Allergic Airways Disease Develops after an Increase in Allergen Capture and Processing in the Airway Mucosa

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Allergic Airways Disease Develops after an Increase in Allergen Capture and Processing in the Airway Mucosa

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Airway mucosal dendritic cells (AMDC) and other airway APCs continuously sample inhaled Ags and regulate the nature of any resulting T cell-mediated immune response. Although immunity develops to harmful pathogens, tolerance arises to nonpathogenic Ags in healthy individuals. This homeostasis is thought to be disrupted in allergic respiratory disorders such as allergic asthma, such that a potentially damaging Th2-biased, CD4+ T cell-mediated inflammatory response develops against intrinsically non-pathogenic allergens. Using a mouse model of experimental allergic airways disease (EAAD), we have investigated the functional changes occurring in AMDC and other airway APC populations during disease onset. Onset of EAAD was characterized by early and transient activation of airway CD4+ T cells coinciding with up-regulation of CD40 expression exclusively on CD11b− AMDC. Concurrent enhanced allergen uptake and processing occurred within all airway APC populations, including B cells, macrophages, and both CD11b+ and CD11b− AMDC subsets. Immune serum transfer into naive animals recapitulated the enhanced allergen uptake observed in airway APC populations and mediated activation of naive allergen-specific, airway CD4+ T cells following inhaled allergen challenge. These data suggest that the onset of EAAD is initiated by enhanced allergen capture and processing by a number of airway APC populations and that allergen-specific Igs play a role in the conversion of normally quiescent AMDC subsets into those capable of inducing airway CD4+ T cell activation. 

inhaled protein allergen. In particular, we suggest that local CD4+ T cell activation is likely to be driven in this model by a population of CD11b+ airway mucosal DC that uniquely express the costimulatory molecule CD40 during the early stages of disease onset. Furthermore, we also present data supporting the conclusion that the enhanced allergen uptake and processing capacity of APC within the airway mucosa is mediated by allergen-specific IgG that aid Ag capture by airway APC populations. These data highlight the need for multiple target strategies in the treatment and prevention of allergic AHR, including interventions that target DC-, T cell-, and Ab-driven pathogenic processes.

Materials and Methods
BALB/c mouse model of experimental allergic airways disease (EAAD)

BALB/c mice were bred specific pathogen free at the Animal Resource Centre (Perth, Australia) and housed under clean conditions on low-dust bedding at the Telethon Institute for Child Health Research (TICHR). BALB/c DO11.10 TCR-transgenic mice recognizing an I-Ad-restricted epitope of OVA (OVA323-339 peptide sequence ISQAVHAAHAE NEAGR) were purchased from The Jackson Laboratory and maintained under clean conditions. All mice were used as females of 8–10 wk of age and given free access to feed and water. As shown in Fig. 1A, mice were sensitized by i.p. injection with 20 μg of OVA (Sigma-Aldrich) suspended in 200 μl (4 mg) of Al(OH)3 (Alum; Alu-gel-S; Serva) on days 0 and 14. Mice were then challenged with either one or three OVA (1/5 w/v in PBS) aerosols delivered with an ultrasonic nebulizer (DeVilbiss Ultra-Neb) for 30 min on consecutive days starting at day 21. Additional groups of mice served as controls: a naive group and a group that received i.p. PBS and was challenged with OVA aerosols using the protocol described above. In other experiments, mice were sensitized by i.p. injection with 10 μg of β-lactoglobulin A (BLG) from bovine milk (Sigma-Aldrich) suspended in 200 μl of Alum (Alu-gel-S; Serva) on days 0 and 14. Animal experiments were approved by the TICHR Animal Experimentation Ethics Committee operating under guidelines in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Cell preparations from lung and conducting airways

Animals were euthanized by i.p. injection of 100 μl of phenobarbitone sodium (Lethabarb; Virbac). Lung and heart were exposed by bilateral thoracotomy and the aorta and inferior vena cava were cut to exsanguinate animals before perfusion of the right ventricle with at least 5 ml of PBS. Thereafter, the peripheral third of the lung was excised (further referred to as lung parenchyma) and airways, including the trachea and the main bronchi (further referred to as main conducting airways), were prepared. Lung parenchyma was chopped into 2-mm slices using a Mcllwain tissue chopper (Mickle Laboratory Engineering) and main conducting airways were manually sliced into thin pieces. Cell isolation procedures were conducted in a solution of 11 mM t-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM NaH2PO4, and 5.5 mM NaHPO4 · 2 H2O (GKN) supplemented with 10% FCS as indicated. Tissue was transferred into 30 ml of GKN-10% FCS containing 1.8 mg/ml collagenase type 4 (Worthington Biochemical) and 0.1 mg/ml DNase I (Sigma-Aldrich) and incubated for 90 min at 37°C in a shaking water bath. Unless indicated otherwise, all subsequent procedures were strictly performed on ice. Tissue was disrupted with a plastic transfer pipette until most of the larger tissue pieces were dispersed. The digest mixture was then passed through a cotton wool filter to remove tissue debris. After one wash in GKN-10% FCS, BSC lysis was performed with NH4Cl and cells were resuspended in fluorescence buffer (PBS containing 0.5% BSA and 0.1% sodium azide) after one wash.

Flow cytometry

All Abs were obtained from BD Pharmingen, unless indicated otherwise. All incubations were performed on ice throughout the procedure and including an Fc block (mouse CD16/CD32) added for 10 min to reduce nonspecific binding. T cells were identified using a combination of Abs against CD3 and CD4, and early activation was measured with an Ab against CD69. APCs were identified using Abs against CD11c, I-A/E (MHC class II), and CD11b, as described previously, and activation was assessed with Abs against CD40, CD80, and CD86 (5). OVA-specific CD4+ T cells were identified in DO11.10 mice using the KJ1-26 mAb (Caltag Laboratories) and naive cells were distinguished from memory cells on the basis of CD44 expression, where naive T cells are CD44low. Relevant isotype control Abs were used throughout to differentiate specific binding from nonspecific binding or background fluorescence. Cell samples were collected using a four-color FACScanlibur or a six-color LSRII (BD Biosciences) and analyzed with FlowJo Software (Tree Star).

