

Clara cell 10-kDa protein inhibits T_H17 responses through modulating dendritic cells in the setting of allergic rhinitis

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Background: T_H17 responses have recently been implicated to play a role in allergic airway diseases, but their local expression in the setting of allergic rhinitis (AR) and their regulation in allergic airway diseases remain unclear.

Objective: We sought to investigate the regulatory role of Clara cell 10-kDa protein (CC10), an endogenous regulator of airway inflammation, on T_H17 responses in the setting of AR.

Methods: Wild-type and homozygous CC10-null mice were used to establish an ovalbumin (OVA)-induced AR model. Human recombinant CC10 was given during sensitization or challenge. T_H17 responses in human subjects and mice were examined by using flow cytometry, quantitative RT-PCR assay, immunohistochemistry, and ELISA. The direct effect of CC10 on T_H17 cells and CD11c⁺ dendritic cells (DCs) was studied by means of cell culture. Adoptive transfer was used to examine the influence of CC10-conditioned DCs on airway inflammation. The regulatory effect of CC10 on the expression of the CCL20 gene was tested by using the BEAS-2B cell line.

Results: Compared with those of control subjects, T_H17 responses were enhanced in the nasal mucosa of patients with AR. CC10-null mice with AR showed enhanced T_H17 responses, and CC10 treatment significantly decreased T_H17 responses. CC10 had no direct effect on *in vitro* T_H17 cell differentiation. CC10 could significantly decrease the expression of OX40 ligand, IL-23, and IL-6 but enhance CD86 and TGF- β expression in DCs. Importantly, CC10 was able to inhibit T_H17 cell polarization in the presence of OVA-pulsed DCs. CC10 pretreatment inhibited T_H17 responses elicited by adoptive transfer of OVA-pulsed DCs. Furthermore, CC10 decreased the

expression of CCL20 in BEAS-2B cells induced by inflammatory cytokines.

Conclusion: T_H17 responses are enhanced in patients with AR, and CC10 inhibits T_H17 responses through modulation of the function of DCs. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Allergic rhinitis, Clara cell 10-kDa protein, dendritic cell, inhibition, T_H17 response

Allergic rhinitis (AR) is a chronic inflammatory upper airway disease that is characterized by intense eosinophil infiltration, mucus hypersecretion, and airway remodeling, which are orchestrated mainly by antigen-specific T_H2 cells and their cytokines, such as IL-4, IL-5, and IL-13.^{1,2} Recent evidence suggests that IL-17A and T_H17 cells might be also involved in AR and other T_H2-mediated allergic airway diseases.³⁻⁵ Compared with wild-type mice, IL-17 receptor-deficient mice exhibit a reduced level of neutrophil and eosinophil recruitment into the airways, and IL-17A-null mice demonstrate reduced T_H2 responses to antigen sensitization.^{3,4} T_H17 cells cooperate to enhance T_H2 cell-mediated eosinophil recruitment into the airways.⁵ In human subjects increased IL-17 levels, T_H17 responses, or both have been found to be associated with chronic rhinosinusitis, allergic asthma, and AR.⁶⁻⁸

In different organs and under different pathophysiologic conditions, T_H17 responses can be regulated by distinct mechanisms. Several molecules, such as platelet-activating factor, retinoic acid, osteopontin, thymic stromal lymphopoietin, and prostaglandin E₂, have been indicated to modulate the differentiation, function, or chemotaxis of T_H17 cells in patients with autoimmune diseases, dermatitis, cancers, or asthma.⁸⁻¹⁰ Despite this progress, the regulation of T_H17 responses in patients with airway diseases, including AR, remains poorly understood. An improved understanding of the cellular and molecular mechanisms regulating T_H2 and T_H17 responses might lead to novel therapeutic strategies for the prevention, control, or both of allergic airway diseases. Clara cell 10-kDa protein (CC10) is an anti-inflammatory and immunomodulatory protein secreted by the epithelial lining of the lung and nose.¹¹⁻¹⁵ Our previous studies have shown that CC10 expression is downregulated in patients with allergic airway diseases, including AR and asthma, and that CC10 suppresses T_H2 responses in these patients.^{11,13,15-18} However, the regulatory mechanisms underlying the inhibitory effect of CC10 on allergic responses remain ill defined, and the effect of CC10 on T_H17 responses is not known.

In this study we examined the involvement of T_H17 responses in the setting of AR and explored whether CC10 could regulate the T_H17 responses in the context of AR. We found that T_H17 responses were exaggerated in patients with AR and that CC10 inhibited T_H17 responses through modulating the function of dendritic cells (DCs).

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Abbreviations used

AR:	Allergic rhinitis
CC10:	Clara cell 10-kDa protein
DC:	Dendritic cell
HDM:	House dust mite
FITC:	Fluorescein isothiocyanate
FPR2:	Formyl peptide receptor 2
NLF:	Nasal lavage fluid
OVA:	Ovalbumin
OX40L:	OX40 ligand
PE:	Phycoerythrin

METHODS**Animals**

Wild-type C57BL/6 mice and homozygous CC10-deficient mice on a C57BL/6 background were obtained, as previously described.¹³ DO11.10 mice on a BALB/c background and naive BALB/c mice were purchased from the Jackson Laboratory (Hancock, Me). All mice were used according to protocols approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

Patients

Twelve patients with AR and 12 control subjects, who participated in a previous study,¹⁷ were included. Further information is provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Inferior turbinate mucosal samples were taken during septoplasty. This study was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology. Informed consent was obtained from every subject.

AR mouse models, tissue preparation, and RNA extraction

AR mouse models induced by ovalbumin (OVA) and house dust mite (HDM) were established, and human recombinant CC10 (R&D Systems, Minneapolis, Minn) treatment during the sensitization and challenge phases was conducted as described elsewhere.^{17,19} OVA-specific T_H17 cells were generated from DO11.10 mice and adoptively transferred to OVA-sensitized mice, as previously described.⁵ Twenty-four hours after the last challenge, nasal lavage fluid (NLF) was collected and subjected to cell counting and flow cytometric analysis, as described previously.^{17,18,20} The sinonasal cavity structure was processed for histologic examination, as described elsewhere.^{20,21} In some experiments respiratory sinonasal mucosal tissue and splenocytes were harvested and subjected to real-time PCR assay, flow cytometry, or both.^{17,18} The kinetic expression of CC10 and IL-17A in mice after OVA challenge was analyzed. More information is provided in the [Methods](#) section in this article's Online Repository.

DC isolation

Splenic CD11c⁺ DCs were obtained by using anti-CD11c magnetic beads (Miltenyi Biotec, Auburn, Calif) and cultured according to the manufacturer's instructions. In some experiments DCs from naive wild-type C57BL/6 mice were treated with 30 ng/mL human recombinant CC10 or PBS for 2 hours with or without a 2-hour pretreatment of the cells with the formyl peptide receptor 2 (FPR2) antagonist WRW4 (50 μmol/L; Tocris Bioscience, Ellisville, Mo).^{11,17} The cells were then pulsed with 60 μg/mL OVA or PBS for 48 hours. The culture supernatants and cells were harvested and subjected to further assays. More information is provided in the [Methods](#) section in this article's Online Repository.

In vitro T-cell polarization and DC-T-cell culture

CD4⁺ T cells were isolated as mentioned elsewhere.¹² *In vitro* T_H17 cell differentiation was conducted, as previously reported, with modifications.⁵ CD4⁺ T cells were polarized under lineage-instructing cytokines with or without CC10 to explore the direct effect of CC10 on T_H17 polarization.^{11,17} For DC-T-cell culture, DCs from naive DO11.10 mice were pulsed with OVA peptide 323-329 (GenScript, Piscataway, NJ) or PBS in the presence or absence of CC10, and then the cells were cultured with autologous naive CD4⁺ T cells. In some experiments the FPR2 antagonist WRW4 (50 μmol/L) was added 2 hours before CC10 treatment. Splenocytes from OVA-sensitized mice were pretreated with or without CC10, followed by stimulation of the cells with OVA and subsequent analysis of their proliferative response by using the Cell Counting Kit-8 (Boster Bio-Technology Company, Wuhan, China).¹¹ More information is provided in the [Methods](#) section in this article's Online Repository.

Adoptive transfer of DCs into naive mice

OVA- or PBS-pulsed DCs with or without CC10 pretreatment were generated, as previously described, with minor modifications, and transferred to naive mice, followed by OVA challenge. For further information, see the [Methods](#) section in this article's Online Repository.²²

BEAS-2B cell culture

BEAS-2B cells (a human bronchial epithelial cell line) were cultured and stimulated, as previously described.¹⁸ Before stimulation, the cells were pretreated with or without CC10. Five hours after cytokine stimulation, cells were harvested and subjected to real-time RT-PCR. For further information, see the [Methods](#) section in this article's Online Repository.

