

Endothelial amine oxidase AOC3 transiently contributes to adaptive immune responses in the airways

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Amine oxidase, copper containing 3 (AOC3, also known as vascular adhesion protein-1 (VAP-1)) is an endothelial adhesion molecule that contributes to the extravasation of neutrophils, macrophages, and lymphocytes to sites of inflammation. However, the role of AOC3/VAP-1 in allergic responses remains unknown. Here, we studied eosinophil and CD4⁺ T-cell recruitment to the airways using AOC3/VAP-1-deficient mice. In an OVA-triggered asthma model, AOC3/VAP-1 slightly contributed to the accumulation of leukocytes in lungs in an age-dependent manner. We then established a new model to kinetically measure recruitment of OVA-specific CD4⁺ T cells to different airway immune compartments during the priming and effector phases of an adaptive immune response. The results showed that in the absence of AOC3/VAP-1, recruitment of antigen-specific CD4⁺ T cells to draining bronchial lymph nodes is reduced by 89% on day 3 after tracheal allergen exposure, but this difference was not observed on day 6. The dispersal of effector cells to lung and tracheal mucosa is AOC3/VAP-1 independent. Thus, in allergic airway reactions, AOC3/VAP-1 transiently contributes to the antigen-specific, CD4⁺ T-cell traffic to secondary lymphatic tissues, but not to airway mucosa or lung parenchyma. Our results suggest a largely redundant function for AOC3/VAP-1 in allergic inflammatory responses of the airways.

Keywords: Adaptive immunity · Adhesion molecules · Airways · Allergy · Leukocyte trafficking



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Introduction

The immunopathogenesis of asthma involves complex cross-talk between airway epithelial cells, dendritic cells (DCs) and other cells of the innate immune system, and T cells. The current paradigm states that primary immune response to allergens leads to epithelial disruption and cytokine production. These stimuli then activate DCs, which travel to the draining bronchial lymph nodes (BrLNs). In the LNs, DCs present antigen to CD4⁺ T lymphocytes, which leads to lymphocyte activation, differentiation, and imprinting, and finally homing to the airways [1]. During the early, IgE-mediated effector phase in asthma, allergen exposure causes mast cell degranulation and release of mediators, which attract Th2 cells to airways. The late T-cell-mediated asthmatic effector response is then dependent on chemokine-driven recruitment of T effector and memory cells and eosinophils to the airways. Although leukocyte migration to airways is a prerequisite for the pathogenesis of asthma, the molecular mechanisms controlling the multistep extravasation cascade of different subsets of leukocytes to airways are not fully understood [2, 3]. It is evident, however, that the adhesion mechanisms in lung are different when compared with most other tissues, since they are only partially dependent of rolling and selectins and mainly take place in capillaries.

Amine oxidase, copper containing 3 (AOC3) is one of the nonclassical endothelial adhesion molecules involved in leukocyte recirculation [4]. It harbors enzyme activity that oxidizes primary amines to produce hydrogen peroxide, aldehyde, and ammonia, which in turn can act as signaling molecules and contribute to leukocyte adhesion [5]. Absence of AOC3, or its inhibition with antibodies or enzyme inhibitors, attenuates inflammatory responses in several different models, including granulocyte influx in LPS-mediated and ischemia–reperfusion triggered models of acute lung injury. In a liver inflammation model, anti-AOC3 antibodies selectively inhibit interactions of Th2 lymphocytes, but not those of Th1 lymphocytes [6]. In humans, AOC3 mediates lymphocyte binding to vessels in allergic skin lesions *in vitro* [7]. However, the *in vivo* role of AOC3 in allergic inflammation in general and its role in lymphocyte traffic to lung remain unknown. Therefore, we used here AOC3-deficient mice to address the contribution of AOC3 in a well-established mouse model of asthma and developed a new protocol for dissecting its involvement during adaptive immune responses in the airways.