Ag-tracking studies

Ag uptake and processing was tracked in vivo by administering a variety of fluorescent conjugates, as described previously (9). OVA-Alexa Fluor 647 (Molecular Probes) was used to track OVA uptake; an Alexa Fluor 647 conjugate of BLG was prepared using a protein labeling kit (Molecular Probes) according to the manufacturer’s instructions. DQ-OVA (Molecular Probes) was used to track OVA processing. A total of 20–50 μg of each conjugate was administered per nasal (p.n.) in a 50-μl volume by gradual application to the nares during halothane or isoflurane anesthesia. Two hours later, the main conducting airways were collected and prepared for flow cytometry as described above. Ag uptake and processing was assessed in various cell types by comparison with a group of mice administered 50 μl of saline p.n. (saline control).

Measurement of AHR

AHR to inhaled methacholine (MCh) was measured using a modification of the low-frequency forced oscillation technique as previously described (10). Briefly, mice were anesthetized, tracheostomized and ventilated using a custom built ventilator (flexiVent; Sciquest) that functioned both as a ventilator and means of measuring respiratory system input impedance (Zrs). Zrs was measured during periods of apnea by applying a 16-s signal containing 19 mutually prime sinusoidal frequencies ranging from 0.25 to 19.625 Hz. The real and imaginary parts of the Zrs spectrum were fit to the constant-phase model allowing calculation of changes in airway resistance (raw), tissue damping (g), tissue elasticity (H) (11). The changes in these parameters were measured in response to 90-s MCh aerosols generated using an ultrasonic nebulizer (UltraNeb; DeVilbiss) ranging in dose from 0.1 to 30 mg/ml.

Specific Ab determination by time-resolved fluorescence assay

Sera were analyzed for the presence of total IgE and OVA-specific IgG2a and IgG1 Abs by time-resolved fluorescence assays. Briefly, 96-well plates (Nunc Maxisorp) were coated overnight at 4°C with 10 μg/ml OVA for determination of OVA-specific IgG1, or with anti-mouse IgE (R35-72; BD Pharmingen) for total IgE measurements. Plates were blocked with 200 μl of 0.5% BSA in Tris-HCl (pH 7.4) for 1 h at room temperature on a plate shaker. For all subsequent steps, a volume of 50 μl/well was used and incubations were performed during 1 h at room temperature unless otherwise indicated. Between steps, plates were washed five times with wash buffer Tris-HCl (pH 7.8) Tween 20. As interassay standards, mouse anti-OVA IgG1 (Hyb 099-09) and monoclonal mouse IgE (BD Pharmingen) were used. Sera were diluted 1/10 in Delfia assay buffer (Wallac Oy). Biotinylated anti-mouse IgE (R35-118) and IgG1 (A85-1; The Antibody Shop) were purchased from BD Pharmingen and used at 2 μg/ml. Streptavidin-conjugated europium (Wallac Oy) was incubated at 1/500 for 30 min and plates were washed eight times thereafter. Delfia enhancement solution (Wallac Oy) was added and plates were agitated on a shaker during 10 min before reading the fluorescence on a Wallac Victor 2 counter (Wallac Oy).

Bromochoduloear lavage (BAL) fluid inflammatory cell counts

Separate groups of sensitized and control mice were euthanized and tracheostomized to collect BAL fluid. The BAL fluid was centrifuged at 2000 rpm for 4 min. Following the removal of the supernatant, the pellet was resuspended in PBS and cells were stained with trypan blue to determine the total cell count and live cell count (trypan blue exclusion). Differential counts were obtained from the cytospin sample, stained with Diffquick stain (LabAids), and examined using light microscopy and counting at least 300 cells.

Passive immunization

OVA-immune serum was collected from OVA-sensitized BALB/c mice 48–72 h after they had been challenged with three OVA aerosols, as described above. Control serum was collected at the same time from BALB/c

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mice that had received two doses of PBS in Alum i.p. (days 0 and 14) but
were not exposed to OVA aerosols. Sera were pooled from at least 10
mice/group before 100–150 μl was administered i.v. to naive BALB/c
mice. Each pool of sera was assayed for OVA-specific IgG1 and total IgE
as described to confirm increased levels in the OVA-immune group and
low levels in the control group.

Statistics
Parametric statistical analysis of data was performed with Prism software
(GraphPad Software) using the unpaired, nonparametric Student t test unle-
less otherwise indicated. Values of p < 0.05 were considered statistically
significant. Lung functional responses in airway resistance and tissue
damping to inhaled MCh at the maximum dose used (30 mg·ml⁻¹) were
expressed as a percentage of the response to saline aerosol and com-
pared using nonparametric ANOVA on ranks and Dunn’s post-hoc test. Responses in tissue elastance were expressed as a percentage of the response to saline, log₁₀ transformed, and compared using ANOVA and Tukey’s post-hoc test.

Results
Characterization of the BALB/c mouse model of EAAD
A BALB/c mouse model of OVA-induced EAAD was adopted to
analyze RT-APC function during the onset of allergic airways in-
flammation. The sensitization protocol consisted of two systemic
(i.p.) administrations of Alum-adsorbed OVA on days 0 and 14
(OVA sensitization), followed by exposure to aerosolized OVA
(referred to as OVA challenge) at different time points (Fig. 1A).
For the assessment of early changes, studies were conducted after
24 h following a single aerosol on day 21, while late and more
chronic changes were investigated 48 h after the third of three
daily aerosols administered on days 21–23. The primary clinical
markers used to define EAAD are shown in Fig. 1, B–E. Lung func-
tional changes were measured using forced oscillometry, a
technique that enables partition of central airways resistance from
peripheral lung parenchymal tissue damping and elastance. Twen-
ty-four hours after a single OVA-aerosol exposure, significantly in-
creased airways resistance, tissue damping, and tissue elastance
were measured as compared with naive control mice (Fig. 1B).
These parameters remained significantly elevated in the more
chronic setting (48 h after three daily aerosols), although in the
case of airways resistance, were starting to decline. The percentage
of eosinophils was significantly increased in BAL fluid 24 h after
a single OVA-aerosol exposure and continued to increase follow-
ning multiple aerosols (Fig. 1C). In terms of Ab responses, total IgE
was measured (rather than OVA-specific IgE) due to the >14-fold higher OVA-specific IgG1 that were shown to interfere with measurement of OVA-specific IgE by ELISA in some groups (data not shown). Serum IgE levels showed an initial significant rise during the systemic sensitization phase before the first aerosol and remained elevated throughout the aerosol exposure period compared with control mice (Fig. 1D). OVA-specific IgG1 Ab production, in contrast, showed an early (but not significant) increase before aerosol exposure, and then further significant increases in response to single and multiple OVA-aerosol challenges ($p < 0.0001$ compared with the pre-aerosol and single-aerosol time point; Fig. 1E). In summary, the salient clinical features of the BALB/c mouse model of EAAD including increased airways resistance, BAL eosinophilia, and induction of serum IgE and IgG1 Abs resemble the hallmark clinical features of human allergic asthma.