Flow cytometric analysis

Flow cytometric analysis was conducted with an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ). DCs and CD4⁺ T cells, splenocytes, and inflammatory cells in NLF were stained with fluorescence-conjugated antibodies (see [Table E1](#) in this article's Online Repository at www.jacionline.org).^{23,24} More information is provided in the [Methods](#) section in this article's Online Repository.

Immunohistochemistry

Immunohistochemical staining was conducted, as previously described.^{17,20} Rabbit anti-human IL-17A and anti-mouse CCL20 (1:200; Beijing Biosynthesis Biotechnology, Beijing, China) and anti-mouse CC10 (1:200; Santa Cruz Biotechnology, Santa Cruz, Calif) antibodies were used as primary antibodies. Numbers of IL-17A⁺, CC10⁺, and CCL20⁺ cells were counted, as previously described.^{17,18}

Quantitative real-time PCR

cDNA was reverse transcribed, and quantitative PCR was performed with specific primer pairs (see [Table E2](#) in this article's Online Repository at www.jacionline.org), as described elsewhere.^{17,21} Relative gene expression was calculated by using the comparative cycle threshold method, as described previously.²⁵

ELISA

Murine cytokine levels in culture supernatants or NLFs were determined by using ELISA, according to the manufacturer's instructions. For further information, see the [Methods](#) section in this article's Online Repository.

Statistical analysis

All results were expressed as means ± SEMs. The Mann-Whitney *U* test was used for paired sets of data. The paired *t* test was used in cell-culture data analysis. The Spearman test was applied to determine correlations. The

P value for significance was set to .05. Data analysis was performed through the application of SPSS software for Windows (SPSS, Chicago, Ill).

RESULTS

Enhanced T_H17 responses and decreased CC10 expression in the setting of AR

Our previous study demonstrated typical T_H2-skewed eosinophilic inflammation in an OVA-induced AR model.¹⁷ In this study we found the percentage of T_H17 cells was increased in NLF from OVA-induced mice with AR in comparison with that seen in control mice (Fig 1, A-C). It was also noted that the IL-17A levels in NLF were increased, in a time-dependent manner, during the development of AR (Fig 1, C), which was accompanied by a time-dependent downregulation of CC10 expression in the nasal mucosa (Fig 1, A). Similarly, enhanced T_H17 responses were also found in an HDM-induced AR mouse model, which simulates human AR more precisely (see Fig E1 in this article's Online Repository at www.jacionline.org). To keep the consistency between *in vivo* and *in vitro* experiments, we used the OVA-induced AR mouse model for further studies.

In human subjects we previously reported the diminished expression of CC10 in nasal mucosa from patients with AR.¹⁷ In the present study the same group of subjects was studied, and immunohistochemical staining revealed increased infiltration of IL-17A⁺ cells in nasal biopsy specimens from patients with AR compared with control subjects (Fig 1, D and E). Flow cytometric analysis demonstrated that the percentage of T_H17 cells was significantly higher in tissue homogenates from patients with AR than in control subjects (Fig 1, F). Moreover, the levels of mRNA expression for both RORC and IL-17A were increased in patients with AR compared with those seen in control subjects (Fig 1, G and H). However, we did not find a correlation between IL-17A local expression levels and the symptom severity (see Fig E2 in this article's Online Repository at www.jacionline.org).

T_H17 cells exaggerate eosinophilic and neutrophilic inflammation in the setting of AR

The transfer of OVA-specific T_H17 cells to sensitized mice significantly enhanced not only neutrophil but also eosinophil recruitment into the nose after OVA challenge compared with that seen in mice with AR without T_H17 cell transfer (see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, although the mRNA expression of IL-4 was not enhanced, the mRNA expression of eotaxin-1 and eotaxin-2 was upregulated in the noses of T_H17 cell-transferred mice (see Fig E3).

CC10 inhibits T_H17 responses in the setting of AR

Our previous report has described an exaggerated T_H2-skewed eosinophilic inflammation with no significant change in the T_H1 response in the nasal mucosa of CC10-deficient mice with AR compared with that seen in wild-type mice with AR.¹⁷ In the present study we concentrated on the potential regulatory effect of CC10 on T_H17 responses in the setting of AR. First, we found that the percentage of T_H17 cells was significantly increased in NLF from CC10-deficient mice with AR compared with that seen in wild-type mice with AR (Fig 2, A, and see Fig E4, A, in this article's Online Repository at www.jacionline.org). We next examined the T_H1, T_H2, and T_H17 responses in the spleen after

allergen sensitization. It was found that in the splenocyte population there was no significant difference in the percentage of T_H1 cells between OVA- and PBS-sensitized mice or between wild-type and CC10-deficient mice (Fig 2, B, and see Fig E4, B). On the contrary, we found that the proportion of T_H17 and T_H2 cells in splenocytes was markedly increased in OVA-sensitized wild-type and CC10-deficient mice compared with that seen in PBS-sensitized control mice and that, significantly, such an increase was more prominent in CC10-deficient mice (Fig 2, C and D, and see Fig E4, C and D).

Human recombinant CC10 was administered to CC10-deficient mice during sensitization to further define the role of CC10 in the sensitization phase. We previously reported that the T_H2 responses and infiltration of eosinophils, lymphocytes, and total inflammatory cells in the nasal mucosa in OVA-challenged mice could be dramatically ameliorated by the administration of CC10 in the sensitization phase.¹⁷ Here we observed a decreased percentage of T_H17 cells in NLF from CC10-treated mice with AR compared with that of PBS-treated mice with AR (Fig 2, E, and see Fig E4, E).

Finally, we investigated whether CC10 has a therapeutic role in the challenge phase. For this purpose, wild-type mice were used. Our previous study has already demonstrated that intranasal CC10 administration before OVA challenge could significantly inhibit T_H2-skewed eosinophilic inflammation in the nasal mucosa.¹⁷ In this study we further revealed that CC10 administration before challenge could dramatically diminish T_H17 cell numbers in NLF from mice with AR compared with numbers in those treated with PBS control (Fig 2, F, and see Fig E4, F).

CC10 suppresses T_H17 responses through affecting DCs

To explore the mechanisms underlying the physiologic function of CC10, we first tested whether CC10 has a direct inhibitory effect on the differentiation of T_H17 cells *in vitro*. We found no inhibitory effect of CC10 on the differentiation of T_H17 cells (Fig 3, A, and see Fig E5 in this article's Online Repository at www.jacionline.org). In addition, we found no significant effect of CC10 on the antigen-induced T-cell proliferative response (Fig 3, B).

Next we examined whether CC10 could modulate T_H17 cell differentiation through DCs. We found that the mRNA expression of IL-23 and IL-6 was significantly upregulated whereas TGF- β mRNA expression was markedly downregulated in spleen CD11c⁺ DCs from CC10-null mice with AR compared with those from wild-type mice with AR (Fig 4, A). No difference in IL-12 expression was found (Fig 4, A). When OVA-pulsed CD11c⁺ DCs generated from naive mice were pretreated with CC10, we found that CC10 enhanced CD86 expression, whereas it decreased OX40 ligand (OX40L) expression and had no significant effect on CD80 expression (Fig 4, B, and see Fig E6, A, in this article's Online Repository at www.jacionline.org). Moreover, we found that TGF- β production was significantly upregulated whereas IL-6 and IL-23p19 production was markedly downregulated when antigen-pulsed DCs were pretreated with CC10 (Fig 4, C). However, the production of IL-12 was not affected by CC10 pretreatment (Fig 4, C).

DCs from naive DO11.10 mice were stimulated with OVA peptide 323-329 in the presence or absence of CC10 and then cocultured with autologous naive CD4⁺ T cells to examine

