Dendritic Cell-Nerve Clusters Are Sites of T Cell Proliferation in Allergic Airway Inflammation

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Interactions between T cells and dendritic cells in the airway mucosa precede secondary immune responses to inhaled antigen. The purpose of this study was to identify the anatomical locations where dendritic cell-T cell interactions occur, resulting in T cells activation by dendritic cells. In a mouse model of allergic airway inflammation, we applied whole-mount immunohistology and confocal microscopy to visualize dendritic cells and T cells together with nerves, epithelium, and smooth muscle in three dimensions. Proliferating T cells were identified by the detection of the incorporation of the nucleotide analogue 5-ethynyl-2'-deoxyuridine into the DNA. We developed a novel quantification method that enabled the accurate determination of cell-cell contacts in a semi-automated fashion. Dendritic cell-T cell interactions occurred beneath the smooth muscle layer, but not in the epithelium. Approximately 10% of the dendritic cells were contacted by nerves, and up to 4% of T cells formed clusters with these dendritic cells. T cells that were clustered with nerve-contacting dendritic cells proliferated only in the airways of mice with allergic inflammation but not in the airways of negative controls. Taken together, these results suggest that during the secondary immune response, sensory nerves influence dendritic cell-driven T cell activation in the airway mucosa. (*Am J Pathol* 2009, 174:808–817; DOI: 10.2353/ajpath.2009.080800)

The inhaled air contains a multitude of environmental antigens varying from harmless particles to dangerous pathogens.¹ The maintenance of local physiological homeostasis thus becomes a challenging task for the organism in which two major control systems play fundamental roles: the immune system and the nervous system. Physical and chemical hazards are detected by sensory nerves,² whereas biological danger signals are recognized by dendritic cells (DCs).³ DCs integrate these signals during antigen presentation to T cells to initiate an appropriate immune response.⁴ This way, the mucosal immunity of the airways accounts for the discrimination between innocuous and harmful antigens.

Failure in this process can result in uncontrolled infection or allergic airways diseases like asthma. In the course of the allergic immune response, bidirectional interactions between DCs and T cells have been observed in the airway mucosa, but not in parenchymal lung tissue.⁵ Allergen-specific T cells and DCs presenting allergen peptides remain in the airways for several weeks after allergen challenge.⁶ Thus, interactions between DCs and T cells in the airways could account for the chronic nature of the disease.

Whereas the inflammatory process results from an immunological disorder, i.e., a hypersensitivity reaction, the symptoms of asthmatic individuals, such as wheezing, cough, and shortness of breath are best explained by allergen-induced changes in neural activity.⁷ In the airway mucosa, the activation of non-myelinated C-fibers containing neuropeptides like calcitonin-gene related peptide (CGRP) leads, via an axon-reflex mechanism, to bronchoconstriction, excessive mucus production, mucosal vasodilation, and plasma exudation.² Besides

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these immediate effects, sensory neuropeptides may directly influence the pulmonary immune response by controlling DC function.^{8,9} As proof for the existence of this mechanism, we previously demonstrated the close proximity between CD11c⁺ DCs and CGRP⁺ sensory nerves in the airway mucosa.¹⁰ Neuropeptides released from adjacent sensory terminals might also affect the activation of T cells either directly¹¹ or by modulating the activity of DCs in contact with T cells.

To address the question if DC–nerve clusters are sites of T cell activation, it is essential to detect proliferating T cells, together with DCs and nerves, in their normal tissue environment. Although previous studies addressing the role of local DC–T cell interactions in asthma demonstrated the existence of DC–T cell clusters on histological level,⁵ the functional analyses were performed on cells isolated by enzymatic disruption of bronchial tissue⁵ or by broncho-alveolar lavage,⁶ from which information about the localization of proliferating T cells and their contacts with other cells is not accessible. To date, no information has been available about the anatomical relationship of locally activated T cells with DCs and nerves in the mucosa of the conducting airways.

In the present study, we applied confocal microscopy in combination with whole-mount immunohistology to study the three-dimensional relationship of mucosal T cells, DCs, and nerves in the context of the epithelium and smooth muscle of the intrapulmonary conducting airways of mice in course of an experimental allergic airway inflammation. The combination of this technique with an *in vivo* proliferation assay, based on the incorporation of a nucleotide analogue into the DNA of dividing cells, provided fundamental information on the localization of recently activated T cells in relation to DCs and sensory nerves.

Materials and Methods

Animals

CD11c-EYFP-transgenic mice (kindly provided by Michel C. Nussenzweig, The Rockefeller University, New York) on a C57BL/6 background were used at 10 to 15 weeks of age. The animals were fed with ovalbumin (OVA)-free laboratory food and tap water ad libitum and held in regular 12-hour dark: light cycles at a temperature of 22°C. All animal experiments were performed in concordance with the German animal protection law under a protocol approved by the appropriate governmental authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit).

