Chapter 18

Clara Epithelial Cell Depletion in the Lung

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Abstract

The bronchial epithelium has been increasingly recognized as an important immunomodulatory compartment in asthma and other lung diseases. Clara cells, which comprise the nonciliated secretory epithelial cells, are an important epithelial cell type with functions in the regulation of lung homeostasis and inflammation. Using naphthalene, Clara cells can be depleted within 24 h and regenerate by 1 month, hence, providing an easy method to study the impact of Clara cells on lung inflammation.

Key words Clara cells, Airway epithelium, Naphthalene, Asthma

1 Introduction

The airway epithelium comprises ciliated and secretory cells arranged in a continuous stratified structure. In mouse and other species, nonciliated bronchiolar Clara cells (CC) are the predominant epithelial cell type secreting both pro- and anti-inflammatory factors [1–3]. It accounts for 70–90 % of the cells in distal airways of many species including mice [4]. In humans, CC represent about 20 % of epithelial cells and have been shown to contribute to cell renewal in the normal conducting airway epithelium [5, 6]. CC respond to activated Th2 cells via the IL-4 receptor-α [7], can differentiate to mucus-producing goblet cells [8], and secrete anti-inflammatory factors such as Clara cell secretory protein (CCSP/CC-10) and eotaxin [9]. CCSP has been shown to counter-regulate the Th2 response in asthma [10]. Moreover, CC are also able to metabolize and detoxify xenobiotics and toxic compounds, such as naphthalene (NA), present in cigarette smoke [11, 12]. Therefore, CC of the airways are uniquely susceptible to injury by metabolizing chemicals into toxic intermediates.

NA is a prominent component of sidestream, whole, and filtered cigarette smoke [7]. For compounds like NA, toxicity is highly dose dependent and cell type and site selective [4, 13]. The toxicity

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of NA requires metabolic activation, catalyzed by cytochrome P450 monooxygenases that cause airway CC swelling, vacuolization, and exfoliation into the lumen of the airways 24 h after injury is initiated [14]. Susceptibility correlates with the presence of cytochrome P450 2F2 (CYP2F2) within CC. Therefore, murine CC are more susceptible to NA-induced cytotoxic injury than other types of airway epithelial cells. CC present in the distal airway are more susceptible at very low doses, with susceptibility extending to proximal airways with higher doses. Detailed time kinetics of NA-induced CC cytotoxicity has been elegantly shown previously [14].

2 Materials

2.1 Mice

1. 8–10-week-old C57BL/6, BALB/c mice or Swiss Webster mice.

2.2 Naphthalene

1. Naphthalene, 20 mg/ml stock (Sigma Aldrich Chemical, Munich, Germany).
2. Corn Oil (Sigma Aldrich Chemical, Munich, Germany).
3. Pentobarbital sodium.

2.3 Reagents for Immunohistochemistry

1. 10 % formalin.
2. Xylene.
3. 100; 90; 80; and 70 % Ethanol.
4. Distilled water.
5. 0.3 % H_2O_2 in methanol.
6. 3 % citrate buffer (pH 6.0).
7. 1 % bovine serum albumin.
8. CC10 antibody (Upstate, Millipore, MA).
9. DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA).
10. Hematoxylin.
11. Horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (Santa Cruz Biotech, CA).
12. 3,3′ diaminobenzidine (DAB) (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA).

2.4 Reagents for OVA Sensitization and Challenge

1. 10 μg of endotoxin-free OVA (grade VI, Sigma-Aldrich) in 200 μl of 1× PBS per mouse.
2. 1 % OVA (grade V, Sigma Aldrich) in 1× PBS.

2.5 Microscope and Software

1. Light microscope (Olympus Europa GmbH, Hamburg, Germany).
2. Cell^F imaging software program (Soft Imaging System GmbH, Muenster, Germany).

3 Methods

3.1 Clara Cell Depletion in Naïve Animals

1. Prepare NA in a 50 ml falcon tube by dissolving 20 mg in 1 ml of corn oil (stock solution). Control animals should receive the same amount of corn oil i.p. It is best to make aliquots of corn oil and freeze to avoid contamination (*see* **Note 1**).
2. Inject animals with either 200 mg/kg of NA dissolved in corn oil (CO) (10 µl of stock solution) or CO alone (10 µl) intraperitoneally (i.p.) (*see* **Note 2**).
3. Sacrifice animals using an overdose of pentobarbital sodium at the time of analysis. Maximal exfoliation of CC is seen from 24 to 48 h after application of NA + CO, with cell death occurring as early as 6 h. CC start regenerating by day 3 and by day 10, about 60 % of CC regenerate. For analysis of CC numbers, immunohistological analysis of the lung by CC staining should be performed.

