Regulation of T_H2 responses by the pulmonary Clara cell secretory 10-kd protein

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Background: Pulmonary Clara cell secretory 10-kd protein (CC10) is a steroid-inducible and potentially anti-inflammatory cytokine, but its direct involvement in the regulation of T-cell responses remains unknown.

Objective: The role of CC10 in the regulation of T_H2 cytokine expression was investigated.

Methods: The levels of cytokine and GATA-3 expression were determined by ELISA and RT-PCR, respectively. Bronchoalveolar lavage fluid cell counts were also determined by using a standard protocol. CC10 expression *in vivo* was determined by immunocytochemistry and Western blotting.

Results: *In vitro*, a significant, dose-dependent suppressive effect of CC10 was found on T_H2 cytokine expression, but not IFN-γ, in splenocytes of antigen-sensitized mice. A similar suppressive effect was also noted in polarized $CD4^+$ T_H2 cells, but not in naive $CD4^+$ T cells. In contrast, CC10 was able to induce IFN-γ expression in naive $CD4^+$ T cells, but not in polarized T_H1 cells. Furthermore, the suppression of T_H2 cytokine expression was concomitant with reduction of a critical transcription factor, GATA-3. Of significance was the finding that although no significant change was found in the decay kinetics of T_H2 cytokine transcripts, a significant decrease in mRNA stability of GATA-3 was seen in CC10-treated cells. *In vivo*, reconstitution of the CC10 gene in CC10-deficient mice resulted in significantly lower levels of T_H2 cytokines, concomitant with a decrease in GATA-3 expression, after challenge with Ag compared with those seen in mock-transduced mice, which are associated with reduced levels of pulmonary eosinophilia.

Conclusion: These results demonstrate, that CC10 plays a direct role in the regulation of T-cell–mediated inflammatory responses. (J Allergy Clin Immunol 2004;114:664-70.)

Key words: *T_H2, cytokine, Clara cell, CC10, asthma*

Abbreviations used
BALF: Bronchoalveolar lavage fluid
CC10: Pulmonary Clara cell secretory 10-kd protein
OVA: Ovalbumin
rCC10: Recombinant pulmonary Clara cell secretory 10-kd protein

Pulmonary Clara cell secretory 10-kd protein (CC10) is a 10-kd protein produced by nonciliated bronchiolar Clara cells and is one of the most abundant soluble proteins in fluids lining the airways.[1] However, the pathophysiologic function of CC10 has not been elucidated. It has been suggested that CC10 is able to antagonize the activity of secretory phospholipase A2.[2] *In vitro* studies[3] have suggested that CC10 may modulate the activity of IFN-γ and TNF-α. Moreover, increased inflammatory responses are prominent features in CC10-deficient mice after lung injury and infection.[4-6] Recently, we and others also found that compared with responses seen in wild-type mice, CC10-deficient mice develop an intensive pulmonary inflammatory response after sensitization and challenge with Ag.[7-9] It is interesting to note that a reduced level of CC10 in human beings has been shown to be associated with asthma.[10,11] Collectively, these studies suggest a role for CC10 as an important endogenous regulator that modulates pulmonary inflammatory responses.

Despite significant progress in characterizing the pathophysiologic role of CC10, the regulatory mechanism remains ill-defined. Also, in the CC10 gene–deficient murine model, a critical question remains whether exaggerated allergic responses seen in CC10-deficient mice are a primary or a secondary effect. To gain further understanding of the role of CC10, we investigated the effect of CC10 on the regulation of T_H2 cytokines and demonstrated for the first time a direct functional link between CC10 and T_H2 cytokine expression *in vitro* and *in vivo*, providing direct evidence supporting the role of CC10 in the regulation of Ag-induced pulmonary allergic inflammation.

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METHODS

Cell culture, *in vitro* T-cell polarization, and cytokine analysis

Spleen cells were isolated from 8-week-old Balb/c mice after 2 weekly intraperitoneal sensitizations with 10 μg ovalbumin (OVA; grade V; Sigma, St Louis, Mo) emulsified in 2 mg alum (Pierce, Rockford, Ill). To obtain $CD4^+$ T cells, splenocytes were isolated from naive Balb/c mice 8 to 12 weeks old to >97% purity with positive selection by using anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, Calif) according to the manufacturer's instructions. For *in vitro* T_H1 and T_H2 polarization,[12] $CD4^+$ T cells were stimulated with 1 μg/mL anti-CD3 and 1 μg/mL anti-CD28 mAbs in the presence of 5 ng/mL IL-12 and 10 μg/mL anti–IL-4 antibody for T_H1 differentiation, or in the presence of 50 ng/mL IL-4, 1 μg/mL anti–IL-12 antibody, and 5 μg/mL anti–IFN-γ antibody for T_H2 differentiation. IL-2, 5 ng/mL, was added 1 day after initial stimulation, and 7 days later, cells were harvested for analysis. Cells, 5×10^5, from each culture condition were stimulated with 1 μg/mL anti-CD3 and 1 μg/mL anti-CD28 mAbs. Splenocytes (4×10^6 cells per condition) from Ag-sensitized mice were stimulated with medium alone or with 25 μg/mL OVA. Before stimulation, the cells were pretreated with or without varying doses of recombinant CC10 (rCC10; 0.3-300 ng/mL or as appropriate) for 2 hours. Endotoxin levels were undetectable in the rCC10 preparation using Kinetic-QCL Chromogenic LAL (Bio Whittaker, Walkersville, Md).