Induction of an Experimental Allergic Airway Inflammation

Two groups of CD11c-EYFP mice were sensitized with 10 µg OVA (Grade VI, Sigma, St. Louis, MO) adsorbed to 1.5 mg Al(OH)₃ diluted in PBS on days 0, 14, and 21 via intraperitoneal (i.p.) injection. Animals in the positive control group (OVA/OVA) were exposed to 1% OVA aerosol

in PBS for 20 minutes on days 27, 28, and 35. The negative control group (OVA/PBS) was exposed to PBS aerosol. This treatment resulted in a pronounced eosinophilic airway inflammation in the OVA/OVA group in contrast with the OVA/PBS group, according to the cytological analysis of the broncho-alveolar lavage fluid (data not shown).

Labeling the DNA of Proliferating Cells with 5-Ethynyl-2'-Deoxyuridine *in Vivo*

The thymidine nucleotide analogue 5-ethynyl-2'-deoxyuridine (EdU) was used for the *in vivo* labeling of the nuclei of dividing cells. Animals in both groups received a bolus i.p. injection of 1 mg EdU on day 35, 3 hours after the aerosol challenge or on day 36, 1 hour before sacrifice.

Tissue Processing and Whole-Mount Immunostaining

Twenty-four hours after the last allergen provocation, the animals were sacrificed with an overdose of i.p. administered pentobarbital. The tissue was processed as described earlier.¹⁰ Briefly, the lungs were inflation-fixed with Zamboni's solution and the main axial pathways of each lobe were microdissected (see supplemental Figures S1A and S1B at <http://ajp.amjpathol.org>). The airways were then washed, permeabilized with 0.3% Triton X-100 in PBS and immunostained as whole-mounts. The left lung and the right inferior lobe were used for the specific antibody staining, and the right middle and post-caval lobes were used for isotype controls. All antibodies were diluted in PBS supplemented with 0.5% bovine serum albumen, 4% mouse serum, and 4% donkey serum. The primary antibody cocktail contained dilutions of the following antibodies: chicken polyclonal antibody to green fluorescent protein (1:500, NB100-1614, Novus Biologicals, Littleton, CO), and rat monoclonal antibodies to CD5 (1:250, ab25392, Abcam, Cambridge, UK) and CGRP (1:400, EUD6401, Acris, Hiddenhausen, Germany). The secondary antibodies were donkey anti-chicken Cy2 (1:200), donkey anti-rat Cy3 (1:400), and donkey anti-guinea pig Cy5 (1:200, all from Jackson ImmunoResearch, West Grove, PA). The samples were finally labeled with phalloidin-Alexa Fluor 680 (1:50) and mounted in Prolong Gold mounting medium (Molecular Probes, Eugene, OR).

Detection of EdU Incorporated by Dividing Cells

EdU-detection in whole-mount specimens was performed using the Click-iT EdU Alexa Fluor 647 Kit (Molecular Probes) after permeabilization and before antibody labeling. The specimens were blocked with PBS/3% bovine serum albumen for 1 hour, incubated with the Click-iT reaction cocktail (prepared according to the manufacturer's instructions) for 5 minutes, and washed with PBS/3% bovine serum albumen for 3 × 10 minutes.

After the labeling with primary antibodies against green fluorescent protein, CD5, and CGRP (as described earlier), the following secondary antibodies were used: donkey anti-chicken Cy2 (1:200), donkey anti-guinea pig Cy3 (1:800, both from Jackson ImmunoResearch) and donkey anti-rat Alexa Fluor 594 (1:200, Molecular Probes).

Confocal Microscopy

Images were acquired using an LSM 510 META (Carl Zeiss, Germany) confocal microscope using $\times 20$ and $\times 40$ (water immersion) objectives and the laser wavelengths 488 nm, 543 nm, and 633 nm for the excitation of the fluorochromes Cy2, Cy3/Alexa Fluor 594 and Cy5/Alexa Fluor 647/Alexa Fluor 680, respectively. Quadruple-stained specimens were scanned in two steps: first, Cy2 and Cy3 (or Cy2 and Alexa Fluor 647) fluorescence were acquired in "channel/multitracking" mode with the appropriate emission filters in the whole Z-stack. In a second step, fluorochromes with overlapping emission spectra (Cy5 and Alexa Fluor 680 or Cy3 and Alexa Fluor 594) were scanned in "lambda" mode using spectral detectors. The two signals were separated using reference spectra taken from single-stained specimens. Combination of the two image stacks resulted in a four-channel three-dimensional dataset.