**FIGURE 2.** Kinetics of T cell activation during allergic airways sensitization. Cell suspensions were obtained from pooled tissue of 10 animals/group for conducting airways and lung parenchyma before analysis by flow cytometry as described in Materials and Methods. Top panels represent percentage frequency of CD3$^+$CD4$^+$ T cells and bottom panels represent percentage CD69 expression on CD3$^+$CD4$^+$ T cells from main conducting airways (left panels) and lung parenchyma (right panels). Bar graphs represent mean ± SEM of data from three to five experiments at the indicated time points: 1 Aero + 12 h = 12 h post 1 aerosol; 1 Aero + 24 h = 24 h post 1 aerosol; 3 Aero + 48 h = 48 h post 3 aerosols. Statistical analyses ($p$) comparing PBS controls (square) to OVA-sensitized animals (closed circle) at the indicated time points following OVA-aerosol challenge were performed by Student’s $t$ test.

**FIGURE 3.** Kinetics and expression of CD11b and CD40 on CD11c$^+$MHCII$^+$ RT-DC during allergic airways sensitization. At the indicated time points after OVA-aerosol challenge, cell suspensions were obtained from pooled conducting airways and lung parenchyma tissue of 10 animals/group in PBS controls (square) and OVA-sensitized mice (closed circle) before analysis by flow cytometry as described in Materials and Methods. (A) Kinetics of CD11c$^+$MHCII$^+$ airway DC, (B) percentage CD11b, and (C) percentage CD40 expression on CD11c$^+$MHCII$^+$ DC in main conducting airways (left panels) and lung parenchyma (right panels) after OVA-aerosol challenge. Bar graphs represent mean ± SEM of data from three to five experiments at the indicated time points: 1 Aero + 12 h = 12 h post 1 aerosol; 1 Aero + 24 h = 24 h post 1 aerosol; 3 Aero + 48 h = 48 h post 3 aerosols. Statistical analyses ($p$) comparing PBS controls (square) to OVA-sensitized animals (closed circle) at the indicated time points following OVA-aerosol challenge were performed by Student’s $t$ test.
**Table I. Summary of the phenotypic characteristics of mouse airway APC**

<table>
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<tr>
<th>APC Type</th>
<th>I-A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD11c</th>
<th>CD11b</th>
<th>CD2</th>
<th>CD16/32</th>
<th>CD54</th>
<th>CD103</th>
<th>CD115</th>
<th>CD205</th>
<th>F4/80</th>
<th>Gr1</th>
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*<sup>a</sup> - , <10%; ±, 11–20%; +, 21–40%; ++, 41–60%; ++++, >61%.
*<sup>b</sup> Approximately 10% of airway B cells are CD11b<sup>b</sup>.
*<sup>c</sup> Macrophages uniformly express low levels of CD11c compared to airway DC.
*<sup>d</sup> Macrophages and CD11b<sup>b</sup> DC uniformly express intermediate levels of CD103 compared to CD11b<sup>b</sup> DC.
*<sup>e</sup> Macrophages and DC expressed low or intermediate levels of Gr1 compared to granulocytes.
*<sup>f</sup> CD11b<sup>b</sup> DC uniformly express low levels of CD11b compared to B cells.

**CD4<sup>+</sup> T cell activation in anatomical compartments of the RT during aerosol allergen challenge**

T cells are central in the pathogenesis of bronchial allergic asthma, with RT CD4<sup>+</sup> T cell activation considered to be a prerequisite event for the initiation of EAAD (12). Given the early physiological changes occurring in the conducting airways and lung parenchymal compartments described above, we examined whether any correlation existed between onset of functional changes and the kinetics of CD4<sup>+</sup> T cell activation within these compartments. Using the mouse model described above, we followed T cell activation kinetics in the RT during allergic airways sensitization, as measured by the expression of the early activation marker CD69 on CD3<sup>+</sup> CD4<sup>+</sup> T cells. Frequencies of total (CD3<sup>+</sup> CD4<sup>+</sup>) and activated (CD3<sup>+</sup> CD4<sup>+</sup> CD69<sup>+</sup>) T cells were determined in both tracheal tissue (referred to as conducting airways) and lung parenchyma at 12 and 24 h after a single aerosol challenge and 48 h after three aerosols. Although no significant changes were observed in the frequency of CD3<sup>+</sup> CD4<sup>+</sup> T cells in either RT compartment (Fig. 2, top panels), a transient and significant increase in CD69 expression on CD4<sup>+</sup> T cells was observed in the conducting airways at 12 and 24 h after a single aerosol and at 24 h after a single aerosol in the lung parenchyma (Fig. 2, bottom panels). Thus, CD4<sup>+</sup> T cell activation occurred early following allergen challenge in the mucosa of the conducting airways (12–24 h), coinciding with the time point at which increased airways resistance was observed in this model (Fig. 1B).