Results and discussion

AOC3 is expressed by airway-associated blood vessels

In wild-type (WT) mice, AOC3 was found to be present in the high endothelial venules (HEVs) in BrLNs (Fig. 1A). The flat-walled endothelium of blood vessels both in the lungs and in the trachea also expressed AOC3. In addition, the smooth muscle cells in vessels and bronchioles were brightly AOC3 positive, whereas

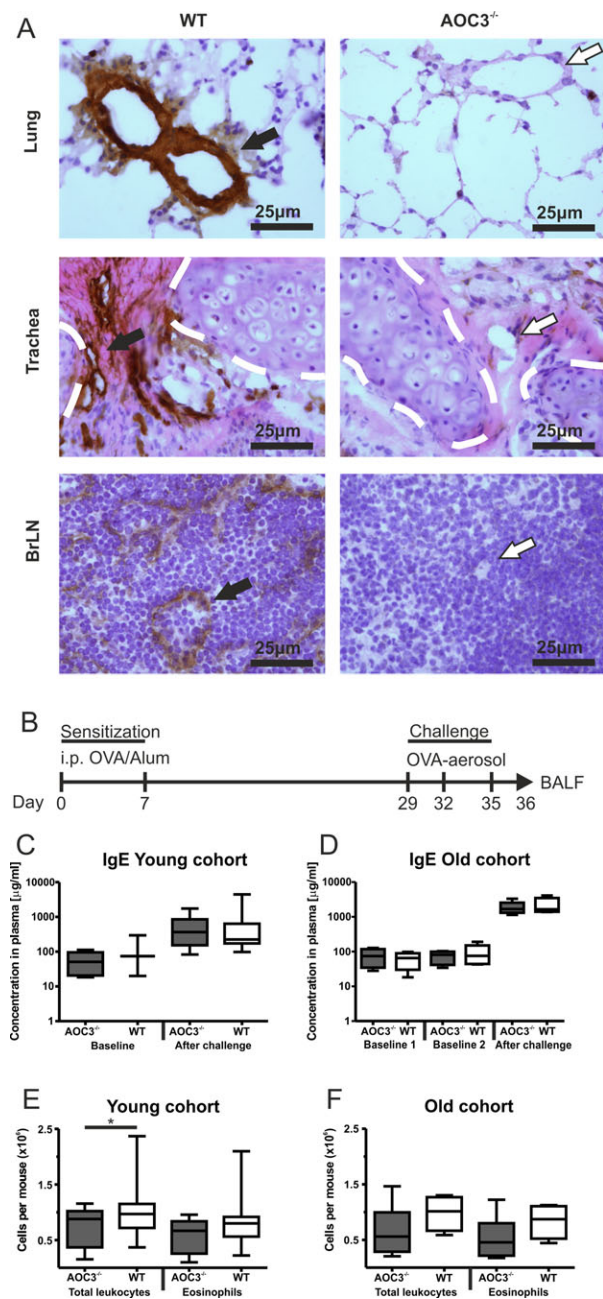


Figure 1. Role of AOC3 in asthmatic inflammation. (A) Expression of AOC3 in the airways. Lung parenchyma, tracheae, and bronchial lymph nodes (BrLNs) from AOC3-deficient or WT mice were immunostained with the monoclonal AOC3 antibody 7–106. Black arrows indicate AOC3-positive vessels; open arrows indicate AOC3-negative vessels in the mutant mice. Dashed white lines indicate tracheal cartilage. Images are representative of four independent experiments. (B) The experimental protocol of OVA-triggered asthma. Mice were first sensitized with OVA, and then challenged via the airways with OVA aerosol over the course of 36 days. (C and D) IgE levels in naïve and OVA-challenged young and old AOC3^{-/-} or WT mice were assessed on day 36 by ELISA. Note: Baseline IgE levels of young mice were mostly undetectable. (E and F) Recruited total leukocytes and eosinophils in BALF of young and old AOC3^{-/-} and WT mice were determined on day 36 by flow cytometry. (C–F) Data shown are from single experiments and presented as median ± interquartile ranges of *n* = 15 mice/genotype (young cohort), and *n* = 8 WT and 7 AOC3-deficient mice (old cohort). Mann–Whitney U-test was used for statistical analysis, **p* < 0.05.