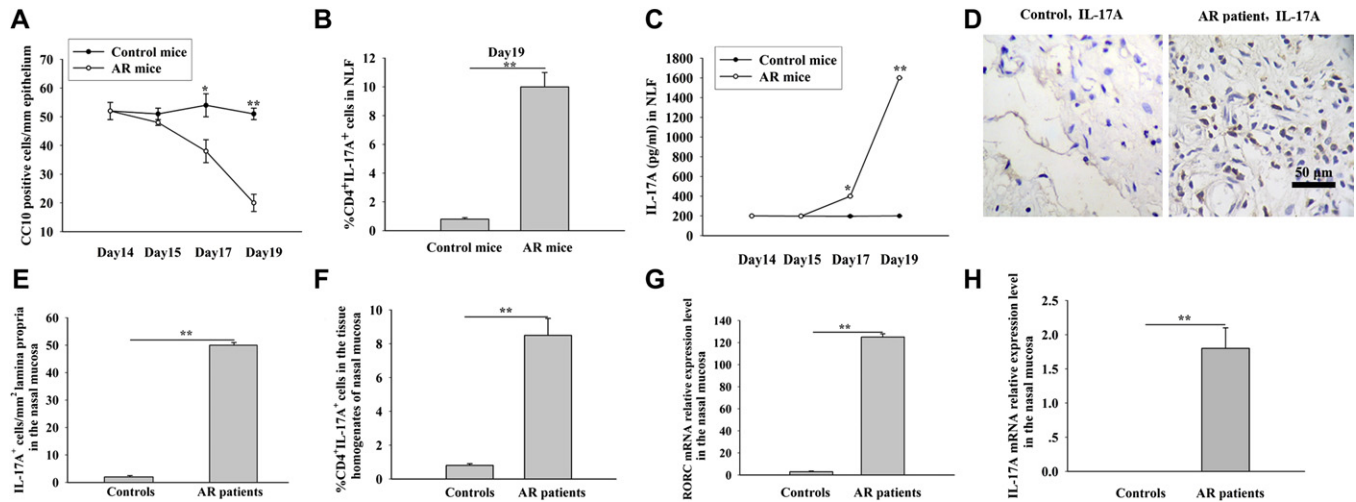


FIG 1. Enhanced T_H17 responses and decreased expression of CC10 in the setting of AR. **A–C**, CC10 expression in the nasal mucosa (Fig 1, A) and levels of T_H17 cells (Fig 1, B) and IL-17A (Fig 1, C) in NLF. * $P < .05$ and ** $P < .01$, OVA-induced mice with AR versus control animals ($n = 8$ –10 mice per group). **D**, Immunohistochemical staining of IL-17A in inferior turbinate tissues. **E–H**, Number of IL-17A⁺ cells (Fig 1, E), percentage of T_H17 cells (Fig 1, F), and levels of RORC (Fig 1, G) and IL-17A (Fig 1, H) mRNA expression in nasal mucosa. ** $P < .01$, patients with AR versus control subjects ($n = 12$ per group).

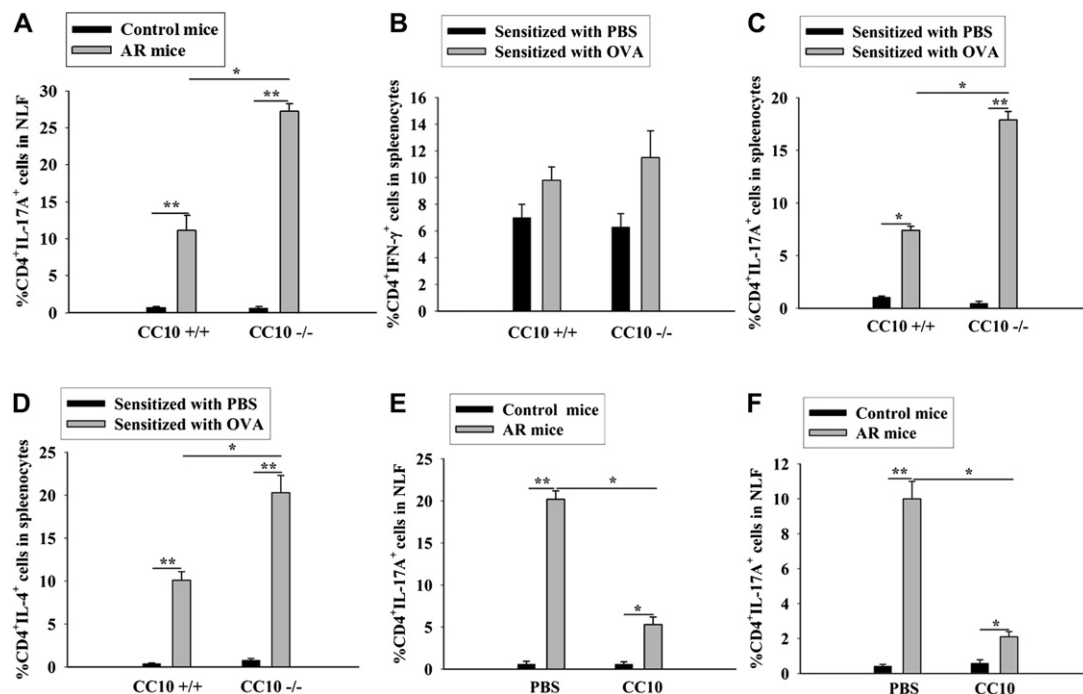


FIG 2. CC10 inhibits T_H17 responses in the setting of AR. **A**, T_H17 cell levels in NLF from mice. **B–D**, Splenic T_H1 (Fig 2, B), T_H17 (Fig 2, C), and T_H2 (Fig 2, D) cell levels in PBS- or OVA-sensitized mice. **E** and **F**, T_H17 cell levels in NLF from CC10-deficient mice treated with CC10 or PBS before sensitization (Fig 2, E) and wild-type mice treated with CC10 or PBS before challenge (Fig 2, F). * $P < .05$ and ** $P < .01$ ($n = 8$ –15 mice per group).

whether CC10 would influence antigen-pulsed DCs and the subsequent generation of T_H17 cells *in vitro*. As expected, we found that the percentage of T_H17 cells was increased in culture with antigen-pulsed DCs when compared with that seen in DCs without antigen stimulation, and a significant reduction in the level of T_H17 cell population was noted when CC10 was added in culture during antigen stimulation of DCs (Fig 4, D, and see Fig E6, B).

Similarly, we found that IL-4 levels were increased in the supernatants of antigen-pulsed DC–T-cell cocultures, and such upregulation was also significantly ameliorated when DCs were treated with CC10 (Fig 4, E). However, IFN-γ levels were not affected by antigen stimulation or CC10 treatment (Fig 4, F).

OVA-pulsed DCs with or without CC10 pretreatment were adoptively transferred into naive mice followed by nasal OVA

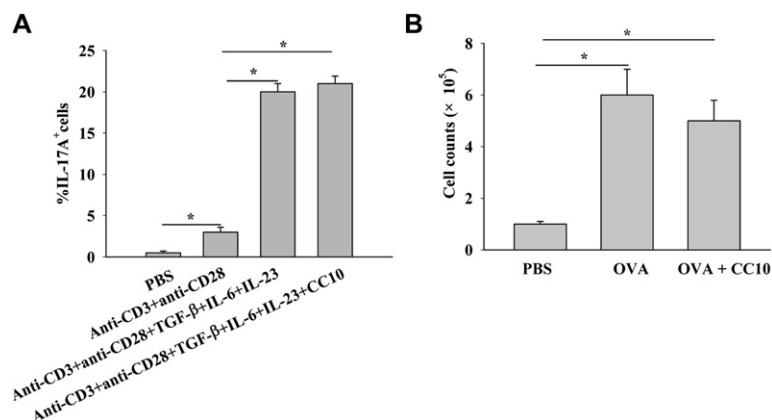


FIG 3. CC10 does not affect cytokine-induced T_H17 cell differentiation. **A**, Generation of T_H17 cells from cytokine-treated naive CD4⁺ T cells with or without CC10 treatment (n = 3-4). **B**, Proliferation assay of OVA-stimulated splenocytes with or without CC10 pretreatment. Pooled spleen cells from 3 OVA-sensitized mice were used, and 3 independent experiments were repeated. *P < .05.

challenge to further investigate whether CC10-modulated DCs can influence T_H17 responses in the context of allergic reactions *in vivo*. OVA-pulsed DC transfer could elicit marked T_H2 and T_H17 responses compared with PBS-pulsed DC transfer. Compared with the mice transferred with DCs without CC10 pretreatment, the mice transferred with CC10-pretreated DCs showed a decreased number of total inflammatory cells, eosinophils, and lymphocytes in NLF (Fig 4, G); a lower percentage of T_H17 cells in NLF (Fig 4, H, and see Fig E6, C); and reduced levels of IL-4 and comparable levels of IFN-γ in nasal mucosa (Fig 4, I and J).

Regulatory effect of CC10 on DC function is not mediated through FPR2

The putative receptors for CC10 remain elusive. Because CC10 exhibits its biological effect through interacting with FPR-like 1 in some human cell types,²⁶ we investigated whether CC10 influenced T_H17 responses through FPR2 (the counterpart of human FPR-like 1 in the mouse) on DCs. We found that FPR2 was expressed on CD11c⁺ DCs (see Fig E7, A, in this article's Online Repository at www.jacionline.org). Furthermore, we found that pretreatment with the FPR2 antagonist WRW4 did not influence the mRNA expression of TGF-β, IL-6, and IL-23p19 in DCs (see Fig E7, B) and T_H17 cell differentiation induced by antigen-pulsed DCs (see Fig E7, C and D). CC10 could dramatically inhibit T_H17-polarizing cytokine expression in DCs and DC-instructed T_H17 cell differentiation, and this effect was not altered by WRW4 pretreatment (see Fig E7, B-D). The efficiency of the FPR2 antagonist WRW4 was confirmed by a control experiment, and the results are shown in Fig E8 in this article's Online Repository at www.jacionline.org.