Image stacks for the quantitative analysis were scanned with an XYZ-resolution of $1024 \times 1024 \times 70$, with dimensions of $325.8 \mu\text{m} \times 325.8 \mu\text{m} \times 35 \mu\text{m}$, respectively, containing the airway epithelium, smooth muscle layer, and nerves. Two image stacks were taken at each out of four airway generation levels labeled as (0), (1–3), (4–6), and (7–9) according to the airway branching points (see supplemental Figure S1C at <http://ajp.amjpathol.org>), as described previously.^{10,12}

Quantitative Image Analysis

Image stacks were analyzed using Imaris 6.1.3. (Bitplane, Zurich, Switzerland). First, surface rendering was performed using optimal threshold settings in the CD11c-EYFP (dendritic cell) and CD5 (T cell) channels via "region growing" that resulted in individual surface objects for every cell in both channels, with an accurate separation of touching cells. "Quality" filter was used for the detection of seed points. Filter settings were optimized by visually comparing the result with the maximum-intensity projection. While DCs were analyzed using the same threshold and filter settings in all datasets, settings for T cells occasionally needed to be modified due to variations in signal intensity, whereas the maximum-intensity projections were used as a benchmark. Cell numbers were automatically calculated from the respective surface objects. To identify and calculate the number of nerve-contacting DCs, a "distance transformation" was performed on the IsoSurface representing CGRP⁺ nerves, which resulted in a "distance" channel encoding the distance of every voxel of the dataset from the nerves. IsoSurfaces of the DCs were then filtered for "zero" distance from the nerves, resulting in the accurate identifi-

cation of nerve-contacting DCs. The same procedure was applied when calculating T cells in contact with DCs or nerves. To identify EdU-positive T cells, T cells with a mean fluorescence intensity above a certain threshold in the EdU-channel were selected.

Pre-Embedding Immunostaining and Electron Microscopy

Zamboni-fixed lungs were sectioned with a vibratome (OTS-5000, Electron microscopy Sciences, Hatfield, PA) at $300 \mu\text{m}$ to expose the airway tree. Slices containing large bronchi were embedded in optimal cutting temperature compound, snap-frozen, and sectioned in a cryostat. Forty-micron-thick sections were collected in PBS containing 10% normal swine serum. Floating sections were incubated with primary antisera (chicken anti-green fluorescent protein, Novus Biologicals, Littleton, CO, diluted 1:8000 and rabbit anti-rat CGRP diluted 1:4000, Peninsula Lab., San Carlos, CA) overnight at room temperature. After a washing step, sections were incubated for 1 hour at room temperature with a peroxidase-conjugated swine-anti-rabbit IgG (1:100, Dako, Hamburg, Germany) and a peroxidase-conjugated donkey-anti-chicken IgG (1:1600, Dianova, Hamburg, Germany). Sections were reacted with 0.0125% 3,3'-diaminobenzidine-hydrochloride (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) in 0.05 M/L Tris-HCl buffer, pH 7.6, containing 0.00225% H_2O_2 . Sections were rinsed again and membranes fixed in 1% aqueous osmium tetroxide for one hour at room temperature. The specimens were then stained en bloc with 1% uranyl acetate in maleate-buffer, pH 5.2, dehydrated and flat-embedded in epon. Sections of approximately 80 nm thickness were cut on an ultramicrotome (Reichert Ultracut E, Leica, Bensheim, Germany), stained with uranyl acetate and alkaline lead citrate, and examined in an EM 902 transmission electron microscope (Zeiss, Jena, Germany). Embedded $40\text{-}\mu\text{m}$ sections and semithin sections were evaluated with a light microscope (Zeiss, Jena, Germany).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical significance of differences in the number of T cells and DCs, as well as in the number and percentage of contacting cells between OVA/PBS and OVA/OVA animals, were analyzed with unpaired t-test using GraphPad Prism 4.03. Differences with $P < 0.05$ were considered as statistically significant.

Results

The Microanatomy of the Interactions between DCs, T Cells, and Nerves

To identify the exact localization of DCs, T cells, and nerves in allergic airway inflammation, we performed confocal microscopy on microdissected airways from

Figure 1. Confocal images showing the spatial relationship of T cells, DCs, and sensory nerves in the course of an allergic airway inflammation. Mice were sensitized to OVA/Alum intraperitoneally on days 0, 14, and 21, and exposed to OVA aerosol on days 27, 28, and 35. On day 36, animals were sacrificed, the lungs were fixed, and the main axial pathway of the left lobe was stained as a whole-mount against EYFP, CD5, and CGRP. **A:** Low-magnification image of the airway mucosa taken at the proximal part of the main axial pathway showing the distribution of CD11c-EYFP⁺ DCs, CD5⁺ T cells, and CGRP⁺ sensory fibers (maximum-intensity projection of 25 optical sections scanned with an interval of 2.7 μ m, beginning from the airway lumen. Scale bar = 100 μ m). **B:** Boxed region in A scanned at higher resolution (100 optical slices with an interval of 0.6 μ m. Scale bar = 50 μ m), **inset** shows more DCs with dendritic morphology and fewer T cells in the mucosa of a PBS-challenged animal without airway inflammation. Scale bar = 50 μ m. **C–E:** Three-dimensional reconstructions of the dataset shown in (B) via surface rendering (DCs, T cells, and nerves) and volume rendering (epithelium and smooth muscle layer, revealed by F-actin staining). **C** and **D** show the luminal side of the airway mucosa. In (**D**), the epithelium was virtually removed (via changing intensity thresholds in the F-actin channel) for a better view of the underlying cells. (**E**) shows the abluminal side of the dataset, where the majority of T cells are located (grid spacing = 20 μ m). **F** and **G:** larger magnifications of the two boxed regions in (**E**) showing a T cell in contact with a nerve-contacting DC (**F**) and a T cell in contact with a DC located at the proximity of nerves (**G**). **H:** Large, rounded DC forming a cluster with many T cells (from an image taken at a different location of the same specimen).