AOC3 was absent from leukocytes and epithelial cells. These data imply that AOC3 has the potential to mediate influx of leukocytes to lungs.

AOC3 mediates leukocyte recruitment to the asthmatic lungs

WT and AOC3-deficient mice were intraperitoneally sensitized with ovalbumin (OVA), and then challenged by OVA aerosol 1 month later (Fig. 1B) [8]. Allergic responses were evaluated by measuring IgE levels, which is a powerful screening parameter for finding allergy-prone mouse mutants. In young 8-week-old mice, the baseline and allergen-induced elevations in plasma IgE levels were comparable between WT and AOC3-deficient mice (Fig. 1C). The levels of IgM and IgG1 in plasma were also increased after the challenge in both genotypes. In contrast, IgA concentration was significantly lower in AOC3-deficient than in WT mice both under resting and challenged conditions (Supporting Information Fig. 1A and B). TNF- α , IL-5, IL-13, and GM-CSF cytokine levels (in general, very low or undetectable) in allergen-challenged WT and AOC3-deficient animals were comparable (data not shown). In the older 24-week-old cohort, the IgE concentration under basal and asthmatic conditions was not AOC3 dependent either (Fig. 1D). These data imply that AOC3 is not needed for plasma cell differentiation or isotype switching during allergic reactions, although there is a minor age-dependent IgA synthesis/catabolism defect in the absence of AOC3.

Leukocyte recruitment to the lungs of allergen-challenged animals was evaluated from bronchoalveolar lavage fluids (BALFs) by flow cytometry. In the younger 8-week-old cohort, total leukocyte recruitment to BALF of AOC3-deficient animals was statistically significantly reduced in comparison with WT mice and a tendency for a reduced eosinophil amount was observed (Fig. 1E). Tendencies for impaired recruitment of leukocytes in the BALF were observed in older AOC3-deficient mice (Fig. 1F). The 24-week-old cohort also showed a tendency for impaired leukocyte (CD45⁺ total leukocytes, eosinophils, neutrophils, B lymphocytes, and T lymphocytes) recruitment to BALF in the absence of AOC3 (Fig. 1F and Supporting Information Fig. 1C and D). The basis of the age-dependent contribution of AOC3 in asthma remains speculative, but implies that the expression and functional dominance of AOC3, its counter-receptors including Siglec-9 and Siglec-10, or other adhesion mechanisms may change during aging. In any case, these data indicate that AOC3 plays a small, but significant, age-dependent role in leukocyte recruitment into asthmatic lungs.

Development of an adoptive transfer model to study antigen-specific T-cell responses in airways

To dissect the potential role of AOC3 in mediating antigen-specific CD4⁺ T-cell responses in the airways in more detail, we developed a new model based on a previously described protocol [9]. Antigen-specific, fluorescent donor cells were obtained by

crossing OT-II mice (in which the majority of CD4⁺ T cells carry a T-cell receptor (TCR) recognizing an OVA peptide) with CAG-DsRed mice (ubiquitously expressing the red fluorophore) and by purifying CD4⁺ lymphocytes from these mice (referred to as OT-II-DsRed mice). The cells were adoptively transferred into recipients, which were primed 1 day later with OVA together with the mucosal adjuvant cholera toxin (CT) via the oropharyngeal route. Lymphocyte recruitment during the priming phase was analyzed on days 3 and 6 (Fig. 2A, Protocol 1). Flow cytometry revealed that the transferred population of DsRed⁺CD4⁺ OT-II T lymphocytes accumulated and divided in the BrLN after priming with OVA without CT adjuvant, and more so with CT but not in those of the PBS-treated control group (Fig. 2B and C). Accumulation of the transferred OT-II-DsRed in mice primed with OVA in the presence of CT resulted in a tendency of more robust lymphocyte recruitment to the lungs compared with OVA alone (Fig. 2D).

