC have the capacity for high-level aeroallergen capture, with the CD11bhi subset dominant under Ag-sensitized conditions. Rather, our data are more consistent with that of Jakubzick et al. (39), who showed that CD11bhi CD103− lung DC preferentially acquire soluble proteins over CD11blo CD103+ DC, the latter preferentially acquiring particulate Ags.

A key finding in this study was that induction of inhalation tolerance was associated with a marked decrease in aeroallergen capture by AMDC and AIM. Tracheal cells were isolated from OVA/alum-sensitized mice 2 h after single p.n. dextran-FITC exposure, and FITC uptake determined by FACS in CD11blo AMDC (upper left panel), CD11bhi AMDC (middle left panel), or CD11blo AIM (lower left panel). Corresponding dextran-FITC uptake by AMDC and AIM isolated from multi-OVA aerosol-exposed mice, in which p.n. dextran-FITC replaced the final OVA aerosol, are shown in the right panels. FACS histograms of dextran-FITC uptake by AMDC and AIM are shown from pools of tracheal tissue of five animals per group, as a representative of three experiments with similar results. OVA-A647 capture by AIM in vivo after multi-OVA aerosol exposure. Tracheal cells were isolated from OVA/alum-sensitized mice 2 h after single p.n. OVA-A647 exposure (left panel) or p.n. OVA-A647 in place of the final OVA aerosol in multi-OVA–exposed mice (right panel). FACS histograms of OVA-A647 uptake by AIM are shown from pools of tracheal tissue from five animals per group, as a representative of three to five experiments with similar results.

**FIGURE 5.** In vivo uptake of inhaled dextrans and OVA by AIM. A, Identification of AMDC and AIM by FACS. Tracheal tissue was isolated from OVA/alum-sensitized mice 2 h after a single p.n. dose of 50 μg/ml 10 kDa dextran-FITC. CD11chi I-A/I-Ehi cells were gated by FACS (left panel, left box) and then gated for CD11blo AIM (lower right panel). AMDC were identified as CD11chi I-A/I-Ehi cells (left panel, right box) and gated as either CD11blo or CD11bhi AMDC. B, Dextran-FITC capture by AMDC and AIM. Tracheal cells were isolated from OVA/alum-sensitized mice 2 h after single p.n. dextran-FITC exposure, and FITC uptake determined by FACS in CD11blo AMDC (upper left panel), CD11bhi AMDC (middle left panel), or CD11blo AIM (lower left panel). Corresponding dextran-FITC uptake by AMDC and AIM isolated from multi-OVA aerosol-exposed mice, in which p.n. dextran-FITC replaced the final OVA aerosol, are shown in the right panels. FACS histograms of dextran-FITC uptake by AMDC and AIM are shown from pools of tracheal tissue of five animals per group, as a representative of three experiments with similar results.
and also airways. This reduction in allergen capture was observed in both AMDC subsets at 2 h after p.n. Ag instillation, although most prominently in the CD11b\textsuperscript{hi} subset, and by 24 h, most allergen-carrying DC had been cleared from the airways of multi-OVA–tolerized mice, although a small amount still persisted in nontolerized mice in both subsets of AMDC. It is interesting to speculate on the fate of aeroallergen captured by CD11b\textsuperscript{hi} and CD11b\textsuperscript{lo} subsets of AMDC. As recent studies have suggested that the former are specialized for direct presentation of Ag to CD4\textsuperscript{+} T cells and the latter for cross-presentation to CD8\textsuperscript{+} T cells in DLN under tolerogenic conditions (21). We found that lower levels of inhaled allergen capture in the airways of tolerized mice closely correlated with persistently reduced amounts of Ag in ADLN for the CD11b\textsuperscript{lo} population of CD8\textsuperscript{+} migratory DC at 24 h after p.n. delivery when compared with nontolerant and allergen-reactive mice, but that accumulation of OVA is delayed, reaching levels equivalent to that of nontolerized controls by 12 (data not shown) to 24 h after p.n. delivery. These data suggest that the deficiency in CD4\textsuperscript{+} T cell proliferation observed in the ADLN of tolerized mice was likely the net result of delayed and persistent changes in the delivery of OVA to the ADLN by migrating AMDC subsets.