The relative levels of cytokine gene expression for IL-4, IL-5, IL-13, and IFN-γ were determined by RT-PCR as previously described.[8] The levels of IFN-γ, IL-4, IL-5, and IL-13 were determined in cell culture supernatants by the use of ELISA kits purchased from eBioscience (San Diego, Calif), BD PharMingen (San Diego, Calif), and R&D Systems (Minneapolis, Minn).

Cell proliferation was assessed by measuring [^{3}H]-thymidine incorporation. Splenocytes (4×10^5 per well) were stimulated as described, and 3 days later, 1 μCi/well [^{3}H]-thymidine was added to the cultures, followed by the counting of radioactivity 18 hours later. The results are expressed as counts per minute.

mRNA stability assay and Western blotting analysis

Polarized $CD4^+$ T_H2 cells were pretreated with increasing doses of rCC10 for 2 hours and stimulated with anti-CD3 (1 μg/mL)/anti-CD28 (1 μg/mL) mAbs for 4 hours, followed by the addition of 5 μg/mL actinomycin D (Sigma). Total RNAs were isolated immediately ($t = 0$) or at 1, 2, 4, 6, and 12 hours after the addition of actinomycin D. Total RNAs were isolated from the cells by using the RNeasy Mini Kit (Qiagen, Valencia, Calif) following the manufacturer's protocol. RT-PCRs with subsaturating cycles (typically 25-30 cycles) for cytokines were performed as described.[8,13] The decay of T_H2 cytokine transcripts for IL-4, IL-5, IL-13, and GATA-3 was assessed by calculating the ratio of densitometric readings of the band intensity for cytokines (or GATA-3) and glyceraldehyde 3-phosphate dehydrogenase from the same cDNA samples at each time point, and a decay curve was generated by plotting the ratios of normalized intensities for each cytokine relative to the respective samples at $t = 0$ as a function of time after the addition of actinomycin D. PCR products were detected by ethidium bromide staining and quantified by video densitometry by using a gel documentation system with a computer containing Image 1.61 software (National Institutes of Health Public Software). Whole cell lysates and immunoblots were prepared, probed with anti–GATA-3 mAb (Santa Cruz Biotechnology, Santa Cruz, Calif), and developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ). After stripping, the blots were probed with antibody to β-actin (Santa Cruz Biotechnology) to demonstrate equivalent protein loading.

Animals, OVA sensitization and challenge

Homozygous CC10-deficient mice (–/–) on C57BL/6 background were obtained from an intercross of heterozygous CC10-deficient mice.[7] All animal procedures were conducted following an animal protocol approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine. Germline transmission of the mutant CC10 allele was identified by PCR as described.[8] Mice were sensitized by intraperitoneal injection of 10 μg OVA (grade V; Sigma) emulsified in 2 mg alum (Pierce, Rockford, Ill) on days 0 and 7. Mice were anesthetized and challenged intratracheally with 5 μg OVA in PBS 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.[8,13] Bronchoalveolar lavage fluid (BALF) and lung tissue specimens were collected 48 hours after challenge, at which time the peak inflammatory response was observed in this model.[8,13] Total BALF cells and differential cell counts were determined as previously described,[8,13] and the levels of BALF IL-4 and IFN-γ were determined by ELISA according to the manufacturer's instructions (BD PharMingen). For analysis of IL-13 level, a commercial kit (R&D Systems) was used. For analysis of GATA-3 gene expression, total RNAs were isolated from the lung homogenates of CC10 gene-transduced or mock-transduced, Ag-challenged mice by using the RNeasy Mini Kit (Qiagen). RT-PCRs of pooled RNAs from the respective groups of mice (n = 5 mice/group) with subsaturating cycles (for GATA-3, 30 cycles; for G3PDH, 23 cycles) were performed as described. The PCR products were detected by ethidium bromide staining and quantified, and the relative level of GATA-3 expression was assessed as described.

Gene construct and transfer

The CC10 coding region gene, including a 291-bp sequence, was amplified from homogenized mouse lung by PCR. The sequence of PCR primer for CC10 used in this study was as follows: sense, 5′-ATGAAGATCGCCATCACAATC-3′; antisense, 5′-TCAGAATC-TTAAATCTTGCT-3′. The PCR products were cloned in a TA vector (Invitrogen, San Diego, Calif), and the sequence of the CC10 coding region was confirmed by sequencing and inserted into the polylinker site of an expression vector, pcDNA (Invitrogen). The resulting plasmids (pCC10) were propagated in *Escherichia coli*, purified through cesium chloride gradient, and confirmed to be free of endotoxin. For mucosal gene transfer, 3 days before the OVA challenge, groups of mice received intratracheal injection of 50 μL pcDNA + lipofectamine (mock; 10 μg plasmid/25 μL PBS + 25 μL lipofectamine) or pCC10 + lipofectamine (pCC10; 10 μg plasmid/25 μL PBS + 25 μL of lipofectamine).