Because after antigen (OVA) challenge OT-II-DsRed cell recruitment to the lungs, but not to the trachea, was markedly increased (not shown), we included house dust mite (HDM) extract and used it in combination with the antigen OVA to enhance the tracheal component of the immune response. After the adoptive transfer, recipients were primed with OVA/CT/HDM on day 0 and challenged with OVA/HDM on days 6 and 7 (Fig. 2A, Protocol 2). Using this protocol, proliferation of OT-II-DsRed cells in the draining BrLNs and recruitment of these cells to the lungs and trachea were all clearly evident in flow cytometric and microscopic analyses. Thus, in the new model, the tracheal co-administration of OVA together with the model allergen HDM and the mucosal adjuvant CT during the priming enhances the accumulation of OT-II-DsRed cells in the lungs and BrLN. Secondary instillation of OVA and HDM at the effector phase further promoted the recruitment of these cells to the tracheal mucosa (Fig. 2E, Supporting Information Video 1). The key improvement in our model was the inclusion of HDM to induce Th2 polarization of T lymphocytes, and to trigger homing into the conductive airways [9]. It has been previously documented that HDM components have the ability to disrupt tight junctions in the epithelial cell layer [10] and to activate them to secrete proinflammatory cytokines [11]. Moreover, recent studies have shown that the local microenvironment during antigen presentation and the anatomical site of antigen administration significantly affect the migratory properties and tissue tropism of effector/memory T cells [12].

AOC3 mediates T-cell homing to BrLNs during the allergic response priming phase

AOC3 was present on endothelial cells in BrLN, lungs, and trachea of WT mice after the allergen sensitization and challenge according to Protocol 2 (Fig. 2A), but there was no clear induction in the expression when compared with the resting state (compare Fig. 3A to Fig. 1A). As expected, specific AOC3 immunoreactivity was completely absent in AOC3^{-/-} mice. Accumulation of OT-II-DsRed cells in the BrLN of challenged mice was inhibited by 89%