CC10 curbs the expansion of T_H17 responses at local sites

The expression of a T_H17 cell chemokine (CCL20) and an expansion factor, IL-23, in the nasal mucosa was explored. The CCL20 protein expression and IL-23 mRNA expression levels were increased in the nasal mucosa of mice with AR compared with those of control mice, and this increase was more prominent in CC10-deficient mice than in wild-type mice (Fig 5, A-D).

Immunohistochemical staining showed that CCL20 was mainly produced by epithelial cells in murine nasal mucosa (Fig 5, A). Similarly, we found the CCL20 and IL-23 mRNA expression was upregulated in nasal mucosa from patients with AR compared with that seen in control subjects (Fig 5, E and F). Furthermore, we found that CC10 could significantly inhibit the upregulation of CCL20 expression in the BEAS-2B cell line induced by IFN-γ, TNF-α, IL-4, IL-1β, and IL-13 (Fig 5, G).

DISCUSSION

A few previous studies have demonstrated increased IL-17 levels in peripheral blood in patients with AR and in NLF in a murine AR model.^{6,27} In the present study we have demonstrated enhanced T_H17 responses in nasal mucosa in the setting of AR not only in human subjects but also in animal models. Furthermore, an adoptive transfer experiment using T_H17 cells demonstrated that T_H17 responses could enhance not only neutrophilic but also eosinophilic inflammation in mice with AR, which might result from the local upregulation of eotaxin-1 and eotaxin-2 expression. These findings are consistent with those found in a mouse model with allergic lower airway inflammation and confirm an effector role of T_H17 responses in the setting of AR.⁵ In this study no correlation between local IL-17A expression and disease severity was found. It should be noted that the sample size was small in our study, and the concomitant nasal septal deviation in our patients with AR could be an additional factor affecting the severity of symptoms. Therefore a larger-scale study of a better defined patient population could more properly address the relationship between IL-17A expression and disease severity.

Despite the progress in our understanding of the pathophysiologic functions of T_H17 cells, the regulation of T_H17 responses, particularly in the context of specific diseases, has been relatively poorly understood. CC10 has been previously demonstrated to inhibit allergic inflammation through dampening T_H2 responses in the setting of AR.¹⁷ In the present study we examined the regulatory function of CC10 on T_H17 responses. We found that local IL-17A levels were increased whereas local CC10 expression was decreased in a time-dependent manner during the development of AR, suggesting an inverse correlation between the expression of

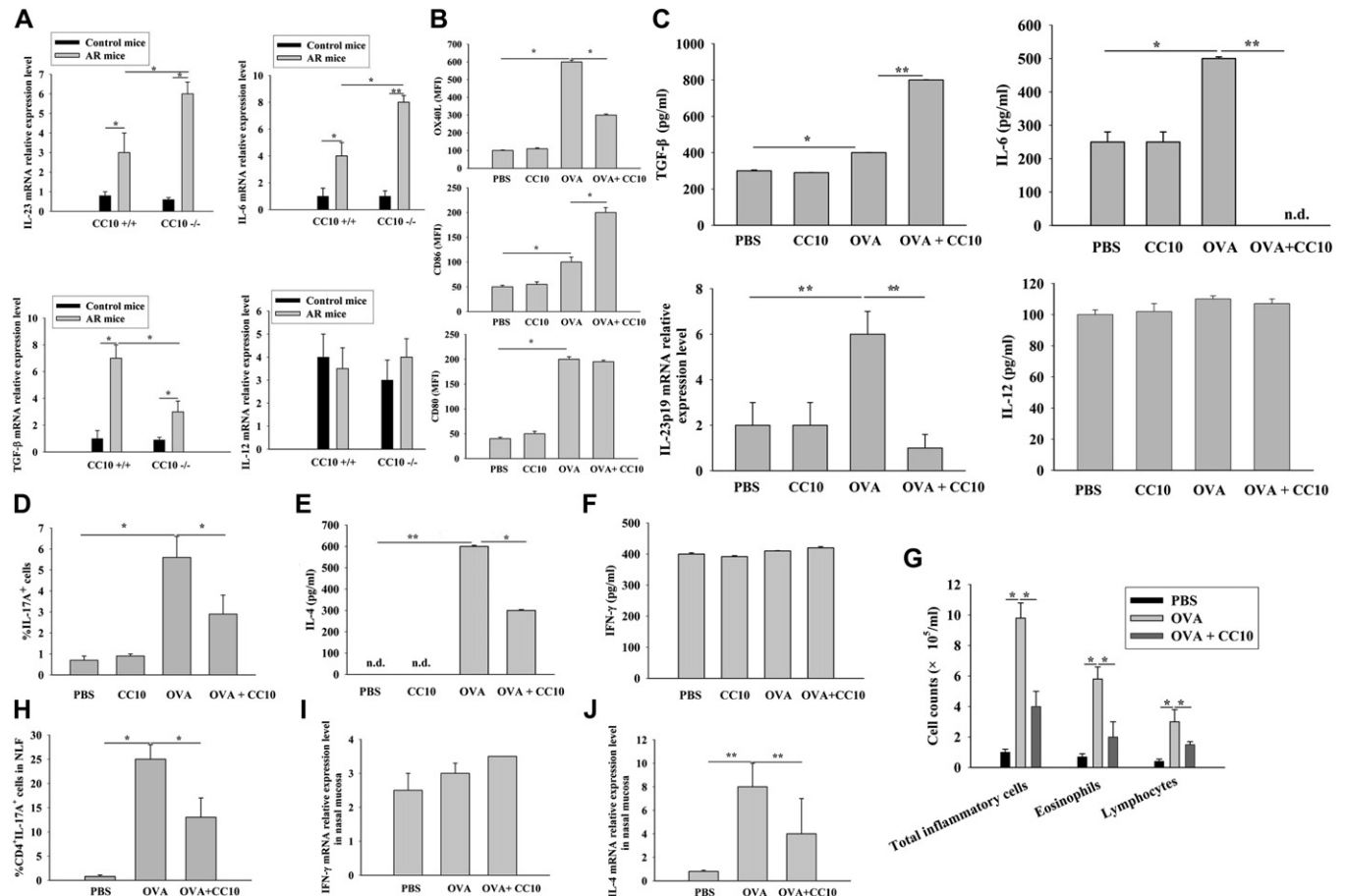


FIG 4. CC10 affects T_H17 responses through modulating DC function. **A**, Relative mRNA expression levels of cytokines in murine splenic CD11c⁺ DCs ($n = 8-15$ mice per group). **B** and **C**, Expression of surface markers on DCs (**B**) and protein levels of cytokines in culture supernatants and IL-23p19 mRNA expression in DCs (**C**). **D-F**, Generation of T_H17 cells (**D**) and IL-4 (**E**) and IFN- γ (**F**) levels in DC-T-cell cocultures ($n = 3-5$). **G-J**, Analysis of infiltrating cells (**G**) and T_H17 cells (**H**) in NLF and IFN- γ (**I**) and IL-4 (**J**) mRNA expression in the nasal mucosa of DC-transferred mice ($n = 8-12$ mice per group). * $P < .05$ and ** $P < .01$.

CC10 and T_H17 responses. Further studies with CC10-deficient mice and the use of recombinant CC10 treatment confirmed that in addition to its well-defined effect on T_H2 responses, CC10 could also suppress T_H17 responses at both the sensitization and challenge phases in the context of AR. Interestingly, the regulatory activity of CC10 on T_H17 responses was mediated through its ability to modulate DC function.

DCs are known to play a primary role in determining the nature of T-lymphocyte differentiation in the face of allergen exposure.²⁸ CD11c⁺ DCs, but not plasmacytoid DCs, have been indicated to perform an essential role in the development of AR.^{29,30} Also, we found that adoptive transfer of OVA-pulsed CD11c⁺ DCs strongly induced nasal eosinophilia and T_H2 and T_H17 responses, supporting the role of CD11c⁺ DCs in the setting of AR. We found that CC10 could significantly inhibit the expression of IL-6 and IL-23, the crucial T_H17 lineage-instructing cytokines,³¹ in antigen-pulsed CD11c⁺ DCs, which might contribute to the inhibitory effect of CC10 on T_H17 differentiation.

IL-23 is a heterodimeric protein consisting of p19 and p40 subunits that promotes survival and proliferation of T_H17 cells.³¹ CCR6 has been shown to be mainly expressed on

T_H17 cells, and CCR6-CCL20 interaction has been shown to be critical in mediating T_H17 cell migration into inflamed tissues.³¹ In the present study we found that IL-23 and CCL20 expression levels were increased locally in both human subjects and mice with AR and that CC10 could inhibit IL-23 and CCL20 expression in mice with AR and inflammatory cytokine-induced CCL20 expression in airway epithelial cells. These results suggest that CC10 can also affect T_H17 cell chemotaxis and local expansion.