**Kinetics of RT-DC subset activation during onset of EAAD**

The above data suggested that early activation of CD4<sup>+</sup> T cells in the mucosa of the conducting airways was a predisposing event for the development of airways hyperreactivity, a key clinical feature of EAAD and human allergic asthma. It is now well-established that RT-DC are important regulators of T cell activation in response to allergen challenge, coordinating both T cell priming in DLN and local T cell activation within the RT (13). Therefore, we next sought to establish whether T cell activation within the RT was accompanied by changes in the frequency of RT-DC subsets in conducting airways and lung parenchyma. Single-cell suspensions prepared from the conducting airways and lung parenchyma were stained for CD11c and I-A/E (MHC class II (MHC II)) and RT-DC were identified as CD11c<sup>+</sup> MHC II<sup>+</sup> by flow cytometry as previously described and shown in Fig. 5A (5). In the conducting airways, a transient but significant increase in the frequency of DC (hereafter referred to as AMDC; Fig. 3A, left panel), accompanied by an increase in the frequency of CD11b<sup>b</sup> AMDC (Fig. 3B, left panel), was observed 24 h after a single aerosol and to a lesser extent at 48 h after three aerosols. Increases were also observed in the lung parenchyma for total (Fig. 3A, right panel) and CD11b<sup>b</sup> RT-DC (Fig. 3B, right panel) at 24 h after a single aerosol, although much greater increases in DC frequency were observed at this site 48 h after three aerosols as compared with conducting airways (Fig. 3A, right panel).

**FIGURE 4.** Subset kinetics and expression of CD40 on airway DC during allergic airways sensitization. At the indicated time points after OVA-aerosol challenge, cell suspensions were obtained from pooled conducting airways tissue of 10 animals/group in PBS controls (□) and OVA-sensitized mice (●) before analysis by flow cytometry as described in Materials and Methods. (A) Changes in frequency of CD11b<sup>b</sup> AMDC and CD11b<sup>b</sup> AMDC and (B) percentage CD40 expression during allergic airways sensitization. Bar graphs represent mean ± SEM of data from three to five experiments at the indicated time points: 1 Aero + 12 h = 12 h post 1 aerosol; 1 Aero + 24 h = 24 h post 1 aerosol; 3 Aero + 48 h = 48 h post 3 aerosols. Statistical analyses (p) comparing PBS controls (□) to OVA-sensitized mice (●) at the indicated time points following OVA-aerosol challenge were performed by Student’s t test.
Interestingly, activation of RT-DC (as indicated by the frequency of cells expressing the costimulatory molecule CD40) was evident at the earliest time point examined in both anatomical compartments (Fig. 3C, left and right panels), preceding changes in RT-DC frequencies described above. However, CD40 expression peaked early and was short-lived in the main conducting airways (Fig. 3C, left panel), contrasting with the more sustained expression of this costimulatory molecule at the later time points in the lung parenchyma (Fig. 3C, right panel). These changes were restricted to up-regulation of CD40, as changes in other costimulatory molecules such as CD80 and CD86 were not observed in the airways at any of these time points (data not shown).

Within the conducting airways, the early transient activation of AMDC described above appeared to correlate with the early activation of CD4⁺ T cells occurring in this anatomical site, prompting us to examine the kinetics of AMDC activation in more detail, especially subsets of these cells identified by the expression of CD11b (see Fig. 5A). We have found these two AMDC subsets differ in their expression of a number of other surface molecules, such as CD103 and F4/80 (Table I). In terms of subset frequencies, only the frequency of CD11b⁻ AMDC was significantly increased 24 h after a single challenge and 48 h after three challenges (Fig. 4A). CD11b⁺ AMDC, however, did not up-regulate CD40 expression at any time point (Fig. 4B, right panel), contrasting with the CD11b⁻ AMDC subset which remained unchanged in terms of cell frequency (Fig. 4A, left panel) but displayed an early and transient up-regulation of CD40 expression 12 h following a single aerosol challenge (Fig. 4B, left panel). A summary of the main phenotypic characteristics of APC populations within murine conducting airways is provided in Table I.

To further investigate the effect of aerosol challenge on the activation status of AMDC, cells from the conducting airways were isolated 12 h after a single aerosol challenge (the time of peak CD4⁺ T cell and AMDC activation in airways) and the expression of CD40, CD80, or CD86 was measured. At the same time, we also considered the expression of these molecules on two other APC populations, airway B cells, and macrophages, that can be identified by flow cytometry (Fig. 5A; see also Table I for a summary of phenotypic features). As previously observed, activation of AMDC was restricted to the CD11b⁻ subset (Fig. 5B, left panel). Furthermore, AMDC activation was restricted to up-regulation of CD40 only, as no changes were observed in the expression of both CD80 and CD86 on both CD11b⁻ and CDC11b⁺ AMDC (Fig. 5B). Moreover, neither CD40, CD80, nor CD86 were

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**FIGURE 5.** Costimulatory marker expression on airway APC during allergic airways sensitization. Twelve hours after OVA-aerosol challenge, cell suspensions were obtained from pooled conducting airways tissue of 10 animals per group in PBS controls (□) and OVA-sensitized mice (■) and analyzed by flow cytometry as described in Materials and Methods. A, Four APC populations (CD11b⁻ AMDC, CD11b⁺ AMDC, B cells, and macrophages) were identified in the airways on the basis of MHC class II, CD11c, and CD11b expression according to the gating strategy shown and as previously described (5). Frequency of CD40, CD80, and CD86 expression during allergic airways sensitization in (B) CD11b⁺ AMDC and CD11b⁻ AMDC and in (C) B cells and macrophages. Bar graphs represent mean ± SEM of data from three to five experiments. Statistical analyses (p) comparing PBS controls (□) to OVA-sensitized animals (■) at the indicated time points following OVA-aerosol challenge were performed by Student’s t test.

Panel B. CD11b⁺ AMDC, however, did not up-regulate CD40 expression at any time point (Fig. 4B, right panel), contrasting with the CD11b⁻ AMDC subset which remained unchanged in terms of cell frequency (Fig. 4A, left panel) but displayed an early and transient up-regulation of CD40 expression 12 h following a single aerosol challenge (Fig. 4B, left panel). A summary of the main phenotypic characteristics of APC populations within murine conducting airways is provided in Table I.