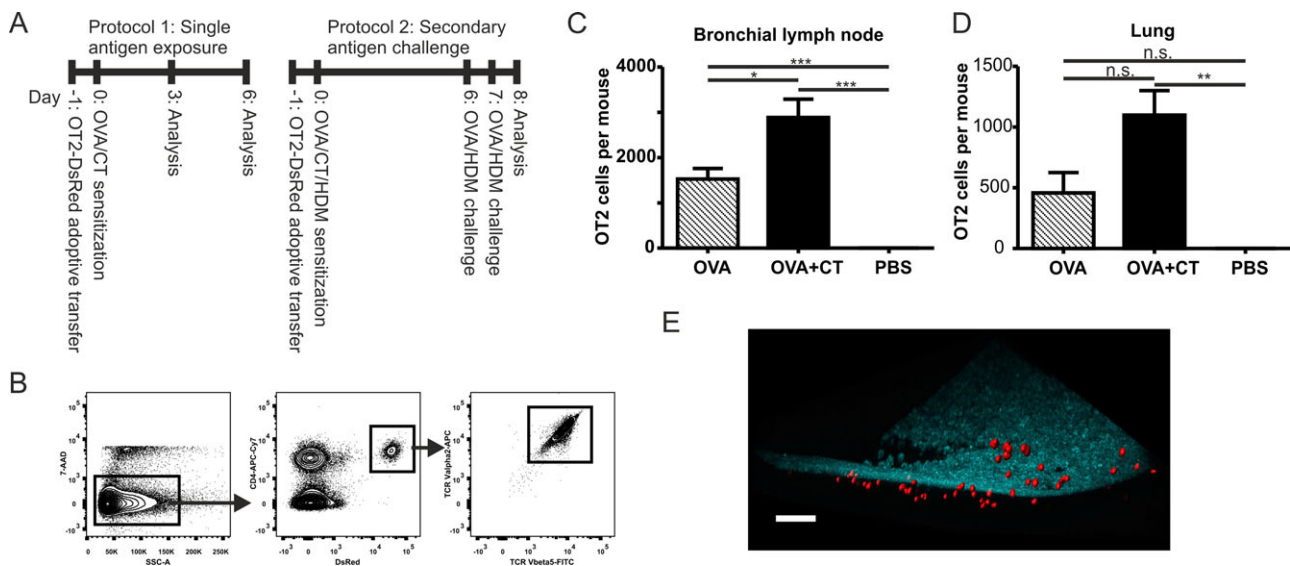


Figure 2. New model of tracking migration of antigen-specific CD4⁺ T cells to airway tissues in vivo. (A) The adoptive transfer protocols. CD4⁺ OT-II-DsRed T cells were adoptively transferred to recipient mice, which were then sensitized, or sensitized and challenged with OVA. (B) Flow cytometric identification strategy for OT-II-DsRed cells from bronchial lymph nodes (BrLNs). The viable (7-AAD negative) cells in the BrLN cell suspensions (left) were gated and analyzed for CD4 and DsRed to identify the transferred CD4⁺ OT-II-DsRed cells (middle). Expression of the transgenic Vα2 Vβ5 TCR was assessed among the gated CD4⁺ OT-II DsRed⁺ cells (right). Dot plots represent five independent experiments. (C and D) Quantification of transferred cells found in the BrLNs and lungs in OVA, OVA + CT, and PBS-primed mice after 3 days (BrLNs) and 6 days (lungs) was performed using flow cytometry. Data are presented as mean + SEM from a single experiment performed with at least four mice per group. Unpaired Student's *t*-test was used for statistical analyses. **p* < 0.05, ***p* < 0.01, and ****p* < 0.005. (E) Detection of transferred OT-II-DsRed cells in trachea of OVA + CT + HDM-primed and OVA + HDM-challenged recipients was performed 8 days postchallenge by two-photon microscopy. The epithelium is visible in cyan due to NADPH autofluorescence and DsRed⁺ lymphocytes are visible in red, beneath the epithelium. Bar, 100 μm. The image represents a single experiment with 13 mice.

in the absence of AOC3 on day 3 (*p* < 0.03), but on day 6 the difference leveled off. The numbers of cells retrieved from the lungs of primarily sensitized AOC3-deficient and WT mice increased from day 3 to day 6 (*p* < 0.002), but no significant differences were seen in lung recruitment of OTII-DsRed cells between AOC3-deficient and WT mice on day 6 (Fig. 3B).

Effector phase of allergen-specific immune response in lungs is AOC3 independent

To analyze the effector phase of the allergic immune response, lungs, BrLNs, and tracheae from the secondarily challenged mice were analyzed 24 h after the second OVA/HDM challenge, on day 8 according to Protocol 2 (Fig. 2A). In the BrLN, there were less antigen-specific T cells also at this phase of the immune response in the absence of AOC3, but the difference was not statistically significant. Accumulation of OT-II-DsRed cells in lungs and trachea of AOC3-deficient and WT animals displayed no significant differences, which is in line with the modest effects seen in the asthma model at day 36 (see above) (Fig. 3C and D). Thus, in contrast to the priming phase, AOC3 does not appear to play a significant role in the effector phase of allergen-specific immune responses in the airways.

In conclusion, we report here that AOC3 is important in mediating lymphocyte homing to BrLNs during the priming phase of allergic airway inflammation. In asthmatic models, AOC3 still modestly

impaired the development of the overall inflammatory response at the effector phase, but the recruitment of antigen-specific T cells to the airways was not AOC3 dependent. Since AOC3 is absent from lymphatics and all leukocytes, but present on HEV and, upon inflammation, in other vessels [13], we speculate that the reduction in the numbers of antigen-specific T cells in the draining BrLNs in the absence of AOC3 is attributed to impaired homing. HEVs in BrLNs also express PNAd but not MadCAM-1, which are the dominant addressins of the peripheral LNs and gut mucosa, respectively. Blocking of PNAd or VCAM-1, which is also expressed in BALT HEVs, efficiently inhibits homing of memory T cells to that location, but do not affect the homing of naïve T- or B cells [14]. Our results would be compatible with a model, in which AOC3 is an important endothelial adhesion molecule for entrance of naïve T cells to BrLNs, and then later on, PNAd and VCAM would take a more central role in this process. Our findings also argue that, in contrast to many other inflammation models, AOC3 blockade is not likely to be effective in controlling allergic airway inflammation. Nevertheless, when studied using a polyclonal anti-AOC3 antibody, which recognizes at least human AOC3 protein produced by both known splice variants of AOC3 [15], we found that AOC3 is expressed at a markedly higher level in the pulmonary vasculature in humans than in mice (Fig. 3E) and, therefore mouse data cannot be directly generalized to humans. Finally, our new model for following antigen-specific T-cell responses in the primary and secondary phases of the immune responses in the different immunological compartments of