Detection of the transduced genes

To analyze the expression of transduced genes, lungs were collected at 48 hours after gene transfer and subjected to immunocytochemistry and Western blotting analysis. Frozen sections (10 μm) of lung samples were made on a TISSUE-TEK II Microtome/Cryostat (Miles, Elkhart, Ind), and immunostaining was performed as previously described.[8] For Western blot analysis, lung homogenates from various groups of mice were run on SDS-PAGE under nonreducing conditions and probed with goat antirabbit CC10 antibody (1:1000), followed by incubation with donkey antigoat IgG conjugated with horseradish peroxidase (1:20,000). The immunoreactive proteins were identified by using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

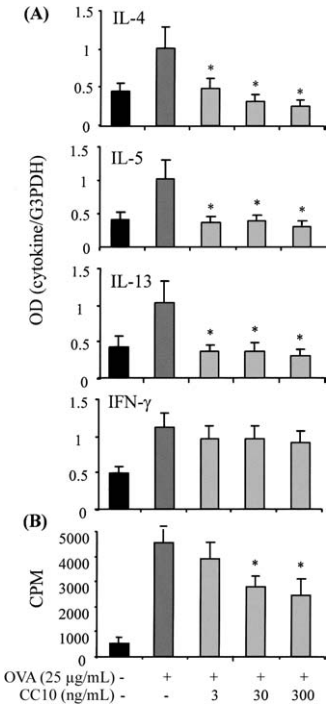

FIG 1. Regulation of cytokine expression and T-cell proliferative response in CC10-treated splenocytes. **A,** RT-PCR analysis of cytokine gene expression. **B,** Proliferation assay. *P < .05 was considered significant versus activated but untreated cells. Pooled spleen cells were used from 3 Ag-sensitized mice in 3 independent experiments. CPM, counts per minute.

Statistics

Differences between groups were assessed by 1-way ANOVA followed by Tukey-Kramer honest significant difference test. P values below .05 were considered statistically significant.

RESULTS

CC10 gene deficiency exacerbates pulmonary inflammation,[8] which is associated with increased levels of T_H2 cytokines, whereas no significant change in the number of infiltrating T cells is seen, suggesting a modulating effect of CC10 on the activation and/or cytokine expression of T cells. To determine the effect of CC10 on Ag-induced T-cell responses *in vitro*, spleen cells from OVA-sensitized mice were treated with varying doses of CC10 for 2 hours, followed by the stimulation of the cells with Ag. The results showed that a significant reduction of T_H2 cytokine gene expression for IL-4, IL-5, and IL-13, but not IFN-γ, was found (Fig 1, *A*). Further, when Ag-induced T-cell proliferative response was examined, a small, but statistically significant, reduction was seen in the presence of 30 ng/mL or more of rCC10 (Fig 1, *B*). It is noted, however, that at a lower dose (3 ng/mL) of CC10, no significant decrease in the proliferative response was observed, whereas in contrast, a significantly reduced level of cytokine gene expression was found, suggesting a differential effect of CC10 on T-cell proliferation and cytokine expression.

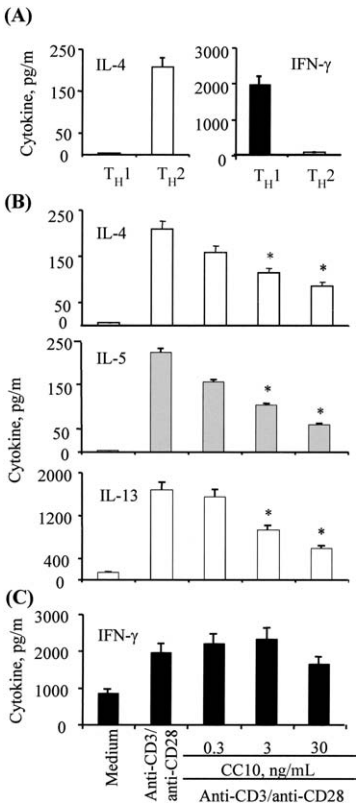

FIG 2. Regulation of T-cell cytokine expression by rCC10. **A,** Dichotomized T_H2 (IL-4) and T_H1 (IFN-γ) cytokine expression in polarized T cells. **B,** The levels of T_H2 cytokine expression in polarized T_H2. **C,** Level of IFN-γ in polarized T_H1 cells. Supernatants were collected 24 hours after stimulation. *P < .05 was considered significant versus activated but untreated cells from 5 independent experiments.