3.2 Immunohistological Analysis

1. Perfuse the lung with PBS via the heart. Insert a cannula into the trachea and fix with a ligature. Inflate and fix the lungs via the cannula by gentle infusion with 10 % formalin. Remove the inflated lungs and store in 10 % formalin. Embed the fixed lung tissues into paraffin and cut into 3 µm sections.
2. Deparaffinize the tissues using xylene and rehydrate in 100–70 % ethanol (10 % steps) for 5 min/concentration and finally in 1× PBS. Remove endogenous peroxidase activity using 1 % hydrogen peroxide in methanol for 30 min. Antigen retrieval can be performed by microwave treatment in 3 % citrate buffer (pH 6.0). However, this step may be optional depending on the antibody. Cool the slides down to room temperature and rinse three times with 1× PBS.
3. After washing in PBS, incubate sections in 1× PBS containing 1 % bovine serum albumin for 30 min, followed by incubation with the polyclonal rabbit antibody directed against Clara cell-specific 10-kDa protein (CC10) in the same solution for 1 h at 37 °C (*see* **Note 3**).
4. Incubate sections with a peroxidase-conjugated anti-rabbit secondary antibody for 30 min at room temperature. Visualize using DAB as the chromogen according to the ABC method following the manufacturer's instructions. All sections may be counterstained with hematoxylin. Sections can be counterstained with DAPI (blue) for detection of all cells.
5. Negative controls without the primary antibody and normal rabbit IgG should be included.
6. Sections can be semiquantitatively analyzed for CC numbers using light microscopy. CC can be counted as CC10-positive cells with nuclear profiles surrounding the proximal or the