Moreover, we found that CC10 was able to enhance the expression of CD86 on CD11c⁺ DCs, suggesting that CC10 can promote the maturation and antigen-presenting function of DCs. Despite exhibiting similar binding affinity to the CD28 ligand, CD80 and CD86 can exhibit different biochemical characteristics that can result in different T-cell functional outcomes.³² Therefore the influence of distinct effects of CC10 on CD86 and CD80 expression awaits further study. Furthermore, DCs are known to express OX40L, which interacts with OX40 on naive T cells and influences T_H2 lineage commitment.³³ Our results indicate that CC10 might suppress T_H2 response through downregulation of OX40L expression on CD11c⁺ DCs. It is also known that IL-12-producing DCs are capable of inducing T_H1

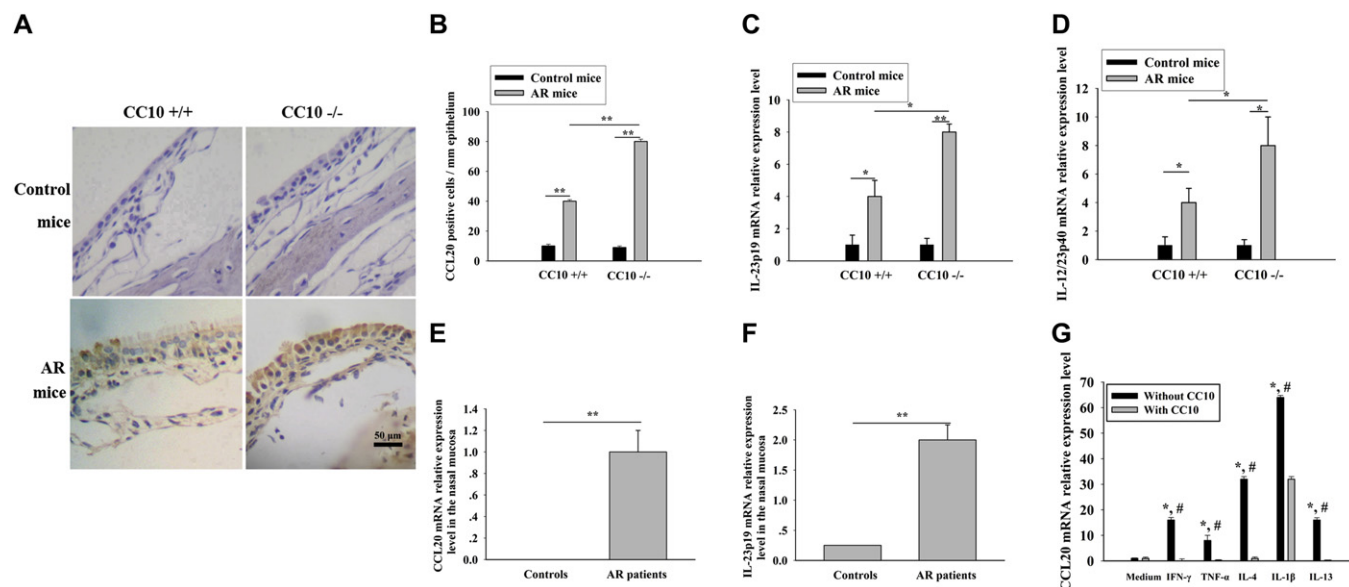


FIG 5. Increased local expression of IL-23 and CCL20 in the setting of AR. **A**, Immunohistochemical staining of CCL20 in murine nasal tissues. **B-D**, Number of CCL20⁺ cells (Fig 5, **B**) and relative mRNA expression of IL-23p19 (Fig 5, **C**) and IL-23p40 (Fig 5, **D**) in murine nasal mucosa. **P* < .05 and ***P* < .01 (*n* = 8-10 mice per group). **E** and **F**, Relative mRNA expression of CCL20 (Fig 5, **E**) and IL-23p19 (Fig 5, **F**) in the nasal mucosa of control subjects (*n* = 12) and patients with AR (*n* = 12). ***P* < .01. **G**, CC10 inhibits CCL20 mRNA expression in BEAS-2B cells. **P* < .05 compared with medium controls, #*P* < .05 compared with the corresponding condition with CC10 treatment (*n* = 3-5).

responses. In the present study we found that CC10 had no obvious effect on IL-12 expression in antigen-pulsed CD11c⁺ DCs, which is in line with the observation that CC10 had no significant influence on T_H1 polarization *in vitro* and *in vivo*. It is possible that CC10 might also affect the engraftment rate and trafficking pattern of DCs to the draining lymph nodes, where DCs present antigens to T lymphocytes and initiate cognate T-cell responses. However, this possibility awaits further investigation.

The putative receptors for CC10 remain elusive. A study indicated that in human subjects CC10 interacts with a low-affinity receptor for formyl peptide.²⁶ Therefore we investigated whether CC10 could influence DC function through FPR2, the mouse counterpart of the human receptor. We found that FPR2 was expressed on CD11c⁺ DCs, but FPR2 antagonist treatment had no influence on CC10's effect on T_H17-polarizing cytokine production in antigen-pulsed DCs and T_H17 cell differentiation instructed by antigen-pulsed DCs, indicating that CC10 does not affect DC function through FPR2. Therefore the intracellular mechanisms underlying the regulatory effect of CC10 on DC function need further investigation.

In conclusion, the evidence from both *in vivo* and *in vitro* studies suggests an inhibitory role of CC10 in T_H17 responses in the setting of AR. CC10 prevents T_H17 polarization through modulation of DC function in terms of costimulatory molecule and cytokine expression. CC10 also affects T_H17 cell chemotaxis and local expansion in the setting of AR. However, the biological signaling pathway, which underlies the function of CC10, needs further investigation. CC10 is relatively small, resistant to proteases, and stable to extremes of heat and pH and can be produced by recombinant methods. These characteristics make CC10 an excellent candidate for clinical development to treat allergic airway diseases.

Clinical implications: CC10 is able to ameliorate allergic airway inflammation in a mouse model of AR through modulating DC functions, resulting in the inhibition of T_H17 and T_H2 responses. CC10 might be an excellent candidate for drug development.

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METHODS

Animals

Wild-type C57BL/6 mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). Homozygous CC10-deficient mice on a C57BL/6 background were obtained from an intercross of heterozygous CC10-deficient mice (kindly provided by Dr A. B. Mukherjee, National Institutes of Health, Bethesda, Md), and germline transmission of the null allele was identified by using PCR, as previously described.^{E1} DO11.10 mice on a BALB/c background and naive BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Me).

Patients' information

Subjects who have participated in a previously published study were involved in the current study.^{E2} Twelve patients (7 male and 5 female patients; mean age, 35 ± 16 years; age range, 26–54 years) with HDM-induced persistent AR and nasal septal deviation and 12 control subjects (8 male and 4 female subjects; mean age, 36 ± 11 years; age range, 28–46 years) with nasal septal deviation alone were included. All patients with AR had typical symptoms of perennial nasal allergy and a positive skin test result to HDM. In this study subjects were excluded if they had received any oral steroid within 3 months before the surgery. Topical steroids and antihistamines were withheld for a minimum of 1 month before the study. None had received antileukotrienes or immunotherapy.

Assessment of symptoms

A symptom questionnaire based on a visual analog scale (VAS) score of 0 to 10 according to severity was used. This focused on 3 major symptoms: rhinorrhea, sneezing, and nasal blockage. A total VAS score was calculated based on the sum of these 3 VAS symptom domains.^{E3}

OVA-induced AR mouse model

An AR mouse model was established, as previously described.^{E2} C57BL/6 mice (6–8 weeks old) were sensitized by means of subcutaneous injection of 0.4 mL of solution containing 10 μ g of OVA (grade V; Sigma, St Louis, Mo) emulsified in 1.6 mg of alum (Pierce, Rockford, Ill) on days 0 and 7. The sensitized mice were challenged nasally by means of administration of 10 μ g of OVA in 40 μ L of PBS (20 μ L per nostril) from day 14 to day 18. Control mice were sensitized with PBS but challenged with OVA. Mice were killed 24 hours after the last challenge. To define the role of CC10 in the sensitization or challenge phase, 2.5 μ g of human recombinant CC10 (R&D Systems) was administered to mice intraperitoneally 2 hours before each OVA sensitization or 1.4 μ g of CC10 in 40 μ L of PBS was administered intranasally (20 μ L per nostril) 2 hours before each OVA challenge, respectively. The control mice were given an equivalent amount of PBS only. The doses of human recombinant CC10 were chosen based on published reports.^{E2,E4} In experiments to study the kinetics of CC10 and IL-17A expression after OVA challenge, 4 time points were studied, including days 14, 15, 17, and 19.