To investigate a possible direct effect of CC10 on T-cell responses and to avoid a potential influence of Ag-presenting cells, mouse polarized T cells derived from naive T cells under polarizing conditions were examined, in which significant induction of dichotomized T_H2 (IL-4) and T_H1 (IFN-γ) cytokines is seen (Fig 2, *A*). Results showed that although no expression of T_H2 cytokines was seen in resting cells, rCC10 at 3 ng/mL and higher inhibits the expression of T_H2 cytokine–secreted proteins (Fig 2, *B*) and transcripts (data not shown) for IL-4, IL-5, and IL-13 in activated cells. In contrast with those seen in Ag (OVA)–stimulated splenocytes, no consistent inhibitory effect of CC10 was seen on anti-CD3/anti-CD28–induced proliferative responses of polarized T_H2 cells in more than 6 independent experiments, suggesting that the inhibitory effect of CC10 on T-cell proliferation may depend on the stimuli used and the cell context.

A control recombinant protein (rIL-17F) purified following the same purification procedure and rCC10 alone in the culture showed no significant modulating activity, and the viability of the cells was more than 95% in all culture conditions. In addition, significant reduction of T_H2 cytokine production is also seen when anti-CD3/anti-CD28 mAbs and rCC10 were added to the culture at the

same time (data not shown), although the potency was less than that seen in the 2-hour CC10 pretreatment protocol. In contrast, the expression of IFN-γ in activated, polarized T_H1 cells was not inhibited by the same doses of rCC10 (Fig 2, *C*). Of interest, when naive $CD4^+$ T cells were analyzed, no significant change in the levels of T_H2 cytokines was noted in rCC10-treated cells (Fig 3). Moreover, CC10 was not able to affect T_H2 polarization, because restimulation of T cells after a 10-day T_H2 polarization condition in the presence of rCC10 (30 ng/mL) resulted in a similar level of IL-4 (153 ± 3.6 [SE] vs 203.3 ± 50 [SE] pg/mL for samples without rCC10 treatment). In sharp contrast with its influence on T_H2 cells, rCC10 showed a significant synergistic effect on the expression of IFN-γ in activated, nonpolarized $CD4^+$ T cells (Fig 3).

One of the mechanisms that may account for the differences in T_H2 cytokine gene expression is altered mRNA stability. It is well established that mRNA stability plays a central role in the regulation of gene expression.[14] To examine whether rCC10 regulates T_H2 cytokine expression posttranscriptionally, analysis of mRNA stability was performed by using a transcription inhibitor, actinomycin D. The results showed a similar decay kinetic of cytokine transcripts for IL-4, IL-5, and IL-13 (data not shown) relative to the culture without the addition of actinomycin D ($t = 0$), suggesting that modulation of T_H2 cytokine gene expression is at the level of transcription.

Data from several published studies have indicated GATA-3–dependent, coordinated regulation of T_H2 cytokines (IL-4, IL-5, and IL-13).[15] To determine whether the suppression of T_H2 cytokines involves the suppression of GATA-3 by CC10, polarized T_H2 cells were pretreated with increasing doses of rCC10 and stimulated with anti-CD3/anti-CD28 mAbs. The results showed that a significant decrease, in a dose-dependent manner, in the level of GATA-3 expression was found after stimulation in cells treated with rCC10 (Fig 4, *A*). Moreover, Western blotting analysis of cell lysates showed a reduced level of GATA-3 proteins in CC10-treated T cells 3 hours after stimulation (Fig 4, *B*). Of significance is the finding that a significantly faster decay of GATA-3 transcripts was seen in rCC10-treated cells compared with untreated cells (Fig 4, *C*). A similar suppressive effect of CC10 on GATA-3 gene expression was also noted in Ag-stimulated spleen cells from Ag-sensitized mice (Fig 4, *D*). These data suggest that the suppressive effect of rCC10 on T_H2 cytokine expression is mediated, at least in part, by a CC10-induced decrease in GATA-3 mRNA stability.

To investigate whether CC10 has a direct influence, *in vivo*, on Ag-induced T_H2 cytokine generation and pulmonary allergic response, mucosal gene transfer experiments using CC10-deficient mice as a model were performed. Results showed first that 3 days after mucosal gene transfer, significant expression of CC10 in the epithelium of pCC10-transduced mice was seen, and no detectable staining was found in mock-transduced mice (Fig 5, *A*). The expression of CC10 in transduced CC10-deficient mice was also examined by Western blotting analysis of lung homogenates. Results showed that the expression of CC10 is clearly evident in mice receiving pCC10, whereas, as expected, no expression of CC10 in the lung homogenates of mock-transduced mice was evident (Fig 5, *B*).

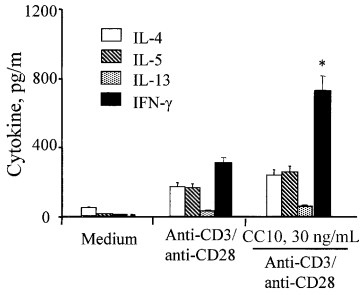

FIG 3. Effect of rCC10 on cytokine expression in nonpolarized, naive $CD4^+$ T cells. *$P < .05$ was considered significant versus activated but untreated cells.