OVA-sensitized and challenged mice. The pan-T-cell marker CD5 was used to detect T cells in conjunction with CGRP⁺ sensory nerve fibers and CD11c-EYFP⁺ DCs in a three-dimensional context using Zamboni-fixed whole-mount preparations. Although CD5 is also expressed on a subset of B cells,¹³ in the airway mucosa all CD5⁺ cells co-expressed CD4 (see supplemental Figure S2 at <http://ajp.amjpathol.org>), suggesting that CD5 can be used to detect helper T cells. In OVA/OVA mice, a pronounced T cell infiltration of the airway mucosa was observed (Figure 1, A and B) along the whole length of the main axial pathway. The CD11c-EYFP transgene revealed the network of DCs in the airway wall. Most DCs showed a rounded shape, compared with those in non-inflamed tissue with a typical dendritic morphology (see inset in Figure 1B). Three-dimensional reconstructions of the confocal data (Figure 1, C–E and supplemental Video S1 at <http://ajp.amjpathol.org>) revealed the exact localization of T cells, DCs and nerves in relation to the epithelium and smooth muscle layer (visualized by phalloidin-staining of F-actin filaments), as well as direct cell–cell contacts. Few DCs with a dendritic shape and nerve fibers were located in the epithelium and above the smooth

muscle layer (Figure 1, C and D). Strikingly, this layer almost completely lacked T cells, which were found in large numbers beneath the smooth muscle (Figure 1E) together with rounded DCs. T cells were either solitary or formed clusters with DCs. We were able to identify DC–T cell clusters in the proximity of CGRP⁺ nerve fibers. DCs involved in these clusters were either directly contacted by nerves (Figure 1F and supplemental Video S2 at <http://ajp.amjpathol.org>) or they were located near nerve fibers (Figure 1G and supplemental Video S3 at <http://ajp.amjpathol.org>) without actual contact. At various locations, large, rounded DCs formed clusters with T cells (Figure 1H and supplemental Video S4 at <http://ajp.amjpathol.org>), independently of nerve fibers. Solitary T cells were occasionally contacted by nerves (not shown).

Quantitative Mapping of Mucosal DCs, T Cells, and Nerves in Allergic Airway Inflammation

To determine how the number of DCs, T cells, their contact and relationship to nerves, changed during the development of an allergic airway inflammation, a three-

Figure 2. Quantitative analysis of the distribution of T cells DCs in the airway wall in the course of an allergic airway inflammation. Two confocal image stacks were taken at each airway generation from (0) to (7–9) and surface objects of each fluorescence channel were generated to estimate the number of DCs (**A**) and T cells (**B**). The results are expressed as number of cells beneath 1 mm² area of airway epithelium. Data are shown as mean $\pm$ SEM (* P < 0.05 and ** P < 0.01 versus OVA/PBS, n = 5). Open bars, OVA/PBS; solid bars, OVA/OVA.

dimensional quantitative mapping along the main axial pathways of OVA-sensitized and -challenged CD11c-EYFP transgenic mice was performed and compared with sham-challenged controls. High-resolution confocal image stacks were taken at four different airway-generation levels of the main axial pathway specified by the bifurcation number of side-branches as described earlier.¹⁰ Three dimensional surface objects were then generated from the confocal image stacks, which enabled the semiautomatic detection of individual DCs and T cells, and analyzed regarding the number of surface objects reflecting the number of cells in the dataset. In a second step, nerve-contacting DCs and DC-contacting T cells were detected as described in Materials and Methods. Finally, the number of T cells in contact with nerve-contacting DCs was determined (see supplemental Figure S3 at <http://ajp.amjpathol.org>).

Figure 2 shows the number of DCs and T cells beneath 1 mm² airway epithelium. Allergic airway inflammation had no effect on the number of DCs in the airway wall (Figure 2A), which were found at a density of $\sim$ 1000 cells/mm² epithelium in both groups. There were also no differences detected between different airway generation

levels. In contrast to DCs, the number of T cells significantly increased at all airway generations (Figure 2B) and showed an increasing center-to-periphery distribution in the inflamed airways, peaking at a density of $\sim$ 1100 cells/mm² epithelium at generation (7–9) of the OVA/OVA group.