HDM-induced AR model

An HDM-induced AR model was established, as described elsewhere, with modifications.^{E5} Briefly, C57BL/6 mice (6–8 weeks old) were immunized by means of subcutaneous injection of 10 μ g of HDM antigen (Greer Laboratories, Lenoir, NC) in 0.1 mL of PBS or PBS alone (control) on days 0 and 7, followed by intranasal challenge with 100 μ g of HDM antigen in 40 μ L of PBS or an equivalent volume of PBS (control; 20 μ L per nostril) from day 14 to day 18.

Generation of OVA-specific T_H17 cells and adoptive transfer

OVA-specific T_H17 cells were generated from splenocytes of DO11.10 mice, as described previously, with minor modifications.^{E6} CD4⁺ T cells were stimulated with OVA peptide 323–329 (GenScript, Piscataway, NJ) in the presence of irradiated BALB/c spleen cells for 3 days with IL-6 (20 ng/mL), TGF- β (1 ng/mL), anti-IL-4 (20 μ g/mL), anti-IFN- γ (20 μ g/mL), and anti-IL-2 (10 μ g/mL) mAbs. Cells were then washed with PBS and

cultured for another 4 days in the presence of IL-23 (10 ng/mL), anti-IL-4, and anti-IFN- γ mAbs without antigen stimulation. The antibodies and cytokines were purchased from R&D Systems. Cytokine profiles were evaluated by means of intracellular staining for IL-4, IFN- γ , and IL-17A. Similar to a previously published study,^{E7} we found that more than 50% of CD4⁺ T cells cultured in T_H17-polarizing conditions expressed IL-17A, but few cells expressed IL-4 or IFN- γ .

OVA-sensitized mice were used as recipients of cultured DO11.10 T_H17 cells. In brief, cultured OVA-specific T_H17 cells (1×10^7 cells per mouse) or PBS controls were adoptively transferred into histocompatible BALB/c mice intravenously.^{E7} Twenty-four hours later, the mice were challenged with OVA intranasally. The numbers of eosinophils, neutrophils, and lymphocytes recovered in the NLF, as well as the mRNA levels of IL-4, IFN- γ , eotaxin-1, and eotaxin-2 in the nasal mucosa, were evaluated, and the histologic analysis of goblet cell hyperplasia was also performed at 24 hours after OVA challenge.

DC isolation

Splenocytes were isolated from naive wild-type C57BL/6 mice or naive DO11.10 mice (8–12 weeks old) to greater than 90% purity with positive selection by using anti-CD11c magnetic beads (Miltenyi Biotec), according to the manufacturer's instructions, to obtain CD11c⁺ DCs. DCs (1×10^6 cells per condition) were cultured in medium consisting of RPMI-1640 supplemented with 10% FCS and 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a humidified incubator with 5% CO₂.

After stimulation of DCs with OVA or PBS, the culture supernatants and cells were harvested and subjected to ELISA and flow cytometric and real-time RT-PCR assays, respectively. In some experiments the FPR2 antagonist WRW4 (50 μ mol/L, Tocris Bioscience) was added 2 hours before CC10 treatment (30 ng/mL).

T-cell isolation

Splenocytes were isolated from naive wild-type C57BL/6 mice or naive DO11.10 mice (8–12 weeks old) to greater than 97% purity with positive selection by using anti-CD4 magnetic beads (Miltenyi Biotec), according to the manufacturer's instructions, to obtain CD4⁺ T cells. Flow cytometry showed that more than 95% of the T-cell fraction coexpressed CD4 and CD45RA.

In vitro T_H17 polarization

CD4⁺ T cells isolated from C57BL/6 mice were stimulated with 1 ng/mL anti-CD3 and 1 ng/mL anti-CD28 mAbs in the presence of 5 ng/mL TGF- β , 10 ng/mL IL-6, and 1 ng/mL IL-23. IL-2 at a concentration of 5 ng/mL was added 1 day after initial stimulation, and 5 days later, cells were harvested for analysis. The antibodies and cytokines were purchased from R&D Systems. The percentage of IL-17A⁺CD4⁺ T cells was examined by using flow cytometry. CD4⁺ T cells were polarized under polarizing condition as above in the presence or absence of 30 ng/mL CC10 to explore the effect of CC10 on T_H17 polarization.^{E2}

DC–T-cell coculture

DCs from naive DO11.10 mice were pulsed with 60 μ g/mL OVA peptide 323–329 (GenScript) or PBS in the presence or absence of CC10 at 30 ng/mL overnight, and then the cells were washed and cultured with autologous naive CD4⁺ T cells for 48 hours. After that, the culture supernatants and cells were collected for further ELISA and flow cytometric assay, respectively. In some experiments, the FPR2 antagonist WRW4 (50 μ mol/L) was added 2 hours before CC10 treatment to determine whether CC10 exhibited its biological effects through FPR2. The efficiency of the FPR2 antagonist was confirmed by a control experiment, in which we found that WRW4 could block the calcium influx in DCs induced by the FPR2 agonist WKYMVM. In brief, murine CD11c⁺ DCs (5×10^5 cells) were isolated from spleens and primed with TNF- α at 37°C for 30 minutes before stimulation with WKYMVM (50 nm, Tocris Bioscience). In some experiments 50 μ mol/L WRW4 was included in a 5-minute preincubation period before the addition of the agonist WKYMVM.

Intracellular calcium influx on WKYMVM stimulation was continuously recorded fluorometrically by using fura-2/AM. The peak $[Ca^{2+}]_i$ value was monitored, and results were expressed as the percentage of inhibition of the increase in $[Ca^{2+}]_i$ values compared with that seen in samples without the addition of the antagonist.^{E8,E9} We found that stimulation with 50 nm of the FPR2 agonist WKYMVM caused a significant increase in $[Ca^{2+}]_i$ values in murine CD11c⁺ DCs, which was markedly inhibited by preincubation with 50 μ M of the antagonist WRW4 (see Fig E8). The percentage of inhibition was approximately 70% $\pm$ 2%, indicating that the FPR2 antagonist WRW4 was functional.

T-cell proliferation assay

Cell proliferation was assessed by using a Cell Counting Kit-8 (Boster Bio-Technology Company), according to the manufacturer's instructions. Splenocytes (3×10^5 cells per condition) from OVA-sensitized mice were stimulated with 60 μ g/mL OVA or PBS for 3 days. Before stimulation, cells were pretreated with or without 30 ng/mL CC10 for 2 hours.

DC adoptive transfer

OVA- or PBS-pulsed DCs with and without CC10 pretreatment were generated, as previously described, with minor modifications.^{E10} CD11c⁺ DCs isolated from naive C57BL/6 mice were pulsed with 200 μ g/mL OVA or PBS overnight with or without a 2-hour pretreatment with CC10 at 30 ng/mL. OVA- or PBS-pulsed DCs diluted in PBS were administered intranasally to naive mice (1×10^6 cells per mouse), and 10 days later, animals were challenged with 1% OVA daily on 3 consecutive days. Twenty-four hours after the last OVA challenge, NLF and sinonasal mucosal samples were obtained for further analysis.

BEAS-2B cell culture

BEAS-2B cells (American Type Culture Collection, Manassas, Va) were grown in Dulbecco modified Eagle medium/F-12 supplemented with 5% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Gibco, Carlsbad, Calif) at 37°C with 5% CO₂ in humidified air. When the cells reached 80% to 90% confluence, medium was changed to serum-free Dulbecco modified Eagle medium/F-12, and the cells were stimulated with IL-4, IL-13, IFN- γ , TNF- α , and IL-1 β at 10 ng/mL (PeproTech, Placentia, Calif) for 5 hours. Before stimulation, the cells were pretreated with or without 30 ng/mL human recombinant CC10 for 2 hours.