To further investigate the effect of aerosol challenge on the activation status of AMDC, cells from the conducting airways were isolated 12 h after a single aerosol challenge (the time of peak CD4⁺ T cell and AMDC activation in airways) and the expression of CD40, CD80, or CD86 was measured. At the same time, we also considered the expression of these molecules on two other APC populations, airway B cells, and macrophages, that can be identified by flow cytometry (Fig. 5A; see also Table I for a summary of phenotypic features). As previously observed, activation of AMDC was restricted to the CD11b⁻ subset (Fig. 5B, left panel). Furthermore, AMDC activation was restricted to up-regulation of CD40 only, as no changes were observed in the expression of both CD80 and CD86 on both CD11b⁻ and CDC11b⁺ AMDC (Fig. 5B). Moreover, neither CD40, CD80, nor CD86 were...
up-regulated on airway B cells or macrophages, indicating that only the CD11b^− subset of AMDC was involved (Fig. 5C). This was also the case for all other time points (data not shown). Thus, analysis of APC activation showed an early and transient up-regulation of CD40 expression that was restricted to CD11b^− AMDC in the main conducting airways. Importantly, this activation coincided with an increase in the frequency of activated CD4^+ T cells in the airways.

**Allergic airways sensitization leads to increased Ag-specific uptake and processing by airway APC**

Because the kinetics of T cell activation and AMDC activation appeared to be closely related, we hypothesized that functional changes in AMDC, especially uptake and processing of Ag, may contribute to T cell activation within the conducting airways during the onset of EAAD. Thus, we sought to determine whether sensitization to OVA specifically increased OVA processing by AMDC, as assessed by the development of DQ-OVA fluorescence measured by flow cytometry, at the time of peak CD4^+ T cell and APC activation in airways (i.e., 12 h postchallenge). In this assay, DQ-OVA does not fluoresce until it is proteolytically cleaved into fragments, which we have previously shown correlates well with Ag processing for presentation by DC in the DLNs (9). In the present study, we collected the main conducting airways from sensitized mice 2 h after p.n. administration of a single DQ-OVA challenge (a time point previously determined to give an optimal in vivo DQ-OVA signal in DC), with an aim to measure Ag processing in situ before AMDC migration out of the tissue. AMDC were identified as above by labeling for CD11b, CD11c, and MHC class II and compared with a saline control to judge the extent of OVA processing (Fig. 6A). There were 2.9-fold more DQ-OVA^- CD11b^− AMDC and 3.6-fold more DQ-OVA^+ CD11b^+ in the airways of OVA-sensitized mice as compared with PBS controls (Fig. 6B). We also observed an increase in DQ-OVA fluorescence in B cells (4.1-fold) and airway macrophages (3.2-fold), suggesting a general increase in processing by a variety of APC in the airways.

To confirm that the increase in DQ-OVA fluorescence was related to Ag capture by each APC population, we administered fluorescent conjugate of OVA (OVA-Alexa 647) p.n. and collected cells from the main conducting airways as described for DQ-OVA above. Compared with DQ-OVA, OVA-Alexa 647 does not require processing to fluoresce and is not affected by the acidic pH of Ag-processing compartment, so it can be detected intracellularly for at least 3 days after administration (data not shown). OVA was rapidly captured by cells in the airways of saline-sensitized mice, such that 15% of CD11b^− AMDC, 54% of CD11b^+ AMDC, and 94% of airway macrophages were OVA^- (Fig. 7A) 2 h after p.n. administration of OVA Alexa 647. In contrast, <1% of airway B cells were able to capture the OVA conjugate. Sensitization with OVA increased uptake by CD11b^- AMDC 5.2-fold, CD11b^- AMDC by 1.7-fold, and airway B cells by 52-fold, and while there was little change the proportion of OVA^- macrophages, they were able to capture larger amounts of OVA as evidenced by an increase in mean fluorescence (Table II). Thus systemic Ag immunization improved Ag-specific uptake by all APC in the main conducting airways, correlating with the increase in DQ-OVA processing observed in the initial experiments.

We next tested the specificity of Ag capture by airway APC by using a second Ag, BLG from bovine milk, a protein which can also act as an allergen in the airways (14). Three groups of mice were sensitized, the first with PBS in Alum, the second with OVA in Alum, and the third with BLG in Alum. On day 21, all three groups were administered 50 μg of BLG-Alexa 647 in 50 μl of saline p.n. and the airways collected 2 h later. As before, a saline control was included to judge the amount of BLG uptake. We observed low levels of BLG uptake in CD11b^- AMDC, CD11b^- AMDC, and B cells from mice sensitized with PBS or OVA in Alum that were increased 12-, 3.7-, and 15-fold, respectively, in mice sensitized with BLG in Alum (Fig. 7B). There was little difference in the proportion of BLG^- macrophages in each of the sensitized groups, but macrophages from BLG-sensitized mice were able to capture larger amounts of BLG than macrophages from PBS- or OVA-sensitized mice (Table II). All of the increases observed for BLG uptake in BLG-sensitized mice compared well with those seen for OVA uptake in OVA-sensitized mice (compare Fig. 7, B with A). Thus, allergic airways sensitization specifically increases the capacity of airway APC populations to capture the sensitizing Ag.