The quantitative assessment of direct cell–cell contacts is shown in Figure 3. The number of nerve-contacting DCs (Figure 3A) was not altered by allergic inflammation. Approximately 9% to 12% of DCs were contacted by nerves. The number of T cells in contact with DCs significantly increased at the airway generation levels (1–3), (4–6), and (7–9) of the inflamed airways, compared with controls (Figure 3B), whereas the percentage of these T cells (varying between 12% and 25%), calculated from the total number of T cells, remained unchanged. Since some of the DCs in contact with T cells were found to be located in the proximity of sensory nerve fibers, we sought to determine the amount of T cells involved in such contacts. The number of T cells in contact with nerve-contacting DCs increased during inflammation, with significant differences between the two groups at the generations (1–3) and (7–9) (Figure 3C).

Figure 3. Quantitative analysis of cell-cell contacts between DCs, T cells, and sensory nerves in the airway wall in the course of an allergic airway inflammation. Contacts are displayed as the number of DCs in contact with nerves (**A**), number of T cells in contact with DCs (**B**), number of T cells in contact with nerve-contacting DCs (**C**), and number of T cells in contact with nerves (**D**), calculated as number of cells in contact beneath 1 mm² airway epithelium. Data are shown as mean $\pm$ SEM (* P < 0.05 and ** P < 0.01 versus OVA/PBS, n = 5). Open bars, OVA/PBS; solid bars, OVA/OVA.

Figure 4. Visualization of proliferating T cells together with DCs and nerves in the airway wall in the course of an allergic airway inflammation. Mice were sensitized to OVA/Alum intraperitoneally on days 0, 14, and 21, and exposed to OVA aerosol on days 27, 28, and 35. Three hours after the last OVA aerosol challenge, animals received 1 mg EdU i.p. On day 36, animals were sacrificed, the lungs were fixed, and the main axial pathway of the left lobe was treated with EdU detection reagents to label EdU-incorporating nuclei. Subsequently, the specimens were stained as whole-mounts against EYFP, CD5, and CGRP. **A–E:** Confocal image taken at the proximal part of the main axial pathway showing the distribution of CD11c-EYFP⁺ DCs (**A**), CGRP⁺ sensory fibers (**B**), CD5⁺ T cells (**C**), and EdU⁺ nuclei of proliferating cells (**D**). **E:** Merged representation of the images shown in **A–D**. (maximum-intensity projection of 80 optical sections scanned with an interval of 0.6 μ m, beginning from the airway lumen. Scale bar = 100 μ m). **Arrows** point at EdU⁺ T cells. **F:** Three-dimensional reconstructions of the boxed region in (**E**) via surface rendering (DCs, T cells, and nerves) and volume rendering (EdU⁺ cell nuclei). T cells are made transparent for the visualization of EdU⁺ nuclei of two T cells (**black arrows**) located on the surface of a large DC, which is contacted by a nerve fiber (**white arrow**, grid spacing = 20 μ m). **G:** Side-view (maximum-intensity projection) of the region between the two dashed lines in (**F**) showing an “extended” cross section of the T cells with EdU⁺ nuclei (**arrows**) associated with a DC contacted by a CGRP⁺ nerve fiber (**double arrow**).

However, the percentage of these cells (lying between 1% and 4%), out of the total T cell pool, did not change. Finally, we determined the number of T cells in direct contact with nerve fibers. Allergic inflammation resulted in an elevation in the total number of nerve-contacting T cells (Figure 3D), with significant differences at the generations (4–6) and (7–9). Approximately 2% to 5% of all T cells were in contact with nerves, without differences between the two groups.

Spatial Interactions of Activated T Cells with DCs and Nerve-Contacting DCs in the Airway Wall

A recently developed method to detect DNA synthesis in proliferating cells, based on the incorporation of the nucleotide analogue EdU and subsequent detection via fluorescence¹⁴ enabled us the visualization of proliferating cells in bronchial whole-mount preparations. This method was used here to identify those T cells in the mucosa that underwent cell division after the last aerosol provocation, possibly reflecting T cells that were locally activated by DCs in the airway wall. For this purpose,

EdU was first applied as a single bolus i.p. injection to the animals 3 hours after the last OVA aerosol challenge and the animals were sacrificed 1 day later, with a time period of 20 hours between the EdU application and tissue analysis. In a second experiment, EdU was applied 1 day after the last OVA aerosol challenge, just 1 hour before the animals were sacrificed and their tissue analyzed. As shown in Figure 4D, a large number of proliferating cells could be detected this way in the airway wall. Although the majority of T cells did not proliferate within this time frame, some EdU⁺ T cells could be identified along the whole length of the main axial pathway. While most EdU⁺ T cells were solitary, some of them were found in contact with DCs (Figure 4, A–E). Since we found an extensive contact between DCs and sensory nerves earlier,¹⁰ it was important to find out whether nerve-contacting DCs were associated with EdU⁺ T cells. Figure 4F shows a three-dimensional reconstruction of a confocal image with a DC in contact with EdU⁺ T cells and a CGRP⁺ sensory nerve fiber in the same time (see supplemental Video S5 at <http://ajp.amjpathol.org>). Although such sites of interaction between DCs, activated T cells, and nerves were not