Flow cytometric analysis

Fluorescein isothiocyanate (FITC)-anti-OX40L, FITC-anti-CD80, and FITC-anti-CD86 antibodies were used to detect the surface markers of DCs, as previously described.^{E11,E12} Cultured CD4⁺ T cells, inflammatory cells in NLF, and splenocytes were stimulated with 12-O-tetradecanoyl-phorbol-13-acetate, ionomycin, and brefeldin A for 4 hours, and then the splenocytes were stained with phycoerythrin (PE)-anti-IFN- γ , allophycocyanin-anti-IL-4, PE-anti-IL-17A, and FITC-anti-CD4 antibodies; the inflammatory cells in NLF were stained with PE-anti-IL-17A and FITC-anti-CD4 antibodies; and the cultured CD4⁺ T cells were stained with only PE-anti-IL-17A antibody, as previously described.^{E13,E7} Antibodies used are listed in Table E1. Species- and subtype-matched antibodies were used as negative controls. Cells were analyzed with an LSRII flow cytometer (Becton Dickinson). For CD11c⁺ DCs, a gating strategy was followed, as described elsewhere.^{E14} Briefly, high light scatter events originating from neutrophils and eosinophils were identified in a side and forward light scattering plot and excluded from further analysis by removal from the display. In addition, low light scatter events appearing as debris were removed. Armenian hamster FITC-IgG, rat FITC-IgG₁, and rat FITC-IgG_{2a} antibodies were used as isotype controls to determine background fluorescence. A CD86, CD80, or OX40L plot was used to detect the surface markers of DCs. For lymphocytes, a gating strategy was followed, as described elsewhere.^{E15} Briefly, an acquisition gate was set according to side and forward light scattering cell properties to collect only the lymphoid population. These cells have low forward light scatter and low right-angle light scatter. Before starting the sample acquisition, the recovery (>85%) and purity

(>85%) of lymphocytes in the light scatter gate were determined, adjusting the light scatter gate to include the maximum number of lymphocytes while reducing number of the other contaminating cells. Rat PE-IgG₁, rat allophycocyanin-IgG₁, rat PE-IgG_{2a}, and rat FITC-IgG_{2a} antibodies were used as isotype controls to determine background fluorescence. A CD4 versus IL-17, CD4 versus IFN- γ , or CD4 versus IL-4 plot gated on lymphocytes was used to select T_H17, T_H1, or T_H2 cells, respectively. For cultured CD4⁺ T cells, an IL-17 plot was used to select T_H17 cells. All the antibodies were purchased from R&D Systems, except for anti-OX40L from MyBioSource (San Diego, Calif).

Cells were first stained with rabbit anti-mouse FPR2 antibody (200 ng/10⁶ cells; Santa Cruz Biotechnology) to examine the FPR2 expression on CD11c⁺ DCs. The cells were then washed in PBS and incubated with FITC-conjugated F(ab)₂ fragments of goat anti-rabbit IgG antibody (1:600, Santa Cruz Biotechnology). After a further wash in PBS, cell-surface fluorescence was analyzed immediately by using flow cytometry. Isotype and unstained controls were also prepared for accurate calibration of the fluorescence-activated cell sorting machine.

ELISA

The murine cytokine levels in culture supernatants or NLFs were determined by using commercially available ELISA kits, according to the manufacturer's instructions. The limits of detection for each assay were as follows: 4 pg/mL for IL-4 and IFN- γ (BD Pharmingen, San Diego, Calif) and 15 pg/mL for TGF- β , IL-6, and IL-12 (Boster Bio-Technology Company).

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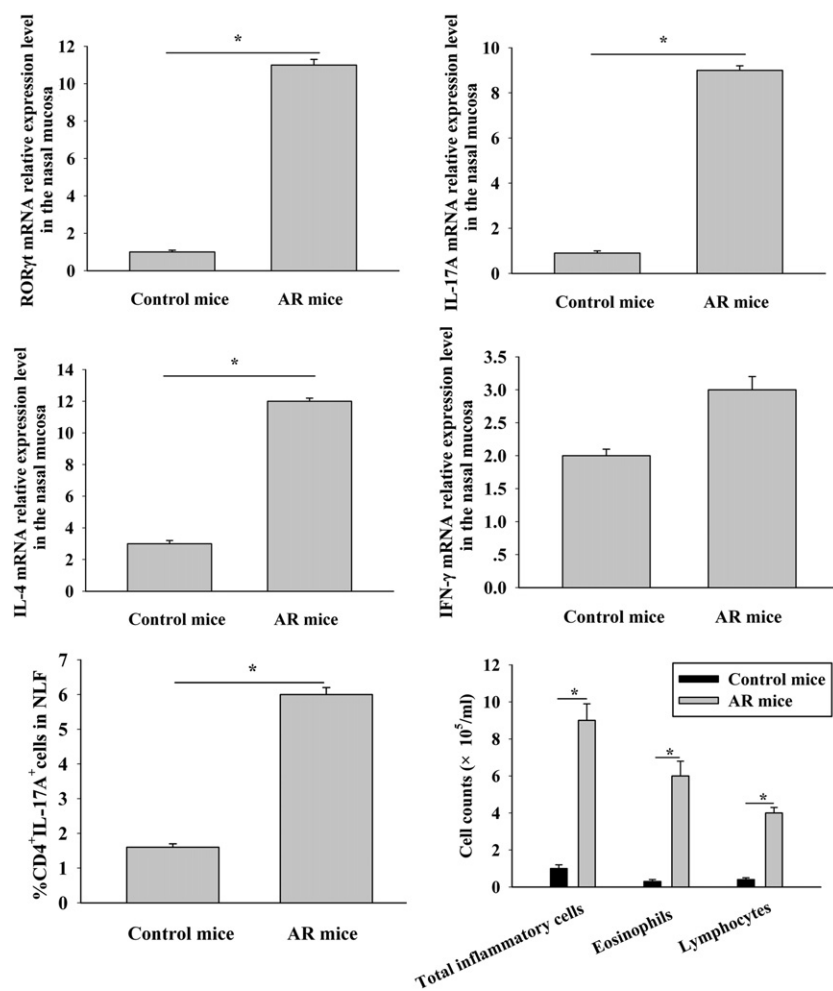


FIG E1. Enhanced T_H17 and T_H2 responses in the nose in mice with dust mite-induced AR. The relative mRNA expression of retinoic acid-related orphan receptor γ t (*ROR γ t*), IL-17A, IL-4, and IFN- γ in the nasal mucosa and the percentage of T_H17 cells and numbers of inflammatory cells in NLF from mice are shown. * $P < .05$ (n = 8-10 mice per group).

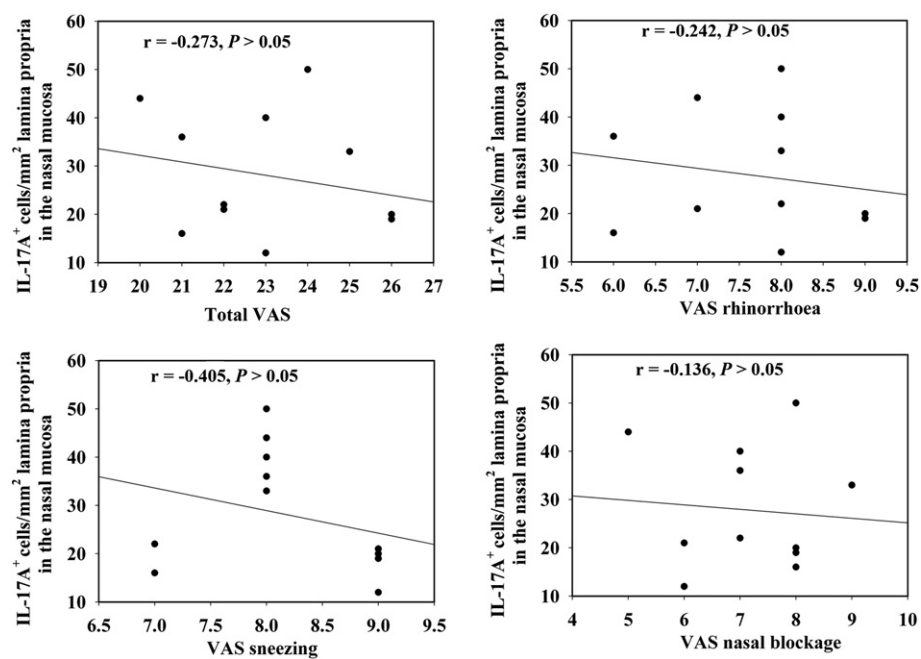


FIG E2. No significant correlation between IL-17A expression in the nasal mucosa and VAS symptom scores in 12 patients with AR.