**Passive immunization increases Ag uptake and processing in the airways**

The allergen-specific increases in uptake and processing by airway APC observed in the above experiments suggested a role for Ag-specific Ig in facilitating allergen uptake and transport into airway

![FIGURE 6](http://www.jimmunol.org/) Sensitization increases Ag processing in airway APC populations. Two groups of five BALB/c mice were sensitized with PBS/Alum (PBS) or OVA/Alum (OVA) on days 0 and 14, then 40 μg of DQ-OVA was administered in 50 μl of saline p.n. on day 21. At the same time, a third group of OVA-sensitized mice (Control) were administered 50 μl of saline p.n. to serve as a control. The main conducting airways were collected 2 h later and prepared for flow cytometry. A, Representative plot showing DQ-OVA fluorescence in CD11b^- and CD11b^- AMDC from mice sensitized with PBS and OVA. B, DQ-OVA fluorescence was elevated in all airway APC populations from OVA-sensitized mice. Mean and SEM are shown for four independent experiments. Statistical analyses were performed comparing PBS controls (□) to OVA-sensitized animals (■) using the Student’s t test.
APC populations. To test this, we collected sera from OVA-sensitized and aerosol-challenged mice and used this to passively sensitize naive BALB/c mice by i.v. transfer. These sera contained high levels of OVA-specific IgG1, increased total IgE (Fig. 1C), and elevated levels of OVA-specific IgE as determined by capture ELISA (J. Burchell and P. A. Stumbles, data not shown). We also collected sera from mice sensitized with PBS in Alum (but were left unchallenged) to act as a control, because these contained low levels of IgE and undetectable levels of OVA-specific IgG1 (data not shown). Mice were administered 100–150 μl of sera i.v. and 24 h later, 20 μg of OVA-A647 plus 40 μg of DQ-OVA was administered p.n. The main conducting airways were isolated 2 h later and Ag uptake was determined for CD11b− AMDC, CD11b+ AMDC, B cells, and macrophages after comparison with a control group of OVA-sensitized mice administered 50 μl of saline p.n. (Control). A, Representative plots showing OVA-A647 uptake vs CD11b in total AMDC from each experimental group are shown in the top panels. B, Three groups of five BALB/c mice were sensitized with PBS/Alum (PBS), OVA/Alum (OVA), or BLG/Alum (BLG) before 50 μg of BLG-A647 was administered in 50 μl of saline p.n. on day 21. The main conducting airways were collected 2 h later and Ag uptake was determined for CD11b− AMDC, CD11b+ AMDC, B cells, and macrophages after comparison with a control group of BLG-sensitized mice administered 50 μl of saline p.n. (Control). Representative plots showing BLG-A647 uptake vs CD11b in total AMDC from each experimental group are shown in the top panels. Results are shown for two independent experiments.

FIGURE 7. Immunization specifically increases Ag uptake by airway APC. A, Two groups of five BALB/c mice were sensitized with PBS/Alum (PBS) or OVA/Alum (OVA) on days 0 and 14, then 50 μg of OVA-A647 was administered in 50 μl of saline p.n. on day 21. The main conducting airways were collected 2 h later and Ag uptake was determined for CD11b− AMDC, CD11b+ AMDC, B cells, and macrophages after comparison with a control group of OVA-sensitized mice administered 50 μl of saline p.n. (Control). A, Representative plots showing OVA-A647 uptake vs CD11b in total AMDC from each experimental group are shown in the top panels. B, Three groups of five BALB/c mice were sensitized with PBS/Alum (PBS), OVA/Alum (OVA), or BLG/Alum (BLG) before 50 μg of BLG-A647 was administered in 50 μl of saline p.n. on day 21. The main conducting airways were collected 2 h later and Ag uptake was determined for CD11b− AMDC, CD11b+ AMDC, B cells, and macrophages after comparison with a control group of BLG-sensitized mice administered 50 μl of saline p.n. (Control). Representative plots showing BLG-A647 uptake vs CD11b in total AMDC from each experimental group are shown in the top panels. Results are shown for two independent experiments.

Passive immunization increases naive T cell activation in the airways after Ag exposure

In our previous studies on the primary response to inhaled Ag, we found that naive T cells were activated in the DLN within 12 h of administration, while activated T cells were not detected in the airways or lungs until day 3 (5, 9). These results contrast with the sensitized animals in the current study, where an increase in the proportion of CD4+ T cells with an activated phenotype were observed in the main conducting airways as early as 12 h after one aerosol challenge (Fig. 2). We hypothesized that this early phase activation in the airways was a direct result of an increase in Ag presentation in situ. To test this hypothesis, we used DO11.10-transgenic mice that express an OVA-specific TCR on the majority of their CD4+
T cells. These mice offer the advantage that a population of OVA-specific naive CD4\(^+\) T cells can readily be detected in the main conducting airways that are not normally present in wild-type animals. These cells are CD44 low and CD69\(^+\) compared with CD44\(^{high}\)CD69\(^+\) memory cells that are found in the RT following infection or specific immunization (9, 17).

DO11.10 mice were passively immunized with OVA immune or control sera as above, and then 100 \(\mu\)g of OVA was administered 24 h later. The main conducting airways and DLN were collected from each group at 16 h and the proportion of OVA-specific CD4\(^+\) cells that were CD44\(^{low}\) and CD69\(^+\) (i.e., recently activated but otherwise naive) was determined by flow cytometry. The results were compared with a third group of unmanipulated DO11.10 to judge any increase in T cell activation. This third group of mice is an important control because our DO11.10 mice have not been bred on a RAG-deficient or SCID background to eliminate expression of endogenous TCR chains that allow dual reactivity with environmental Ags and promote memory T cell production (18, 19).

Approximately 2% of transgenic TCR\(^+\), OVA-specific CD4\(^+\) T cells were CD44\(^{low}\)CD69\(^+\) in the DLN of unmanipulated DO11.10 mice, while up to 16% these cells in the airways were CD44\(^{low}\)CD69\(^+\) (Fig. 9), presumably due to activation by environmental Ags (18, 19). After p.n. OVA immunization, an increase in T cell activation in the DLN of both groups of sera recipients was observed, but was significantly higher in mice passively sensitized with OVA-immune sera (Fig. 9, left panel). We also saw a significant increase in T cell activation in the airways of DO11.10 mice administered OVA-immune sera, whereas mice administered control sera resembled nonimmunized mice (Fig. 9, right panel). Thus, the increase in OVA uptake and processing mediated by passive immunization lead to an increase in OVA presentation.