Figure 5. Quantitative analysis of EdU⁺ T cells and their contacts with DCs and nerve-contacting DCs in course of an allergic airway inflammation. EdU was applied 20 hours or 1 hour before sacrifice and tissue analysis. Four confocal image stacks were taken at the proximal half of the main axial pathway and surface objects of DCs, T cells, and nerves were generated to identify the proportion of cells involved in contacts. Inside T cells, mean fluorescence intensity of the EdU channel was measured to identify EdU⁺ T cells. **A:** Number of EdU⁺ T cells in the airway wall, calculated as cell number beneath 1 mm² airway epithelium. **B:** Percentage of EdU⁺ T cells in contact with DCs, calculated from the total number of T cells in contact with DCs. **C:** Percentage of EdU⁺ T cells in contact with nerve-contacting DCs, calculated from the total number of T cells in contact with nerve-contacting DCs. **D:** Percentage of EdU⁺ T cells in contact with DCs without nerve contact, calculated from the total number of T cells in contact with DCs without nerve contact. Data are shown as mean $\pm$ SEM (* P < 0.05 and ** P < 0.01 versus OVA/PBS, n = 6, #no events detected). Open bars, OVA/PBS; solid bars, OVA/OVA.

frequent, they could be found along the whole length of the main axial pathway (Figure 4G).

The quantitative analysis of EdU⁺ T cells and their contacts with DCs and nerve-contacting DCs is shown in Figure 5. This analysis was limited to the proximal half of the main axial pathway, where CGRP⁺ nerves could be found at the highest density. The total number of EdU⁺ T cells was significantly elevated in the inflamed airways (Figure 5A) in case of both time intervals (20 hours and 1 hour) between EdU-application and tissue analysis. However, the frequency of these cells, calculated from the total T cell number, did not change and varied between 4% and 5%. Since airway mucosal DCs have previously been shown to locally interact with T cells, we determined the frequency of EdU⁺ T cells in contact with DCs and, more specifically, with nerve-contacting DCs. The percentage of EdU⁺ T cells in contact with DCs, calculated from the number of all DC-contacting T cells, showed an elevation in the inflamed airways (Figure 5B), which was significant when EdU was applied 1 hour before analysis. Strikingly, EdU⁺ T cells in contact with nerve-contacting DCs were only found in the inflamed airways (Figure 5C) and their frequency was approximately 8% or 2% out of all T cells involved in such contacts, with EdU applied 20 hours or 1 hour before analysis, respectively. These sites provide an anatomical basis for sensory neuropeptides like CGRP to influence the local activation of T cells

indirectly via modulating DC activity. In support of this possibility, the expression of calcitonin receptor-like receptor, a component of the CGRP receptor complex, by CD11c⁺ DCs could be detected using conventional immunohistology (see supplemental Figure S4 at <http://ajp.amjpathol.org>). Finally, to determine whether T cells in contact with nerve-contacting DCs preferentially get activated in allergic inflammation, we also measured the frequency of EdU⁺ cells among the T cells clustered with DCs that are not contacted by nerves. Indeed, when EdU was applied 20 hours before analysis, the percentage of these cells did not show any change in the inflamed airways, compared with controls (Figure 5D). However, when EdU was administered just one hour before analysis, this parameter showed a tendency to increase in the inflamed airways.

Visualization of DC–Sensory Nerve Contact Sites Using Immuno-Electron Microscopy

The contact between sensory fibers and DCs clustered with proliferating T cells, as detected by confocal microscopy, does not reveal the actual distance between the axonal and DC cell membranes. Thus, to visualize DC–nerve contact sites on the ultrastructural level, we per-

Figure 6. Electron microscopic representation of DC-sensory nerve proximity sites. Pre-embedding immunohistochemistry for CGRP and EYFP followed by transmission electron-microscopy. **A:** A dendritic cell process (**asterisk**) is situated next to a nerve fiber bundle being surrounded by a perineurial sheath (**arrow**) containing myelinated (m) and non-myelinated (nm) axons (smc = smooth muscle cells, e = epithelium). **B:** An unlabeled process of a non-neuronal cell (**asterisk**) establishes direct contact with the plasma membrane of a dendritic cell (DC; n = nucleus of the dendritic cell; el = elastic lamella in the epithelial basal membrane; sc = secretory epithelial cell). **C:** Both CGRP-immunoreactive (**asterisk**) and non-reactive axons (**arrows**) are observed within the same Schwann-cell covering at close proximity to the cell body and processes of a DC. Collagen fibrils (c) are still interposed between the axons and the dendritic cell, whereas a non-neuronal cell process was in intimate contact with the DC (**inset**) (n = nucleus of the DC; **arrowhead** = cis-terna of endoplasmic reticulum in the non-neuronal cell process).

formed pre-embedding immunostaining followed by electron microscopy.