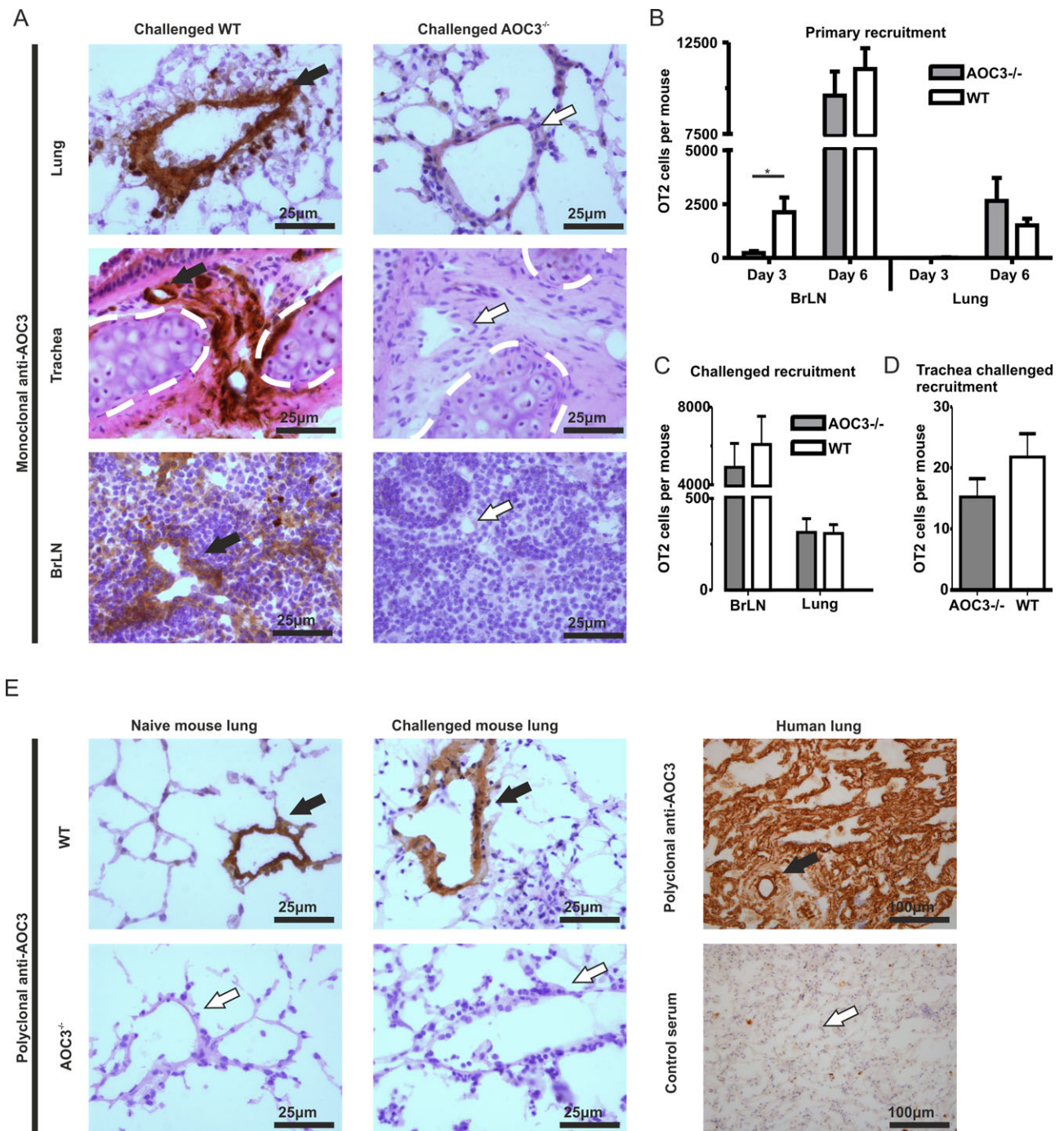


Figure 3. Role of AOC3 in antigen-specific T-cell accumulation in various airway-associated compartments. (A) Expression of AOC3 in the lung parenchyma, mucosa-associated blood vessels between tracheal cartilage rings, or in bronchial lymph nodes (BrLNs) in the adoptive transfer model was analyzed by 7–106 immunostaining in challenged WT and AOC3^{-/-} mice on day 8, post-OVA/HDM. Black arrows indicate AOC3-positive vessels, open arrows indicate AOC3-negative vessels, and dashed white lines indicate tracheal cartilage. Images represent three independent experiments. (B–D) The recruitment of transferred OT-II-DsRed cells during (B) the primary immune response (day 3: $n = 5$ mice/genotype, day 6: $n = 7$ WT and 6 AOC3^{-/-} mice) and (C) the effector phase ($n = 7$ WT and 6 AOC3^{-/-} mice) in BrLNs and lungs was quantified by flow cytometry. (D) The recruitment of OTII-DsRed cells during the effector phase in the trachea was quantified by two-photon microscopy ($n = 7$ WT and 6 AOC3^{-/-} mice). (B–D) Data shown are from single experiments and are presented as mean $\pm$ SEM. Mann–Whitney U -test was used for statistical analyses. * $p < 0.05$. (E) A polyclonal anti-AOC3 antibody cross-reacting with human and mouse was used to compare AOC3 expression in human versus mouse. Black arrows indicate AOC3-positive vessels, and open arrows indicate AOC3-negative vessels in mutant mice and in human lung stained with a nonimmune control serum. Images represent three independent experiments.

airways should be generally useful for further dissection of allergic inflammation in the respiratory tract.