Our data indicate that AMDC numbers were increased after both single- and multi-OVA exposure, with no obvious alteration in the ratio of CD11b\textsuperscript{hi} to CD11b\textsuperscript{lo} cells, indicating that reduced aeroallergen capture was not a consequence of reduced or biased AMDC subset recruitment. However, AMDC from the airways of tolerized mice did show a decreased capacity for ex vivo CD4\textsuperscript{+} T cell stimulation when compared with single-OVA aerosol-exposed mice, and DC grown from bone marrow precursors of tolerized mice showed a reduced maturation profile, including reduced MHC class II expression and capacity to stimulate CD4\textsuperscript{+} T cells in vitro either as fresh cells or after LPS maturation. Lambrecht et al. (40) have previously shown that bone marrow cells from sensitized and aeroallergen-exposed rats have increased numbers of DC progenitors, and suggest this as a mechanism for the increased numbers of DC observed in the airways during allergic airways inflammation. Our data showing increased DC numbers in the airways of both single- and multi-OVA mice is consistent with this; however, we now show that induction of aero-tolerance leads to a decreased OVA-presenting capacity of DC grown from bone marrow precursors of multi-OVA–tolerized mice. Previous studies have suggested that cytokines such as TGF-\textbeta can alter the stimulatory activity of DC and generate a “tolerogenic” DC phenotype and promote long-term allograft survival on adoptive transfer into mice (41–43). In this regard, we have previously shown markedly increased levels of TGF-\textbeta in lavage fluids of multi-OVA–tolerized mice (13), and in this article, we postulate that TGF-\textbeta may be acting systemically to alter DC function and/or maturation in the bone marrow of tolerized mice. The possible mechanisms of actions of TGF-\textbeta on BMDC function remain unclear; however, in vitro studies have shown that TGF-\textbeta exposure reduces the allostimulatory activity of human BMDC and blocks the final maturation step of DC in murine BMDC cultures, thus causing an immature population of DC (44, 45). TGF-\textbeta appears to upregulate IL-10 and downregulate IL-12 production, along with NF-\kappaB activation, and may act at the transcriptional level to inhibit MHC Class II expression (46, 47). Future in vivo adoptive transfer studies will investigate the potential for TGF-\textbeta to alter DC development from BM or airway precursors as a potential mechanism for tolerance induction, as well as local activity of this cytokine on AMDC subset function.

An outstanding question from this study is the Ag specificity of the tolerogenic T cell response induced after multi-OVA exposure. Because of technical reasons, we were not able to address this directly; however, our in vivo dextran uptake experiments suggest a generalized reduction in inhaled molecular uptake by DC and macrophages after induction of tolerance that would affect immune responsiveness to any innocuous inhaled Ag (Fig. 5). The tolerogenic response is likely to be specific only for innocuous inhaled allergens, however, as even low-level activation of AMDC through Toll receptor engagement or other innate stimuli, such as would be the case with house dust mite extracts, for example, will promote an immunogenic response through upregulation of MHC and costimulatory molecules (as reviewed in Refs. 1, 24).

