**Cutting Edge: Altered Pulmonary Eosinophilic Inflammation in Mice Deficient for Clara Cell Secretory 10-kDa Protein**

Li-Chen Chen, Zhongjian Zhang, Allen C. Myers and Shau-Ku Huang

*J Immunol* 2001; 167:3025-3028; doi: 10.4049/jimmunol.167.6.3025

[http://www.jimmunol.org/content/167/6/3025](http://www.jimmunol.org/content/167/6/3025)
Clara cell secretory protein (CC10) is a steroid-inducible protein, and its in vivo function is currently unclear. The role of CC10 in modulation of pulmonary allergic inflammation was examined in mice deficient for the CC10 gene. Wild-type and homozygous CC10-deficient mice were sensitized with an Ag, OVA, and challenged with either OVA or saline. When compared with those seen in wild-type mice, a significantly higher level of pulmonary eosinophilia was found in Ag-sensitized and challenged CC10-deficient mice. Significantly increased levels of Th2 cytokines IL-4, IL-5, IL-9, and IL-13 were also found in CC10-deficient mice. In addition, an increased level of eotaxin, but not RANTES, was also seen in CC10-deficient mice. No significant difference was observed in the level of a Th1 cytokine, IFN-γ, between different groups of mice. These results provided the first in vivo evidence that CC10 plays a role in the modulation of pulmonary allergic inflammation. The Journal of Immunology, 2001, 167: 3025–3028.

It has been established that dysregulation of the IgE response and Th2-associated inflammatory network underlies the etiology of, and perpetuates the persistence of, airway inflammation, leading ultimately to the expression of allergic asthma (1–3). Data from several gene knockout/transgenic mouse studies, together with molecular analysis of individual genes, provide in vivo evidence for the importance of Th2-associated eosinophilic inflammation in the expression of bronchial hypersensitivity (4–11). Furthermore, these allergic responses seen in Ag-sensitized and challenged mice could be reversed by the treatment of mice with several Th2-inhibitory cytokines (12–14). These studies thus establish a general phenotypic feature of allergic inflammation. However, it is becoming apparent that the expression of allergic inflammation involves a complex array of molecular and cellular interactions. The exact sequence of events leading to allergic inflammation is still unclear, and the importance of pulmonary resident cells, such as Clara cells, in the pathophysiology of pulmonary inflammation remains to be defined.

Clara cell secretory 10-kDa protein (CC10 or CCSP) is a homodimeric protein produced by nonciliated bronchiolar cells (Clara cells) and is one of the most abundant proteins in the lining fluid of airways (15). However, the pathophysiological function of CC10 in the lung has not been elucidated. CC10 has been shown to be able to inhibit chemotaxis and phagocytosis of neutrophils and monocytes, respectively (16). CC10 also inhibits fibroblast chemotaxis, which is related to a blockade of the secretory phospholipase A2 (PLA2; Ref. 17). Similarly, studies have suggested that CC10 is able to modulate the activity of IFN-γ (18). To investigate the role of CC10 in controlling the development of Ag-induced allergic inflammation, we have used an established mouse model of eosinophilic inflammation. The results showed that when compared with those seen in wild-type mice, CC10-deficient mice, following sensitization and challenge with Ag, develop intensive eosinophilic inflammatory response, which is associated with elevated levels of Th2 cytokines and eotaxin.

Cutting Edge: Altered Pulmonary Eosinophilic Inflammation in Mice Deficient for Clara Cell Secretory 10-kDa Protein

Li-Chen Chen,*† Zhongjian Zhang,‡ Allen C. Myers,* and Shau-Ku Huang*#2

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, MD 21224; †Division of Allergy, Asthma, and Rheumatology, Department of Pediatrics, Chang Gung Children’s Hospital, Taoyuan, Taiwan, Republic of China; and ‡National Institute of Child Health and Development, National Institutes of Health, Bethesda, MD 20892

Received for publication June 5, 2001. Accepted for publication July 23, 2001.

Materials and Methods

Animals

Wild-type (+/+) C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous CC10-deficient mice (−/−) on C57BL/6 background were obtained from an intercross of heterozygous CC10-deficient mice (Ref. 19; kindly provided by Dr. A. B. Mukherjee, National Institutes of Health, Bethesda, MD), and germline transmission of the mutant CC10 allele was identified by PCR as described (Ref. 19; Fig. 1A). Homozygous CC10-deficient mice (−/−), 6–8 wk of age, were used.