Of significance is the finding that mice receiving 10 μg pCC10 demonstrated significantly reduced levels of IL-4, IL-13, and IL-5 (Fig 5 *C, D,*, and *E,* respectively), but not BALF IFN-γ (Fig 5, *F*). The reduction of T_H2 cytokines was associated with a significantly decreased level of GATA-3 gene expression (Fig 5, *G*). Moreover, the percentage (Fig 6, *A*) and total number (Fig 6, *B*) of eosinophils in the BALF were significantly reduced in pCC10-transduced mice compared with mock-transduced mice. In contrast, the number of BALF lymphocytes (Fig 6, *B*) showed no significant difference between these 2 groups of mice.

DISCUSSION

In this report, we provide evidence that rCC10 is able selectively to inhibit the expression of T_H2 cytokines and a critical T_H2 transcriptional factor, GATA-3. Also, the suppression of GATA-3 expression is mediated, at least in part, by CC10-mediated alteration of mRNA stability. In addition, the results of *in vivo* studies demonstrated that mucosal CC10 gene transfer was able to inhibit pulmonary T_H2 cytokine production and eosinophilia seen in CC10-deficient mice, providing evidence for a functional role of CC10 in limiting pulmonary T_H2-associated allergic responses. It is of interest to note that intratracheal administration of recombinant IFN-γ induces the expression of CC10,[16] and the level of CC10 can be induced by IL-13 *in vitro*,[17] suggesting an interaction between CC10 and the inflammatory cytokine network.

Gene-deficient models, together with limited *in vitro* studies, have suggested anti-inflammatory and immunomodulatory activities of CC10, but the molecular mechanisms by which CC10 limits pulmonary inflammation remain to be defined, and the functional link between CC10 and the immune system needs to be established. In the case of allergic asthma, elevated levels of T_H2 cytokines typify the allergic inflammation,[18] which may

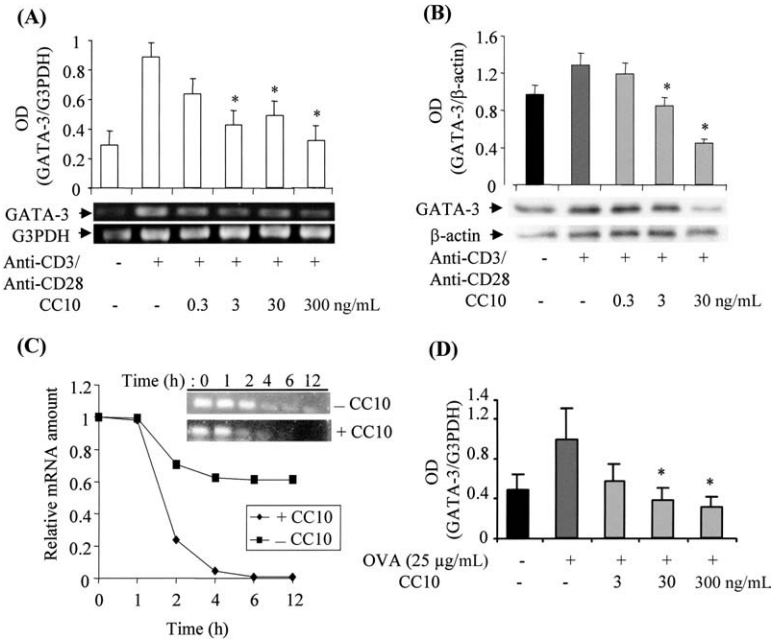

FIG 4. Regulation of GATA-3 expression in CC10-treated cells. **A,** RT-PCR analysis of GATA-3 gene expression in polarized T_H2 cells. **B,** Western blotting analysis of GATA-3 expression in polarized T_H2 cells. Cell lysates were isolated 3 hours after stimulation from each condition as indicated. **C,** Decay kinetics of GATA-3 transcripts. *$P < .05$ was considered significant versus activated but untreated cells from 3 independent experiments. **D,** RT-PCR analysis of GATA-3 gene expression in splenocytes of Ag-sensitized mice as described in Fig 1.

involve a combination of inflammatory cell-specific and tissue-specific regulatory processes. However, the molecular mechanisms governing the mucosal T_H2 responses are still not understood. Recent studies have established that GATA-3 is the key factor for T_H2 development.[19-21] GATA-3 has been shown to regulate IL-5 and IL-13 expression directly and play a more global role in regulating T_H2 cytokines.[22,23] Ectopic expression of GATA-3 induced T_H2 differentiation under conditions that otherwise polarized toward T_H1 development.[20,21] The results of the current study suggest a direct role of CC10 in the regulation of T-cell responses, leading to a strong possibility that CC10 is able to limit the extent of the effector function of T_H2 cells via the suppression of GATA-3–dependent T_H2 cytokine generation. The ability of CC10 to inhibit T_H2 cytokines through inhibition of GATA-3 expression may provide a general mechanism to redirect or switch local T-cell responses. Several studies in mice have shown that newly differentiated T_H2 cells still have a flexible phenotype, but once polarization is completed by repeated stimulation under polarizing conditions, this reversibility is lost.[24,25] The results of our study provide the first evidence that rCC10 is able to suppress T_H2 cytokine expression in polarized T_H2 cells. This is reminiscent of the activities of IL-12, wherein restimulation of polarized T_H2 cells in the presence of IL-12 induced a shift toward T_H0/T_H1 phenotypes, accompanied by suppression of GATA-3 expression.[26]