The ultrastructural immunolabeling procedure resulted in the same type of electron dense reaction product in both DCs and in CGRP-immunoreactive nerve fibers, and distinction was based on morphological criteria. DCs were first identified by their size and presence of a cell nucleus in plastic-embedded 40 μm -thick sections by light microscopy. Such regions were excised, re-embedded, and re-sectioned at 0.5 μm for orientation in light microscopy (see supplemental Figure S5 at <http://ajp.amjpathol.org>) and at 80 to 90 nm for electron microscopic evaluation. In the electron microscope, cells containing a nuclear section profile and electron dense reaction product in the cytoplasm were identified as DCs, and their processes were traced over serial sections wherever possible. The labeling intensity of ultra thin sections cut close to the surface of the 40 μm -thick cryosection was high and decreased toward the depth of the tissue section.

DCs were observed in close proximity to nerve fiber bundles still being surrounded by a thin perineurial sheath. These nerve fiber bundles contained both myelinated and non-myelinated axons (Figure 6A). Along their course, such nerve fiber bundles lost their perineurial sheath and unmyelinated axons were incompletely surrounded by Schwann-cell processes, so that areas of their surface were exposed to the interstitial spaces. Both CGRP-immunoreactive and non-reactive axons were observed within the same Schwann-cell covering. Such terminal axon regions were observed in close proximity (0.3 μm) to cell bodies or processes of DCs, albeit other cells process or collagen fibrils were still interposed between

the axons and the DC (Figure 6C). Unlabeled processes of not identified non-neuronal cells, however, had direct plasma membrane contacts to DCs (Figure 6B).

Discussion

In allergic asthma, repeated encounter with inhaled allergen leads to the local activation of Th2 lymphocytes, which promote the recruitment of eosinophils and mast cells into the airway wall, resulting in airway hyperreactivity and chronic inflammation.⁵ Airway DCs play an essential role in this process by locally presenting antigenic peptides to T cells.⁶ The recruitment of DCs into the airways during the secondary immune response to inhaled antigen critically depends on airway sensory innervation.⁹ The neuropeptide CGRP, abundantly produced by airway sensory C-fibers, acts as a chemoattractant on DCs⁸ and influences the DC-driven proliferation of T cells *in vitro*.¹⁵ Since most functional studies on DC-T cell interactions in allergic asthma and on the neural control of DCs were performed on cells isolated from tissue or generated *in vitro*, the exact anatomical sites of “productive” interactions between DCs, T cells, and nerves remain unknown. We have previously demonstrated the extensive contacts between CGRP⁺ sensory nerves and CD11c⁺ DCs in the airway wall, providing an anatomical basis for the neural control of DC activity.¹⁰ The aim of the present study was to identify those locations at which interactions of T cells with DCs may result in their activation and this interaction is potentially influenced by sensory nerves. For this purpose, we performed a three-dimensional quantitative mapping of T cells, DCs, and their interaction with nerves

along the intrapulmonary airways (which is the primarily involved anatomical compartment in asthma) of mice during an ongoing airway inflammation. The use of confocal microscopy on bronchial whole-mount preparations enabled us to observe the entire mucosal network of DCs, T cells, and nerves. Although many important markers of DCs, like CD11c, and T cells, like CD3, CD4, and T cell receptor- β , were undetectable after fixation with Zamboni's solution (which was essential for the detection of CGRP), this problem was overcome by using the CD11c-EYFP transgenic mouse to visualize DCs and the pan T cell marker CD5 (which, in the airway wall, co-localized with CD4) to detect T cells. For the quantitative analysis of confocal data a completely novel approach was used here, based on three-dimensional reconstruction via surface-rendering and subsequent semiautomatic measurements, yielding highly accurate cell enumeration and detection of cell-cell contacts. At this point it must be noted that an analysis on the distribution of cells and their contacts in the wall of the intrapulmonary conducting airways has not been performed before in a way like in the present study, which makes a comparison between this study and other reports extremely difficult.

Consistent with earlier reports,^{5,16} repeated exposure to OVA aerosol in this model led to an increase in the number of T cells in the airway wall, which was most pronounced in the distal part of the main axial pathway, suggesting the predominant involvement of distal airways in allergic inflammation. This increase was also reflected in the number of T cells involved in contacts with DCs and nerves. The number of DCs in the airway wall did not change after the last allergen challenge. Although in most studies an increase in the number airway DCs was observed,^{17,18} some reports selectively focusing on airway mucosal DCs found that this increase of DC number is transient and limited to the initial phase of airway inflammation.¹⁹ Whereas DCs were found both in the epithelial layer and beneath the smooth muscle, the vast majority of T cells were located beneath the smooth muscle layer, and contacts between DCs and T cells were only found in this compartment. Up to one quarter of all T cells were clustered with DCs, which at one time point, given the dynamic characteristics of DC-T cell interactions,²⁰ might underestimate the number of T cells that actually get in contact with DCs in course of the allergic response. One tenth of the total DC population had contact to sensory nerve fibers and up to 4% of T cells were found in clusters with these DCs. Assuming a dynamic equilibrium between the solitary T cells and T cells involved in such contacts, there is a considerable possibility for the indirect neural control of T cell activation via neuropeptides like CGRP exerting their effects on DCs.⁸ This assumption is also supported by our finding that DCs express calcitonin receptor-like receptor, which, in association with the receptor activity modifying protein 1, acts as a receptor for CGRP.²¹ However, since the association of calcitonin receptor-like receptor with receptor activity modifying protein 2 constitutes a receptor for adrenomedullin instead of CGRP, further studies on the subtype of receptor activity modifying protein on DCs will be necessary to determine the specificity of calcitonin receptor-like receptor. In

addition to the DC-mediated effect of sensory neuropeptides on T cell activation, a direct neural influence on T cells is suggested by a further 2% to 5% of T cells directly contacted by nerves.¹¹