Materials and methods

Mice and reagents

OT-II mice [16] and DsRed mice [17] were purchased from Jackson laboratories. These two strains were crossed and the F1 litter was used in the experiments as a lymphocyte donor. PCR detection was used to control that the crossed animals express the appropriate OT-II transgene. AOC3-deficient mice [18] have been back-crossed to the C57Bl/6J background. The experimental protocols were approved by the Local Ethical Committee.

Lyophilized HDM was from Greer, EndoGrade OVA was from Hyglos, CT was from Sigma-Aldrich, and alum inject was from Pierce. The Restain Quick-Diff Kit was from Reagen and Optimum Cutting Temperature compound (OCT) was from Sakura. The Ketamine was from Intervet, and Xylazine and Pentobarbital were from Orion Pharma.

Anti-mouse mAbs for the following antigens along with the appropriate isotype controls were from BD Biosciences (Franklin Lakes, NJ, USA): TCR V α 2, TCR V β 5, CD3 ϵ , CD4, CD5, CD8 α , CD11b, CD11c, CD19, CD25, CD45, CD45R(B220), CD62L, CD103, Ly6C, Gr1, and H2-Kb SIINFEKL multimer. CD16/32 Fc-block was from BD Biosciences. 7-AAD viability stain was from eBioscience. Monoclonal rat IgG for mouse-IgE was from the Binding Site. Murine IgE standards, biotinylated rat anti-mouse-IgE, and BD OptEIA Reagent Set B were all from BD Biosciences. 7-106 [19] is a rat anti-mouse AOC3 mAb and Hermes-1 [20] (rat anti-human CD44) is a negative control. Rabbit anti-rat IgG-HRP and DAB chromogen were from Dako. Rabbit anti-mouse/human AOC3 pAb, which recognizes both splice variants of AOC3, has been described [21].