![FIGURE 8. Passive immunization with immune sera increases Ag uptake and processing by airway APC. Two groups of 5–10 BALB/c mice were passively immunized with 100–150 \(\mu\)l of sera from PBS- or OVA-sensitized mice (Control sera, immune sera, respectively). Twenty-four hours later, 20 \(\mu\)g of OVA-A647 and 40 \(\mu\)g of DQ-OVA were administered in 50 \(\mu\)l of saline p.n. A, Representative plots showing OVA-A647 and DQ-OVA fluorescence for CD11b\(^+\) AMDC from each group of sera recipients compared with naive mice administered 20 \(\mu\)g of OVA-A647 and 40 \(\mu\)g of DQ-OVA alone (no DQ-OVA, no OVA-A647, respectively). B and C, OVA uptake (B) and OVA processing (C), respectively, by CD11b\(^-\) AMDC, CD11b\(^+\) AMDC, B cells, and macrophages after comparison with a control group of naive mice administered 50 \(\mu\)l of saline p.n. (Nil). Mean and SEM are shown for three results from two independent experiments. Statistical analyses were performed comparing control sera (□) to immune sera (■) using the Student’s t test.](http://www.jimmunol.org/)

**Table II. OVA uptake by airway macrophages 2 h after p.n. administration**

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![FIGURE 9. Passive immunization enhances T cell activation in the airways and DLN. Two groups of DO11.10 mice were passively immunized with 100–150 \(\mu\)l of sera from PBS- or OVA-sensitized mice (control sera, immune sera, respectively). Twenty-four hours later, each group was administered 100 \(\mu\)g of OVA in 50 \(\mu\)l of saline p.n. The airways and DLNs were collected 16 h later and the proportion of OVA-specific CD4\(^+\) T cells that were CD44\(^{low}\)CD69\(^+\) (i.e., recently activated but otherwise naive) was determined by flow cytometry and compared with naive DO11.10 mice (Nil). Mean and SEM are shown for three independent experiments. Statistical analyses were performed comparing control sera (□) to immune sera (■) using the Student’s t test.](http://www.jimmunol.org/)

![Table II. OVA uptake by airway macrophages 2 h after p.n. administration](http://www.jimmunol.org/)
Discussion

Allergic inflammatory airways diseases such as atopic bronchial asthma are a group of immunoinflammatory disorders of the central conducting airways and parenchymal lung tissue. Data from experimental mouse models indicate that the pathogenesis of these diseases is dependent on the actions of IL-4 and IL-5 and productive activation of Th2-biased CD4+ T cells (20–24). Given the central role of CD4+ T cell activation, recent attention has focused on professional APC such as RT-DC as key regulators of T cell activation during the induction of EAAD. Previous studies have demonstrated that induction of inflammation is dependent on DC and that myeloid DC derived from bone marrow or the RT have the capacity to prime Th2 responses to inhaled Ags (7, 25, 26). In addition, RT-DC have been shown to accelerate the translocation of antigenic signals to DLNs and to interact with CD4+ T within the airway mucosa before the onset of disease (9, 12, 27, 28).

In the current study, we have extended these earlier studies to investigate in detail how RT-DC populations capture and process inhaled allergen within the RT during the early phases of EAAD onset. Our initial data showed that an early event occurring at the inception of the clinical symptoms following allergen challenge was the appearance of CD69+CD4+ T cells within the tissue of the conducting airways and lung parenchyma. These data are in agreement with recent studies showing a peak of 24 h for T cell recruitment into human airways following allergen challenge although mouse studies have shown a later peak for T cell recruitment into BAL fluid using a similar model of EAAD, perhaps reflecting a difference in the kinetics of T cell recruitment into tissue compartments as opposed to the airway lumen (29, 30). Nevertheless, these data are consistent with the conclusion that the early stages of allergic inflammation are an allergen-specific, CD4+ T cell-mediated event.

The observed increased proportion of activated CD4+ T cells within the airways after OVA challenge may be accounted for by several, nonmutually exclusive mechanisms. First, recently activated CD4+ T cells may rapidly migrate from lymph nodes into the airways at the time of Ag exposure. On this point, we have detected activated T cells in draining and non-DLNs early after inhaled Ag delivery, which might be attributable to recirculation of activated cells from either the RT or from the DLN. However, we have failed to detect DC bearing inhaled Ag in the non-DLN at any stage, so it is likely these activated T cells are derived from known sites of Ag exposure (i.e., RT and DLN) (9).

Second, changes in the activation status of local APC populations may provide the necessary costimulatory signals to exceed the threshold requirements for local memory T cell activation within the airway mucosal tissue. On this basis, in the current study we analyzed the activation status of various APC populations within the RT, including AMDC subsets residing within the airway mucosa. The early appearance of activated CD4+ T cells at this site coincided with an increase in the frequency of activated CD11b+AMDC expressing the CD40 (but not CD80 or CD86) costimulatory molecule. As previously reported, other rodent studies have identified changes in numbers and activation status of key APC populations such as AMDC during the onset of allergic airways disease and suggest that this process may be driven by local DC-CD4+ T cell interactions occurring early after Ag inhalation (12, 25–27). Furthermore, our data suggest that CD4+ T cell activation within conducting airways was, in part, driven by activation of CD11b−AMDC. The phenotype of CD11b−AMDC is compatible with that of a CD103+ epithelial-derived DC previously described in the mouse lung and we have shown that CD11b−CD8a low DC are one of the major Ag-bearing cell types in the DLN following p.n. exposure to OVA (6, 9). Although the underlying mechanism of CD11b−AMDC activation was not directly addressed in the current study, one hypothesis that this may in part be driven by interactions between allergen-bearing CD11b−AMDC and OVA-specific memory CD4+ T cells within the airway epithelium. Alternatively, CD40 up-regulation may be mediated by interaction of OVA-Ig immune complexes with FcRs on DC, as previously demonstrated by Regnault et al. (31). To support this, we have preliminary data to show that CD40 is up-regulated on both CD11b− and CD11b+AMDC (but not airway B cells or macrophages) in an Ag-specific manner 12 h after p.n. delivery of OVA into mice that had been passively sensitized with OVA-immune serum (M. E. Wikstrom, unpublished data). CD40-CD40L interactions have been shown to be important in regulating DC migration into DLNs following cutaneous Ag delivery (32). An absence of CD40L signaling has been shown to inhibit the onset of allergic airways inflammation in gene knockout mice, and we have preliminary data to show that CD40L blockade during allergen sensitization interrupts the development of eosinophilia and Ig production in EAAD (Ref. 33 and P. A. Stumbles and L. Graca, unpublished data). Together, these data support the conclusion that CD40 expression by CD11b−AMDC may be an early checkpoint for T cell activation and initiation of airways inflammation in EAAD.