It is an important question whether direct cell-cell contact is necessary for the action of CGRP on DCs. On the ultrastructural level, we found CGRP⁺ unmyelinated axons in the proximity of DCs, without membrane contact. The shortest distance between DCs and sensory fibers was 0.3 μm , with only extracellular matrix interposed between the two membranes. In contrast to classical neurotransmitters involved in synaptic neurotransmission, neuropeptides released from terminals of sensory C-fibers can diffuse in tissue and exert their effects in a paracrine fashion to regulate target cells many micrometers away.²² Thus, although DCs had no actual membrane contact with CGRP⁺ nerves, they can still be efficiently regulated by CGRP.

Since T cell surface activation markers did not work reliably in our model, we used an alternative method to study the possible connection between DC-T cell contacts and T cell activation. Firstly, proliferating cells were visualized by the administration of EdU¹⁴ three hours after the last aerosol provocation and 20 hours later the animals were sacrificed. T cells with an EdU-positive nucleus had an active DNA synthesis within this time period, meaning that they either just started or already underwent cell division. This setup enabled us the assessment of T cell proliferation in vivo without the need for transferring ovalbumin-specific T cell receptor-transgenic CD4 positive T cells, which would have inevitably compromised the physiological immune response. Although we were primarily interested in the local activation of T cells in the airway mucosa, we cannot exclude the possibility that EdU⁺ T cells proliferated elsewhere, most likely in the draining lymph nodes, before their recruitment to the airway mucosa. For this reason we performed a second experiment, in which EdU was administered 1 day after the last aerosol provocation and 1 hour later the animals were sacrificed. We suggest that in these latter settings, EdU-positivity of T cells more accurately reflected their proliferation in the airway mucosa. Although not more than 5% of all T cells had an EdU positive nucleus, in allergic inflammation these cells occurred at higher frequency in the population of DC-contacting T cells, compared with the non-inflamed situation, meaning that T cells clustered with DCs preferentially get activated in allergic inflammation. Most importantly, activated T cells clustered with nerve-contacting DCs were only found in the inflamed airways. Taken together, these data suggest that, if EdU positive T cells represent those getting activated in the airway mucosa, this activation is potentially mediated by DCs presenting their antigen under the influence of sensory nerves.

In summary, this work presents a novel method to study the distribution of DCs, T cells, and nerves, three key cellular elements of the asthmatic response, along the intrapulmonary airways in a three-dimensional quantitative fashion. Allergen provocation resulted in an elevation of T cell number in the airway wall, with no changes in DCs. Extensive interactions between DCs and T cells occurred beneath the smooth muscle layer of the airway

Figure 7. The proposed model of DC-T cell-nerve interactions in the inflamed airway mucosa. DCs form two networks in the airway wall: one just beneath the epithelium and a second one, located deeper, beneath the smooth muscle. Some DCs in both networks are contacted by sensory C-fibers, as demonstrated earlier.¹⁰ DCs beneath the epithelium capture inhaled antigens by extending their pseudopods into the airway lumen and then migrate to the deeper layers of the airway wall to present their antigen to resident T cells beneath the smooth muscle layer. This local T cell activation, which results in subsequent T cell proliferation, can be influenced by sensory fibers contacting the DCs via the release of the neuropeptides CGRP and SP.

wall. A fraction of T cells were found in contact with DCs located at the immediate proximity of sensory nerve fibers. This T cell population divided only in the inflamed airways, in contrast to those clustered with DCs without nerve contact. The data presented here will form the basis of future imaging studies addressing the dynamics and outcome of interactions between these three central components of asthma pathology.

The observations of this work suggest the following model of the local events during the secondary immune response to inhaled antigen (Figure 7), which occur parallel with the events that take place in the regional lymph nodes. Dendritic cells, forming a network beneath the epithelium, collect inhaled antigens from the airway lumen and migrate to the deeper layers of the airway wall, beneath the smooth muscle, where they present their antigenic cargo to newly recruited T cells. These local DC-T cell interactions are modulated by neuropeptides released from sensory fibers “innervating” DCs. Subsequently, T cells involved in such contacts begin to proliferate locally to fulfill their effector function. It will be an important task of future studies to determine the exact outcome and molecular mechanisms of interactions between DCs, T cells, and sensory nerves in the airways.

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