Although a direct effect of CC10 on the expression of T_H2 cytokines in polarized T_H2 cells is suggested, it is possible that this is a result of an induction of IFN-γ in CC10-treated cells. We think that this is an unlikely event, because the *in vitro* polarization protocol requires the presence of anti–IFN-γ and anti–IL-12 antibodies. However, we cannot rule out a potential effect of CC10 on the T_H1 development through the induction of IFN-γ in naive T cells. Further work is needed to test this hypothesis. Also, a small but significant reduction of Ag-induced T-cell proliferation was seen in CC10-treated splenocytes from Ag-sensitized mice. However, when polarized T_H2 cells were tested and stimulated with combined anti-CD3/anti-CD28 mAbs, no consistent inhibitory effect of CC10 was noted. This is a puzzling finding, and the underlying mechanism for this differential effect is unclear. It is noted that the levels of T_H2 cytokines and GATA-3 expression were similarly inhibited regardless of the cells and the stimuli used in the assay. It is known that the use of a combined anti-CD3/CD-28 stimulation is potent, bypassing the Ag presentation and maximizing the T-cell responses through the direct engagement of TcR/CD3 complex and a costimulatory pathway, CD28. It is known to induce maximal proliferative response, which is different from that seen in the Ag-induced T-cell response involving Ag-presenting cells and T-cell interaction. This difference may have been the cause of differential response of the cells to CC10 treatment, but a detailed study must address this question specifically.

The finding that CC10 influences the T_H2 cytokine expression only in polarized T_H2 cells, not naive T cells, further suggests its importance in controlling local T_H2 responses. The reason for differential regulation of naive

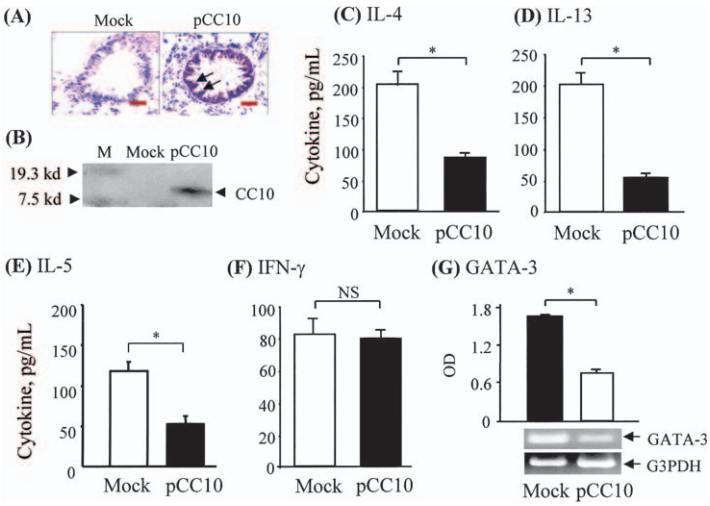

FIG 5. Transduced CC10 expression and inflammatory parameters. **A,** Immunocytochemical analysis of CC10 expression. The arrows indicate the expression of CC10 in the epithelium. Scale bar represents 50 μm. **B,** Western blotting analysis. The protein size markers are also shown. The levels of BALF **(C)** IL-4, **(D)** IL-13, **(E)** IL-5, and **(F)** IFN-γ. **G,** RT-PCR analysis of GATA-3 gene expression. N = 4 to 6 mice per group in 3 independent experiments.

versus polarized T cells by CC10 is currently unclear. Although the molecular nature of the putative receptor for CC10 remains elusive, the levels of CC10 receptor expression at different stages of T-cell differentiation and development may determine the extent of the inhibitory effect of CC10. The detailed mechanism regulating the GATA-3 mRNA stability is also unknown. Decreased stability of GATA-3 transcripts in CC10-treated cells may represent a primary effect of CC10 through the induction of destabilizing factors in the nuclei of activated T cells, or alternatively, it may be a secondary effect through, perhaps, the suppression of IL-4–dependent GATA-3 expression in polarized, CC10-treated cells. Further work is required to identify the molecular basis of CC10-mediated GATA-3 suppression. Also, a potential CC10-induced modification of known transcriptional factors involved in T_H2 cytokine and IFN-γ gene expression needs further investigation. The current study provides a significant experimental basis, and in the future, these data together with identification of the putative CC10 receptor and its signaling will ultimately link the signaling pathways and gene regulation. It is unclear, however, whether the reduction of GATA-3 expression *in vivo* is a result of a direct effect of CC10 on GATA-3 suppression *in vivo*, or a decrease in the number of infiltrating eosinophils, or the decrease in the number of infiltrating Ag (OVA)–specific T cells. Analysis of each individual Ag-specific T cell would be informative.