OVA challenges

OVA challenges were performed at the German Mouse Clinic [22]. AOC3-deficient and WT mice, aged 8 (15 + 15) and 24 weeks (8 + 7) at the beginning of the experiments, were subjected to the same protocol in two independent experiments. In both experiments, the KO and WT mice were age-matched.

The mice were vaccinated with 10 μ g OVA and 2 mg Alum i.p. on days 0 and 7, then challenged with 1% OVA aerosol on days 29, 32, and 35, and sacrificed on day 36 (Fig. 2A). Blood samples for ELISA measurements were drawn on prior to treatment and on day 36. Mice were sacrificed and BALF samples were collected on day 36.

Total plasma IgE was analyzed by immunoassay isotype-specific sandwich ELISA. Ninety-six -well plates were coated with 10 μ g/mL anti-mouse-IgE, followed by 1:10 diluted serum samples and standards, secondary antibodies, and ELISA reagents. The plates were read at 450 nm.

BALF samples were stained for flow cytometry to identify leukocyte subsets. The experimental protocol and gating strategies have been previously described in detail [23].

Adoptive transfer studies

Donor OT-II-DsRed mice were sacrificed at the age of 6–8 weeks, and spleen and LNs were collected. Single-cell suspensions were prepared and a magnetic negative selection kit to purify CD4⁺ T cells was used to enrich a population of red-fluorescent CD4⁺ T-cells, expressing the TCR with specificity for MHC-II-bound OVA. A total of 10⁶ CD4⁺ OT-II-DsRed cells were adoptively transferred to AOC3-deficient or WT recipients by tail-vein injection. In some experiments, the transferred cells were labeled with CFSE for evaluation of cell proliferation [24]. On the second day, 100 μ g OVA, 100 μ g HDM, and 1 μ g CT in 50 μ L PBS were applied to the airways under ketamine-xylazine anesthesia to prime the transferred cells toward the Th2 phenotype. In some experiments, mice were challenged under anesthesia via the airways by 50 μ g OVA and 50 μ g HDM on days 6 and 7 and sacrificed on day 8 by pentobarbital overdose.

Both the systemic and pulmonary circulations were extensively perfused with ice cold PBS to flush out all intravascular leukocytes. Thereafter, right upper lung lobes, BrLN, and tracheae were collected. Transferred cells in the trachea were quantified by making 3D stacks from three locations within each trachea with a two-photon microscope (Leica TCS MP5 with two MP lasers and an OPO unit) using protocols modified from those previously described [25]. Single-cell suspensions from BrLN and lungs were prepared and stained for flow cytometry.

Human lung samples

Human lung samples were collected for immunohistochemistry as previously described [26].

Phenotype analyses

Cell preparations were analyzed with an LSRII flow cytometer (BD Biosciences) and Flowing Software v2.5 (University of Turku Cell Imaging Core, Turku, Finland, www.flowingsoftware.com) or FlowJo v10 (TreeStar Inc., Ashland, OR, USA).

Immunohistochemistry

Mouse lungs were inflated with PBS/OCT. All samples were snap-frozen in OCT, cut into 5 μ m thick sections, and fixed in acetone. Sections were blocked with 5% normal serum, and incubated with primary mAbs at 20 μ g/mL or polyclonal antibodies at 1:1 000. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ and anti-rat IgG-HRP or anti-rabbit IgG-HRP were used at 1:40 as

second-stage reagents. DAB chromogen was used for detection and samples were counterstained with H&E.

Statistical analyses

Data are presented as median $\pm$ interquartile ranges or mean $\pm$ SEM and analyzed by Mann–Whitney rank sum test, Kruskal–Wallis test, and the unpaired Student's *t*-test, as appropriate.

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Abbreviations: **AOC3:** amine oxidase, copper containing 3 · **BrLN:** bronchial lymph node · **CT:** cholera toxin · **HDM:** house dust mite · **HEV:** high endothelial venule · **VAP-1:** vascular adhesion protein-1

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