Third, allergic sensitization increased the capacity of all airway APC subsets analyzed, including CD11b+ and CD11b−AMDC, B cells and airway interstitial macrophages, to capture and process inhaled Ag in an Ag-specific manner. The extent to which individual APC populations can contribute to local T cell activation within the RT is not clear; however, we have previously shown that B cells, but not macrophages, isolated from both lung parenchyma and main conducting airways efficiently present Ag and activate T cells in vitro suggesting that B cells may also be potential contributor to T cell activation within the airway mucosa (5). The Ag specificity of the up-regulated OVA uptake and processing by airway APC subsets in this model raised the possibility that elevated levels of allergen-specific IgG1 and/or IgE could play an opsonizing role by mediating enhanced allergen capture. This was confirmed by the observation that enhanced uptake of allergen by a range of airway APC subsets could be reproduced in naive mice by passive transfer of serum from OVA-immunized mice. In contrast to allergen uptake, however, enhanced allergen processing was not observed in CD11b−AMDC (which was the case in the active immunization model), suggesting that Ab alone cannot fully recapitulate the enhanced Ag handling in the airway mucosa observed after active induction of EAAD. Passive immune serum transfer also increased CD4+ T cell activation in the airways and DLNs of DO11.10 TCR-transgenic mice, demonstrating that the increases in OVA uptake and processing detected early in the airways lead to an increase in Ag presentation and subsequent T cell activation. It will be important to determine whether any of the T cell activation we observed in the airways DO11.10 mice can be attributed to local Ag presentation and activation of recirculating naive T cells. Regardless, our data are consistent with previous studies indicating that both IgG1 and IgE are capable of mediating allergic airways inflammation and altered airways function (34).

A characteristic feature of the Ab response of the BALB/c model of EAAD was the production of high levels of both OVA-specific IgE as well as IgG1, raising the question of the relative contribution of each isotype to the observed changes in APC function following passive serum transfer. DC have been shown to express the high-affinity IgE receptor FcεRI on their surface that can capture IgE bound to allergen (35). Earlier studies have proposed a role for IgE in enhanced allergen uptake and processing.
and recently Kitamura et al. (37) have shown that the high-affinity IgG receptor FcγRI on APC populations was crucial during the sensitization phase for the development of allergic airway inflammation (36, 37). Although mice deficient for the FcRI common γ-chain (FcγRI−/−) produced comparable IgE and IgG levels to wild-type mice, AHR and other inflammatory indicators were reduced in these animals; reconstitution of FcγRI−/− animals with wild-type bone marrow DC partially, and with spleen cells completely restored the allergic airways inflammation in this model (37). Furthermore, Oshiba et al. (34) have shown that passive transfer of anti-OVA IgE and IgG1, but not IgG2a and IgG3, resulted in increased airways reactivity and BAL fluid eosinophilia following OVA challenge.

We found that heat-inactivated serum retained the ability to mediate the observed functional changes in the airway APC populations observed with intact serum, indicating that at least in the context of the EAAD model, IgG1 is the dominant contributor to passive APC sensitization (data not shown). In some respects this is not surprising, as in our hands IgG1 is by far the predominant Ab isotype induced following OVA/Alum immunization and may be the principal APC-sensitizing agent in BALB/c mice. Nevertheless, other studies have shown Ag processing through FcγRI-mediated uptake is 1000 times more effective than allergen presented less, other studies have shown Ag processing through FcγRI expression is up-regulated on DC and seemingly correlates with elevated IgE levels, while treating asthmatic animals with anti-IgE reduced the exacerbation rate and reduced steroid requirements (39, 40). Others have also proposed a role for the low-affinity IgE receptor (FcεRI or CD23) in the pathogenesis of atopy and allergic airways disease in humans and mice (41–45). In allergic patients, FcεRI expression is up-regulated on DC and seemingly correlates with elevated IgE levels, while treating asthmatic animals with anti-IgE reduced the exacerbation rate and reduced steroid requirements (39, 40). Others have also proposed a role for the low-affinity IgE receptor (FcεRI or CD23) in the pathogenesis of atopy and allergic airways disease in humans and mice (41–45).

In summary, previous studies have implicated transient up-regulation of the T cell-activating properties of local airway APC populations in the initiation of pathogenic CD4+ T cell responses to inhaled Ag in EAAD, involving in particular increased costimulatory expression by AMDC (12, 25, 27). The trigger for activation of these AMDC functions was shown to be provided via interaction with OVA-specific Th-memory cells. The present study provides supporting evidence for this hypothesis via the demonstration of CD40 up-regulation on AMDC, but importantly extends this concept to include parallel up-regulation of the Ag-uptake and -processing properties of AMDC. In our model, this process could be replicated by transfer of circulating Ag-specific IgG1 that acted to promote the retention and processing of specific Ag within the airway mucosa. It is also possible that IgE may have contributed to this effect, however, we were unable to demonstrate this directly in these experiments. We have previously argued that the tight regulation of local Ag processing is critical in the maintenance of immunological homeostasis (8). The current data support a mechanism whereby high levels of circulating allergen-specific Ig can contribute toward the breakdown of local immunoregulation these in situ regulatory such that local Ag-processing and -presenting function is enhanced beyond the threshold required for local T cell activation. Current immunotherapeutic strategies for atopic asthma focus primarily on desensitization of allergen-specific CD4+ Th cells. However, the present findings suggest that strategies targeting Ab-dependent, Ag-processing pathway through the combined approaches of neutralization of circulating IgG and IgE, B cell-targeted therapies and the functional blockade or modulation of FcεRI expression, also have potential for limiting the persistence of this disease.

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Disclosures
The authors have no financial conflict of interest.

References