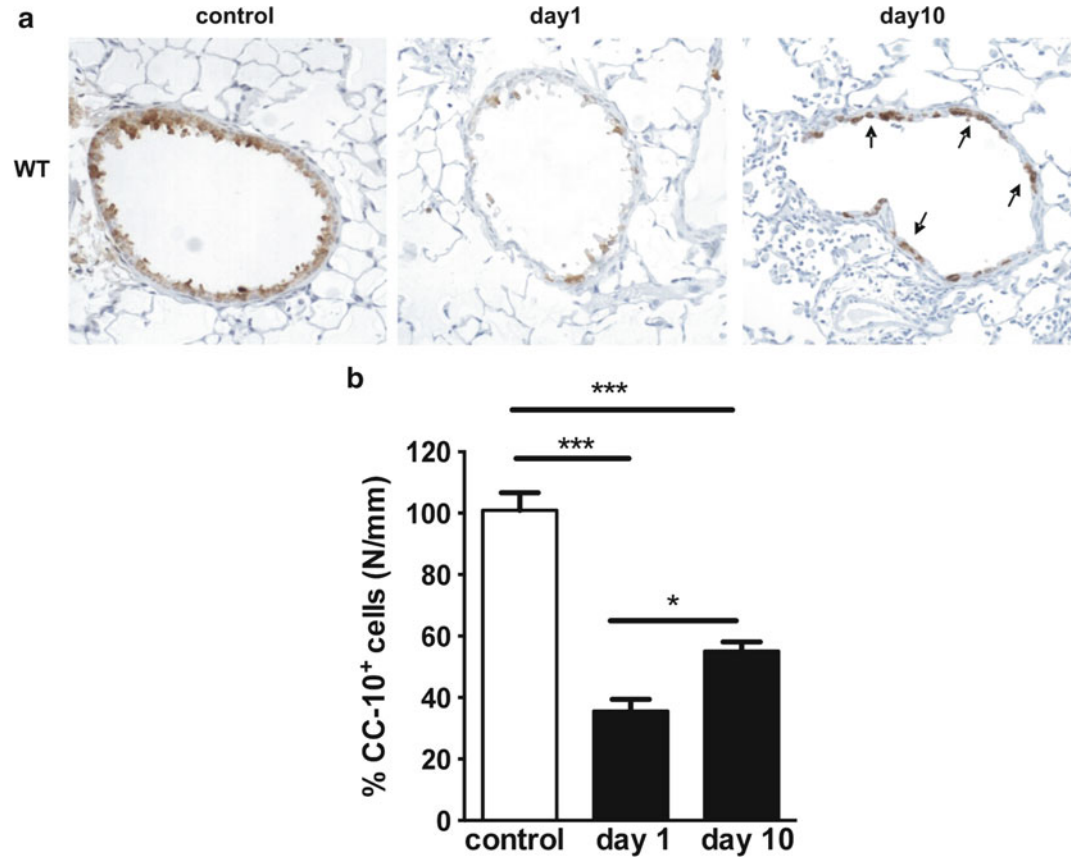

Fig. 1 Clara cell depletion. (**a**) CC10 staining was performed in lung sections of corn oil (control) and NA-treated mice (day 1 and day 10). Mice were sacrificed 24 h post NA treatment for CC10 staining. The CC10-positive cells stained with anti-CC10 antibody are diaminobenzidine (DAB) positive (*brown*) against the hematoxylin counterstain (*blue*). (**b**) Quantification of CC10-positive cells in the airways of corn oil (control) and NA-treated animals at day 1 and day 10. The graph represents CC10-positive cells with nuclear profiles per mm of the basement membrane, normalizing CC in the control group as 100 %. Results represent the mean ± SEM of at least six animals. ***$P < 0.001$. *$P < 0.05$. These data are representative of three experiments. Figure 1b, reproduced with permission of the European Respiratory Society. Eur Respir J February 2012 39:429–438; published ahead of print August 4, 2011, doi:10.1183/09031936.00197810

distal airways/mm of the basement membrane (Fig. 1). At least ten similar airways are counted per mouse using the software Cell^F (or a similar software) linked to the light microscope (*see* **Note 4**).

3.3 Clara Cell Depletion in Asthma

1. Sensitize two groups of mice with subcutaneous (SC) injections of 10 μg of endotoxin-free OVA (grade VI) in 200 μl of PBS and in another two control groups with sham injections of PBS on days 0, 7, and 14 (*see* **Note 5**).
2. On day 16, i.p. inject one group of SC OVA-sensitized mice and one group of mice receiving sham injections of PBS with

200 mg/kg of NA dissolved in CO. The dose depends on the amount of desired Clara cell exfoliation (*see* **Note 6**).

3. On days 26, 27, and 28, subject the SC OVA-sensitized and PBS-injected mice for 20 min with either aerosolized 1 % OVA (grade V) or aerosolized PBS, as appropriate.
4. Sacrifice mice 24 h after the last challenge or, if performing airway hyperreactivity (AHR) measurements, 48 h after the last challenge.
5. Harvest the lungs and conduct the immunohistochemical analysis as described under Subheading 3.2.

4 Notes

1. NA is a toxic irritant and is flammable. Hence, care must be taken while handling NA by wearing gloves, lab coat, face mask, and eye protection. Solutions should be prepared in a chemical fume hood. Prepare a fresh solution every time. Perform mouse injections under the hood.
2. Usually 200 mg/kg should be optimal to efficiently denude CC in proximal and distal airways; however, quantities may be optimized for the experiment. Additionally, female and male mice have been shown to vary in response.
3. The precise dilution of CC10 antibody should be tested for each lot.
4. CC counted on the basis of immunohistochemistry by staining nuclear profiles positive for CC10 antigen revealed an ~65 % reduction in CC10+ cells at day 1 post NA + CO administration. This number went to 40 % after 10 days (Fig. 1b). However, doses and time points can be manipulated based on the experimental goals.
5. Although this method has been described for subcutaneous murine models of asthma, given the many other experimental protocols for induction of asthma, including the i.p. sensitization, the route of airway exposure, and the variety of antigens available for asthma induction, protocols may be changed to suit specific experimental goals. However, since NA is toxic to the mouse, a minimum rest period of 5–10 days should be included post NA application before challenge with allergen.
6. CC in the distal airways are more susceptible to NA than cells in the proximal airways. Additionally, sex-based differential responses to NA have also been reported. Ideally, a dose–response for the strain and sex of mice used should be established in the lab prior to beginning large-scale experiments.

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