CC10 is constitutively expressed in the fluid lining the airways, and the level of CC10 in normal healthy subjects varies widely.[27] Reduced levels of CC10 have been found in patients with asthma and in smokers,[10,11,28] suggesting a multifactorial influence involving environmental factors or a genetic basis[29-31] of the level of CC10 in the airways. To investigate the direct effect of CC10 on inflammatory parameters, we elected to use a CC10-deficient mouse

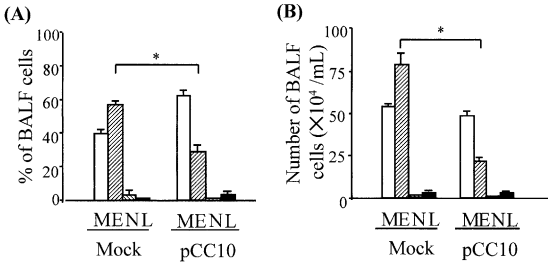

FIG 6. Significant decrease in **(A)** the percentage and **(B)** the total number of BALF eosinophils in pCC10-transduced, CC10-deficient mice. *M,* Macrophage; *E,* eosinophil; *L,* lymphocyte; *N,* neutrophil.

model and reconstitution experiments to avoid potential confounding variables, including variations in the level of endogenous CC10 before and after the acute inflammatory response in wild-type mice. Although the mucosal gene transfer approach does not allow a direct assessment of the quantitative relationship *in vivo* between the level of CC10 and various inflammatory parameters, a dose-dependent suppression of T_H2 cytokine expression by CC10 is seen *in vitro*. Therefore, there is a strong possibility that the level of CC10 determines the extent of inflammatory responses at the mucosal site. Therefore, it would be of interest to examine whether administration of rCC10 is able to modulate pulmonary allergic responses in Ag-sensitized and Ag-challenged wild-type mice. To accomplish this, a large quantity of rCC10 is required to assess dose-response relationship and its efficacy adequately, an additional limitation of the current study. Also, the kinetic variation in the level of endogenous CC10 after acute Ag challenge is uncertain at this time and remains a critical question to be addressed to understand fully the pathophysiology of CC10 in the lung. The conclusion

that can be drawn in the current study is that CC10 is able to modulate T_H2 responses *in vitro* and *in vivo* in this mouse model.

Interestingly, results from CC10-gene knockout studies of different models[4-6] have also shown alterations in proinflammatory cytokines. It is tempting to speculate that CC10 may play a general role in protecting the host from inflammatory insults, and its differential activity may depend on the nature and the type of inflammatory responses. This possibility may suggest, therefore, that a common regulatory pathway mediated through CC10 is involved in various inflammatory responses, and that different inflammatory parameters may be differentially regulated. Indeed, CC10 has been shown to be able to interact with fibronectin with high affinity,[2] although the pathophysiological significance of this potential interaction in the lung is unclear at present. Also, overexpression of CC10 in a nonsmall cell lung cancer cell line, A549, induces a marked reduction in invasiveness and decreased expression of the corresponding matrix metalloproteinases in the CC10-transfected cell lines.[32] Therefore, the novel role of CC10 in the regulation of T-cell responses documented in this report has a strong implication for our understanding of the role of CC10 in various disease states in which T-cell immunity is critically involved.

In conclusion, the evidence from both *in vivo* and *in vitro* studies suggests a direct role of CC10 in the regulation of Ag-induced pulmonary allergic inflammation and implicates a potential therapeutic utility. Also, these results provide a basis for defining the precise molecular mechanism by which CC10 interacts with the proinflammatory cytokine network and limits lung inflammation and potentially other diseases *in vivo*.