In addition to DC, the airway environment contains other cell types with Ag capture potential, including a prominent population of interstitial macrophages that express intermediate levels of CD11c and high levels of CD11b and F4/80 (30, 48). These macrophages show a high capacity for inhaled protein capture and processing in vivo, and although current dogma states that macrophages do not migrate to DLN, a recent study has challenged this concept (49). During the course of this study, we noted that OVA capture by AIM was also inhibited after induction of inhalation tolerance. Further investigation showed that this was not restricted to OVA, but could also be observed when inhaled dextrans of small (10 kDa) and medium (40 kDa) molecular mass were used in place of OVA. Dextran are soluble, complex sugar molecules whose endocytosis by DC and other APCs is mediated by the mannose receptor (CD206), a pattern-recognition receptor with similarities to c-type lectin receptors such as DEC205, that aids the capture of glycosylated proteins and allergens by DC and other APCs (50–52). Although we observed expression of CD206 on airway macrophages and AMDC, little change in the expression levels of CD206 was observed during the induction of inhalation tolerance (data not shown). This suggested that other mechanisms may be operating to limit Ag availability within the airway microenvironment to cells with endocytic activity, including AMDC and macrophages. This was supported by confocal imaging of inhaled fluorescent OVA in the airways of multi-OVA–tolerized mice, which showed a different distribution pattern to OVA in the airways of nontolerant, single-OVA–exposed mice: in multi-OVA–tolerized mice, inhaled OVA was distributed throughout the epithelium and submucosa, whereas in nontolerant mice, the inhaled OVA stayed predominantly on the epithelial barrier surface where it would presumably be more readily available for uptake by intraepithelial AMDC, as supported quantitatively by our flow cytometry studies.

The tight junctions between adjacent polarized airway epithelial cells form a physical barrier and normally provide the first line of defense against inhaled viruses, which can disrupt tight junction integrity to aid viral pathogenesis (53). Tight junction barrier
function is maintained by several junctional proteins, including intracellular zona occludens (ZO), extracellular occludins and claudins, and basolateral desmosomal adhering junctions that form intercellular junctions between epithelial cells that regulate passage of macromolecules (54). Altered expression of tight junction proteins, including reduced levels of ZO-1, have been observed in human asthma and may contribute to inflammatory cell and protein influx during inflammation (55, 56). In this study, we observed qualitatively by confocal microscopy that ZO-1 expression was downregulated after induction of inhalation tolerance (J.T. Burchell and P.A. Stumbles, unpublished observations). Together, our data suggest the possibility that the changes in allergen distribution and capture by APC in the airway mucosa may be influenced by changes in epithelial barrier integrity: intact tight junctions trap and focus inhaled allergen to the epithelial surface where it is readily available for uptake by intraepithelial AMDC. In contrast, changes in tight junction integrity during tolerance induction allow rapid penetration of aeroallergen into the submucosa. This would cause rapid dilution and removal of allergen via lymphatics, reducing local concentrations of Ag available for uptake by resident APC, with the net effect being reduced availability of Ag for presentation to T cells in the airways and ADLN, as we demonstrated quantitatively by flow cytometry for OVA uptake by AMDC and in vivo OVA-specific CD4+ T cell proliferation in ADLN and airways.

In summary, these data show that induction of physiological and immunological tolerance to a defined aeroallergen results in hypersensitiveness of OVA-specific CD4+ T cells mediated through attenuation of OVA capture by CD11b+CD103+ and CD11b+CD103+ AMDC subsets. This resulted in reduced delivery of aeroallergen to the DLN and reduced proliferation of allergen-specific CD4+ T cells in the DLN, a response that could be restored by airway delivery of Ag-loaded and activated BMDC. The maturation and Ag-presenting capacity of DC grown from bone marrow isolated from tolerized mice was also inhibited, suggesting that the induction of tolerance may provide systemic signals to the bone marrow to inhibit DC maturation, whereas the capture of OVA and nonrelated dextran by mucosal macrophages was also inhibited through a mechanism involving alterations in epithelial barrier tight junction integrity and Ag availability at the mucosal surface. Together, these data illustrate the multifactorial nature of tolerance induction at airway mucosal surfaces, and suggest that control of aeroallergen access to AMDC and other airway APCs during aeroallergen challenge may be an important regulatory mechanism for activation of naive allergen-specific CD4+ T cells.

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Disclosures

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References


Supplementary Figure 1: Down-regulation of MHC Class II expression on CD11b\textsuperscript{hi} CD11c\textsuperscript{hi} BMDC grown from OVA-sensitized and multi-OVA-aerosol exposed mice.