Sensitization, challenge, bronchoalveolar lavage (BAL), and histology

Mice (n = 4/group) were sensitized by i.p. injection of 10 μg OVA (grade V; Sigma, St. Louis, MO) emulsified in 2 mg alum (Pierce, Rockford, IL) on days 0 and 7. Mice were anesthetized and challenged intratracheally with 5 μg OVA or received PBS alone in a total volume of 50 μl on day 21 after the initial sensitization. Intratracheal challenge was performed as previously described (6, 12). BAL fluid (BALF) and lung tissue specimens were collected 48 h after challenge. Total BALF cells and differential cell counts were determined as previously described (6, 12). The lungs were inflated with 10% formalin in PBS (pH 7.4), postfixed in 10% formalin in PBS for 24 h, cryopreserved in 18% sucrose in PBS for 18–24 h, and

1 Cutting Edge: Altered Pulmonary Eosinophilic Inflammation in Mice Deficient for Clara Cell Secretory 10-kDa Protein

Copyright © 2001 by The American Association of Immunologists

3 Abbreviations used in this paper: CC10, Clara cell secretory 10-kDa protein; PLA2, phospholipase A2; BALF, bronchoalveolar lavage fluid.
embedded in OCT (Tissue-Tek; Sakura Finetec, Torrance, CA). Frozen sections (10 μm) were made on a TISSEU-TEK II Microtome/Cryostat, and stained with Giemsa stain solution (Electron Microscopy Sciences, Fort Washington, PA). The numbers of differentially stained cells within a 1-mm² area in perivascular and peribronchial regions from three fields of tissue sections from each mouse were counted using light microscopy at a magnification of ×400.

Analysis of gene expression and the level of BAL cytokines

Total RNA was isolated from lung tissues using Trizol (Life Technologies, Gaithersburg, MD) following the manufacturer’s protocol. RT-PCRs for cytokines (IFN-γ, IL-4, and IL-5; Refs. 6 and 13), and primers (sense and antisense, respectively) for the following cytokine/chemokine genes: IL-9, 5'-ATGTAGGCCATGAGGTCCAC (494 bp). Amplification conditions for all reactions were 95°C for 45 s, 55–60°C for 50 s, and 72°C for 50 s for subsaturating cycles, typically between 25 and 30 cycles. The intensities of PCR products on 2% ethidium bromide-containing agarose gels with optimized exposure were evaluated by OpiQuant Acquisition and Analysis (Packard Bioscience, Meriden, CT). The levels of BALF IL-4 and IFN-γ were determined by ELISA according to the manufacturer’s directions (BD PharMingen, San Diego, CA). For analysis of IL-13 level, a commercial kit (R&D Systems, Minneapolis, MN) was used.

Data analysis

Data are expressed as means ± SEM unless otherwise indicated. Differences between groups were assessed by one-way ANOVA followed by the Tukey-Kramer honest significant difference test. A value of p < 0.05 was considered statistically significant.

Results

CC10 deficiency exacerbates OVA-induced pulmonary eosinophilia

The number and percentage of BALF eosinophils from wild-type (+/+) and homozygous CC10-deficient (−/−) mice were first examined. Inflammatory cells in BALF were assessed at 48 h post-challenge, at which time the peak inflammatory responses had occurred (6, 12). A significant increase of BALF eosinophil numbers and percentages was found in Ag-sensitized and challenged wild-type mice compared with those seen in Ag-sensitized mice challenged with saline (Fig. 1A, B and C; and D). In sensitized CC10-deficient (−/−) mice, a significantly increased level of eosinophilia in both the number (>2-fold) and percentage of BALF was seen following Ag challenge (Fig. 1, C and D) compared with...
those seen in Ag-challenged wild-type mice (Fig. 1, Bd and C). The number of BAL eosinophils from saline-challenged mice did not vary in either group.

These findings were corroborated by histological examinations of the lungs. First, lungs from sham-challenged wild-type and CC10-deficient mice contained few, if any, inflammatory cells (Fig. 1E, a and c). Second, OVA-challenged lungs of wild-type mice showed signs of inflammation with a dominant eosinophil infiltrate (53.2 ± 7.2/mm²) in the perivascular and peribronchial regions (Fig. 1Eb). Scattered mononuclear cells (6.4 ± 1.2/mm²) comprising mainly monocytic cells (monocyte/macrophage) and lymphocytes were also seen. Significantly, lungs from challenged CC10-deficient mice demonstrated extensive infiltration of eosinophils (242.4 ± 57.2/mm²), and increased monocytic cells (34.4 ± 2.6/mm²) were also observed.

No significant difference was seen in the number and percentage of BALF lymphocytes between challenged wild-type and CC10-deficient mice (Fig. 1, C and D), whereas a few lymphocytes (9.2 ± 2.7/mm²) were seen in the peribronchial region. Consistent with previous studies (6, 13), neutrophils were not prominent in mice sensitized and challenged with Ag and were rare in challenged CC10-deficient mice in the BALF (Fig. 1, C and D) and in tissue sections (neutrophils, <2/mm²) of both wild-type and CC10-deficient mice after challenge.