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REFERENCES

1. Singh G, Katyal SL. Clara cells and clara cell 10 KD protein (CC10). Am J Respir Cell Mol Biol 1997;17:141-3.
2. Mukherjee AB, Kundu GC, Mantile-Selvaggi G, Yuan CJ, Mandal AK, Chattopadhyay S, et al. Uteroglobin: a novel cytokine? Cell Mol Life Sci 1999;55:771-87.
3. Dierynck I, Bernard A, Roles H, De Ley M. Potent inhibition of both human interferon-r production and biologic activity by the Clara cell protein CC16. Am J Respir Cell Mol Biol 1995;12:205-10.
4. Hayashida S, Harrod KS, Whitsett JA. Regulation and function of CCSP during pulmonary Pseudomonas aeruginosa infection in vivo. Am J Physiol Lung Cell Mol Physiol 2000;279:452-9.
5. Harrod KS, Mounday AD, Stripp BR, Whitsett JA. Clara cell secretory protein decreases lung inflammation after acute virus infection. Am J Physiol 1998;275:924-30.
6. Johnston CJ, Mango GW, Finkelstein JN, Stripp BR. Altered pulmonary response to hyperoxia in Clara cell secretory protein deficient mice. Am J Respir Cell Mol Biol 1997;17:147-55.
7. Zhang Z, Kundu GC, Yuan CJ, Ward JM, Lee EJ, DeMayo F, et al. Severe fibronectin-deposit renal glomerular disease in mice lacking uteroglobin. Science 1997;276:1408-12.
8. Chen LC, Zhang Z, Myers AC, Huang SK. Cutting edge: altered pulmonary eosinophilic inflammation in mice deficient for Clara cell secretory 10-kDa protein. J Immunol 2001;167:3025-8.
9. Wang SZ, Rosenberger CL, Espindola TM, Barrett EG, Tesfaigzi Y, Bice DE, et al. CCSP modulates airway dysfunction and host responses in an OVA-challenged mouse model. Am J Physiol Lung Cell Mol Physiol 2001;281:1303-11.
10. Shijubo N, Itoh Y, Yamaguchi T, Imada A, Hirasawa M, Yamada T, et al. Clara cell protein-positive epithelial cells are reduced in small airways of asthmatics. Am J Respir Crit Care Med 1999;160:930-3.
11. Shijubo N, Itoh Y, Yamaguchi T, Sugaya F, Hirasawa M, Yamada T, et al. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. Lung 1999;177:45-52.
12. Noah SB, Martha MM, Timur OY, Linda SP, Gary WH. Altered IL-4 mRNA stability correlates with Th1 and Th2 bias and susceptibility to hypersensitivity pneumonitis in two Inbred strains of mice. J Immunol 2000;169:3700-9.
13. Li XM, Schofield BH, Wang QF, Kim KH, Huang SK. Induction of pulmonary allergic responses by antigen-specific Th2 cells. J Immunol 1998;160:1378-84.
14. Ross J. mRNA stability in mammalian cells. Microbiol Rev 1995;59: 423-50.
15. Murphy KM, Reiner SL. The lineage decisions of helper T cells. Nat Rev Immunol 2002;2:933-44.
16. Magdaleno SM, Wang G, Jackson KJ, Ray MK, Welty S, Costa RH, et al. Interferon-gamma regulation of Clara cell gene expression: in vivo and in vitro. Am J Physiol 1997;272:1142-51.
17. Kim S, Shim JJ, Burgel PR, Ueki IF, Dao-Pick T, Tam DC, et al. IL-13-induced Clara cell secretory protein expression in airway epithelium: role of EGFR signaling pathway. Am J Physiol Lung Cell Mol Physiol 2002;283:67-75.
18. National Institutes of Health. Global strategy for asthma management and prevention. NHLBI/WHO workshop report. Publication no. 1995; 95. Bethesda (MD): National Institutes of Health; 1995.
19. Ouyang W, Lohning M, Gao Z, Assenmacher M, Ranganath S, Radbruch A, et al. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity 2000; 12:27-37.
20. Zhang WP, Falvell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89:587-96.
21. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, et al. Inhibition of Th1 developmental mediated by GATA-3 through an IL-4 independent mechanism. Immunity 1998;9:745-55.
22. Zhang DH, Yang L, Ray A. Cutting edge: differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. J Immunol 1998;161:3817-21.
23. Zhang DH, Yang L, Cohn L, Parkyn L, Homer R, Ray P, et al. Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. Immunity 1999;11:473-82.
24. Murphy E. Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. J Exp Med 1996;183:901-13.
25. Perez VL, Lederer JA, Lichtman AH, Abbas AK. Stability of Th1 and Th2 populations. Int Immunol 1995;7:869-75.
26. Smits HH, van Rietschoten JG, Hilkens CM, Sayilir R, Stiekema F, Kapsenberg ML, et al. IL-12-induced reversal of human Th2 cells is accompanied by full restoration of IL-12 responsiveness and loss of GATA-3 expression. Eur J Immunol 2001;31:1055-65.
27. Shijubo N, Honda Y, Itoh Y, Yamaguchi T, Kuroki Y, Akino T, et al. BAL surfactant protein A and Clara cell 10-kDa protein levels in healthy subjects. Lung 1998;176:257-65.
28. Shijubo N, Itoh Y, Yamaguchi T, Shibuya Y, Morita Y, Hirasawa M, et al. Serum BAL Clara cell 10 kDa protein (CC10) levels and CC10-positive bronchiolar cells are decreased in smokers. Eur Respir J 1997;10:1108-14.
29. Zhang Z, Zimonjic DB, Popescu NC, Wang N, Gerhard DS, Stone EM, et al. Human uteroglobin gene: structure, subchromosomal localization, and polymorphism. DNA Cell Biol 1997;16:73-83.
30. Choi M, Zhang Z, Ten Kate LP, Collee JM, Gerritsen J, Mukherjee AB. Human uteroglobin gene polymorphisms and genetic susceptibility to asthma. Ann N Y Acad Sci 2000;923:303-6.
31. Laing IA, Hermans C, Bernard A, Burton PR, Goldblatt J, Le Souef PN. Association between plasma CC16 levels, the A38G polymorphism, and asthma. Am J Respir Crit Care Med 2000;161:124-7.
32. Zhang Z, Kundu GC, Panda D, Mandal AK, Mantile-Selvaggi G, Peri A, et al. Loss of transformed phenotype in cancer cells by overexpression of the uteroglobin gene. Proc Natl Acad Sci U S A 1999;96:3963-8.