**Increased cytokine and chemokine responses after Ag challenge in CC10-deficient mice**

The relative levels of gene expression for IL-4, IL-5, IL-9, IL-13, RANTES, eotaxin, and IFN-γ were assessed from lung homogenates of CC10-deficient and wild-type control mice 48 h after Ag or saline challenge. The level of gene expression was normalized to the abundance of G3PDH mRNAs. Results (Fig. 2) showed that although no significant change was seen in the level of gene expression for IFN-γ, the levels of IL-4, IL-5, IL-9, and IL-13 were markedly increased in CC10-deficient mice after sensitization and challenge with OVA when compared with wild-type control mice (+/+; p < 0.05). The level of gene expression for a CC chemokine, RANTES, was unchanged in either wild-type or CC10-deficient mice (Fig. 2). In contrast, the level of eotaxin gene expression was increased in the lungs of CC10-deficient mice (+/−) when compared with wild-type mice (+/−) (Fig. 3). When compared with that seen in wild-type mice, a significantly increased level of BALF IL-4 (p < 0.05) proteins was detected in OVA-challenged CC10-deficient mice (Fig. 3). Of significance, the level of BALF IL-13 in challenged CC10-deficient mice was increased >3-fold (p < 0.005) compared with that of challenged wild-type mice (Fig. 3, B).

**FIGURE 2.** Increased cytokine and chemokine mRNAs in CC10-deficient mice. A, Representative PCR products for cytokines, chemokines, and G3PDH as indicated. B, Relative level of gene expression. The intensity of each PCR product was normalized to that for G3PDH for each sample. Significant difference from wild-type (+/+), p < 0.05.

**FIGURE 3.** Increased concentrations of cytokines in the lungs of CC10-deficient (−/−) mice. Cytokine concentrations for IL-4 and IL-13 were increased in the BALF from CC10-deficient (−/−) vs wild-type (+/+). Significant difference from wild-type (+/+) mice: *, p < 0.05; and **, p < 0.005.
wild-type mice. The level of IFN-γ, however, showed no difference in all groups of mice (Fig. 3).

Discussion

Despite the relative abundance of CC10 in the BALF, the physiological function of CC10 remains to be defined. This study provides, for the first time, in vivo evidence that CC10 deficiency exacerbates pulmonary eosinophilic inflammation, which is associated with increased levels of inflammatory cytokines IL-4, IL-5, IL-9, and IL-13 as well as an eosinophilic chemokine, eotaxin. The differential responses of wild-type and CC10-deficient mice to Ag suggest an important role of CC10 and pulmonary resident cells, Clara cells, in the pathophysiology of pulmonary allergic inflammation. The finding that pulmonary eosinophilic inflammation of CC10-deficient mice is significantly altered suggests that CC10 plays a role in limiting alveolar influx of inflammatory cells, particularly for eosinophils and, to a lesser degree, the monocytic population, and cytokine responses during the course of Ag challenge.

In this study, no significant change in the level of IFN-γ was observed, suggesting an alternative pathway leading to the enhanced expression of Th2 cytokines seen in CC10-deficient mice. The increased levels of Th2 cytokines may reflect an increase in the number of infiltrating T cells or a sustained expression of Th2 cytokines from activated T cells and mast cells, two major sources of Th2 cytokines (3, 20). Although there is a trend for increase in the number of lymphocytes in the BALF was seen (Fig. 1D), suggesting a modulating effect of CC10 on the expression of Th2 cytokines, particularly for IL-13. Significant up-regulation of eotaxin gene expression was seen in CC10-deficient mice 48 h after challenge, which may further facilitate and sustain eosinophil influx. Although eotaxin gene expression occurs early after Ag challenge (21), no significant difference can be seen in the level of eotaxin in wild-type mice 48 h after Ag challenge, and after 48 h the level may have returned to the baseline.

Recently, gene-targeted mouse models of CC10 have been described (22, 23). Monocytic and neutrophilic infiltration was more extensive in the lung parenchyma of CC10-deficient mice after adenoviral infection, which was associated with up-regulation of IL-6 and TNF-α (22). In a hyperoxic lung injury model, survival of CC10-deficient mice was reduced compared with control mice (23). Expression of the proinflammatory cytokines IL-3, IL-6, and IL-1 was increased in the lungs of CC10-deficient mice. A reduced level of CC10 in the BALF in humans has been shown to be associated with asthma (24). Furthermore, although to a lesser degree, heterozygous CC10-deficient mice also showed increased levels of inflammatory parameters compared with the wild-type mice (data not shown). It has been suggested that secretory granules in Clara cells of CC10-deficient mice (strain 129) are abnormal or absent (25). Although it is unclear at present whether the function of Clara cells is altered in this model, potentially altered Clara cell function may also contribute to the observed increase in lung inflammatory responses in CC10-deficient mice.

The results from various models of pulmonary inflammation suggest, therefore, that the secretory products of lung epithelial cells are important modulators of lung inflammation and that the protective effect of CC10 is not restricted to a particular type of inflammation. The precise molecular mechanisms by which CC10 limits lung inflammation in vivo remain to be determined. Further studies are needed to gain further understanding of the role of CC10 and Clara cells in protection against pulmonary inflammation.

Acknowledgments

We thank Dr. Anil Mukherjee for scientific input and advice during the course of this study and Jei Lin for excellent technical assistance.

References