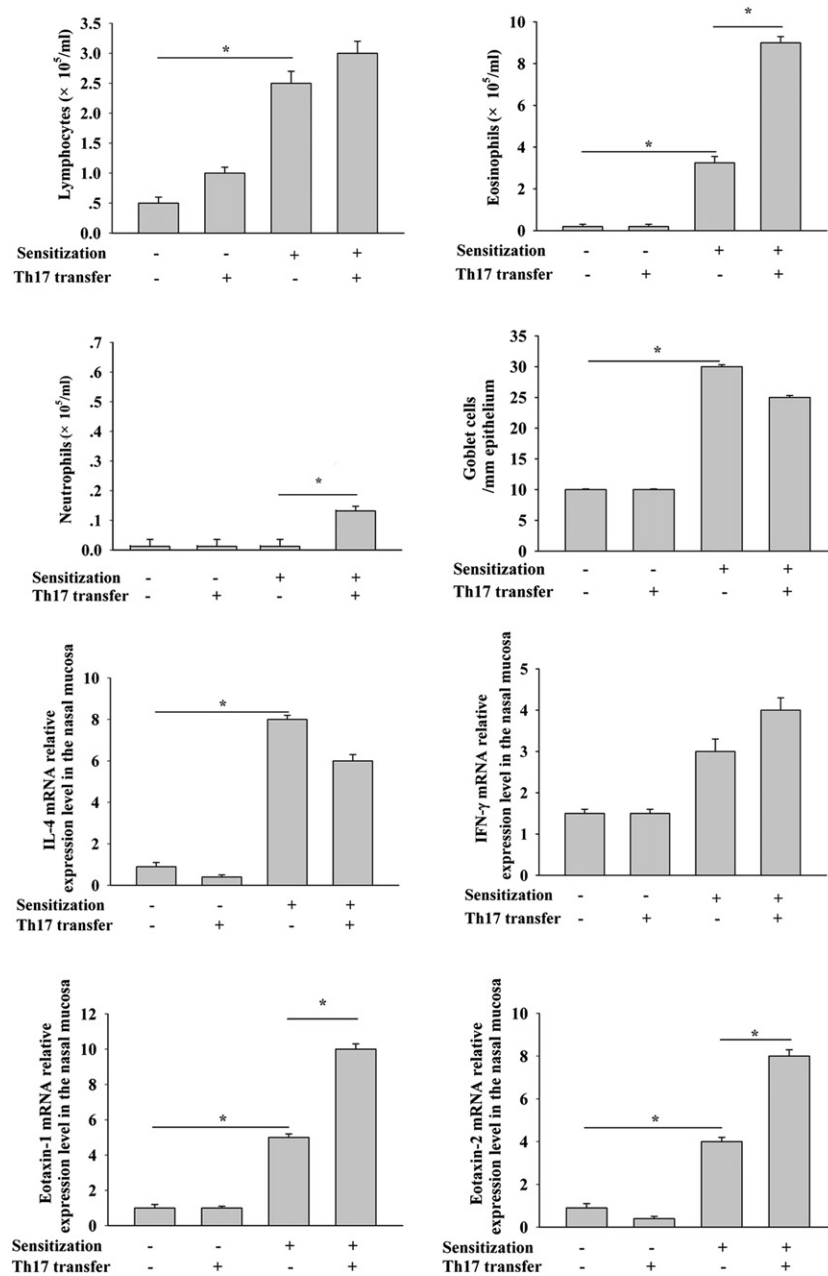


FIG E3. Antigen-specific T_H17 cells enhance antigen-induced eosinophil and neutrophil recruitment into the upper airways of sensitized mice. OVA- or PBS-sensitized mice were transferred with OVA-specific T_H17 cells or PBS controls. The number of inflammatory cells in the NLF and the goblet cell hyperplasia and mRNA expression of cytokines in the nasal mucosa were evaluated. * $P < .05$ ($n = 4$ mice per group).

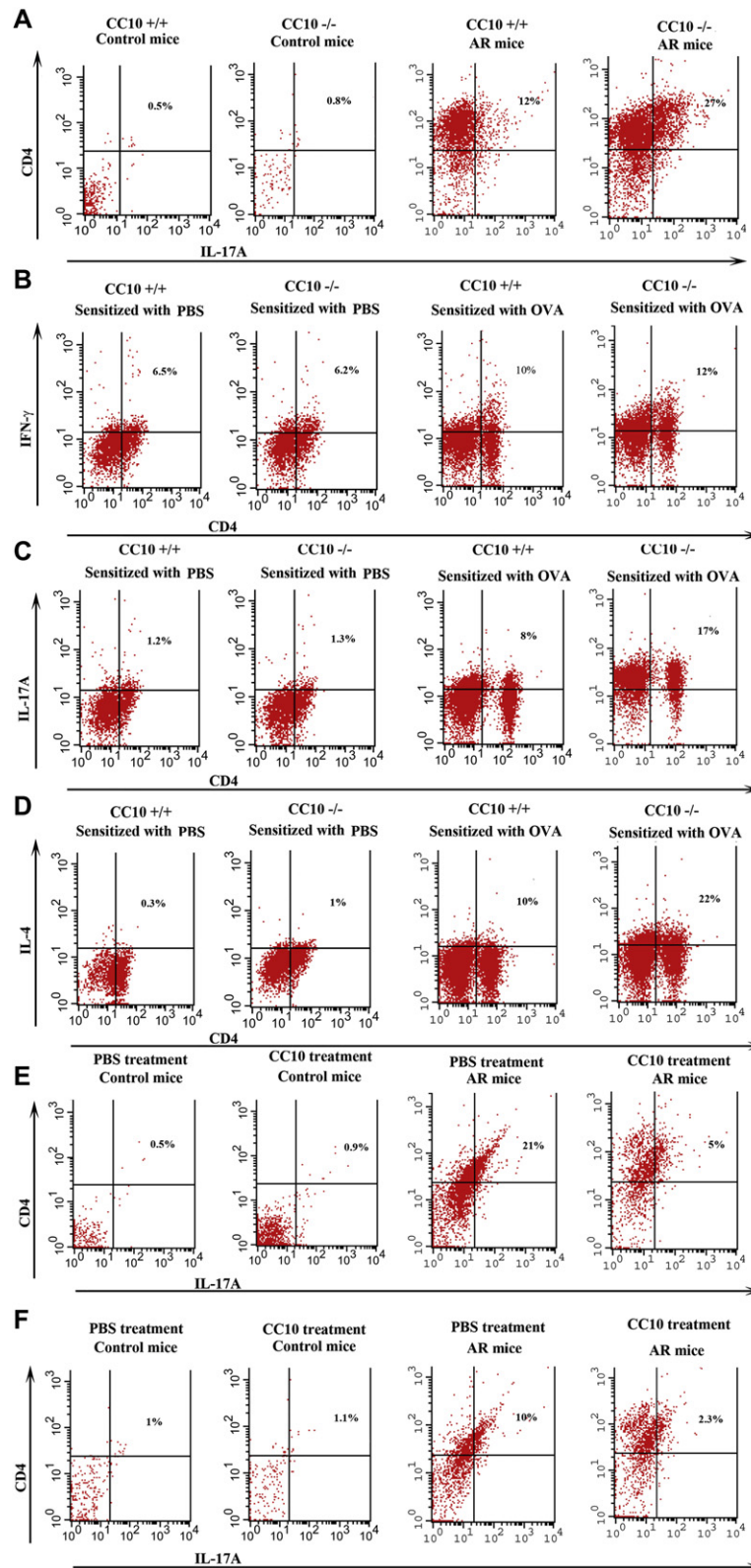


FIG E4. CC10 inhibits T_H17 responses in the setting of AR. **A**, A representative result of flow cytometric experiments of T_H17 cells in NLF from mice. **B-D**, Representative results of flow cytometric experiments of splenic T_H1 (Fig E4, B), T_H17 (Fig E4, C), and T_H2 (Fig E4, D) cells in PBS- or OVA-sensitized mice. **E**, A representative result of flow cytometric experiments of T_H17 cells in NLF from CC10-deficient mice with CC10 or PBS treatment 2 hours before sensitization. **F**, A representative result of flow cytometric experiments of T_H17 cells in NLF from wild-type mice with CC10 or PBS treatment 2 hours before challenge.

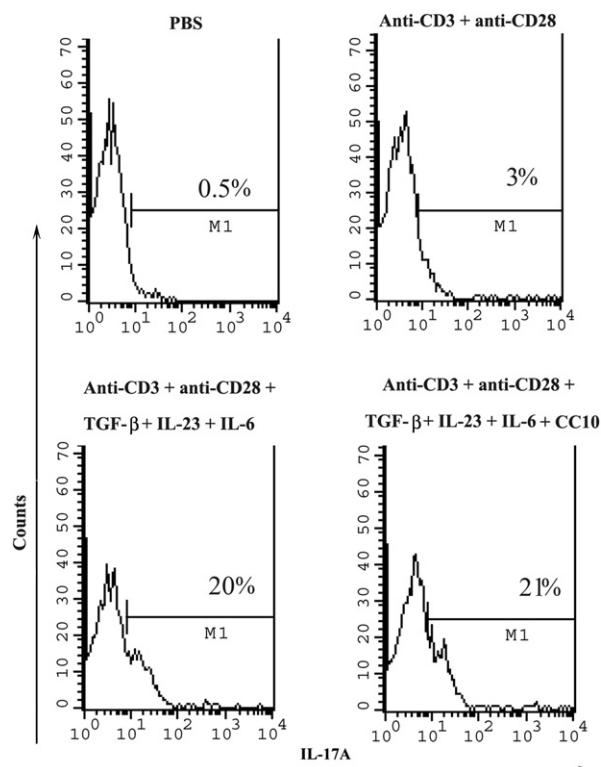


FIG E5. CC10 does not affect cytokine-induced *in vitro* T_H17 cell differentiation. Representative results of flow cytometric experiments of T_H17 cells differentiated from naive $CD4^+$ T cells in the presence of polarizing cytokines with or without CC10 treatment.

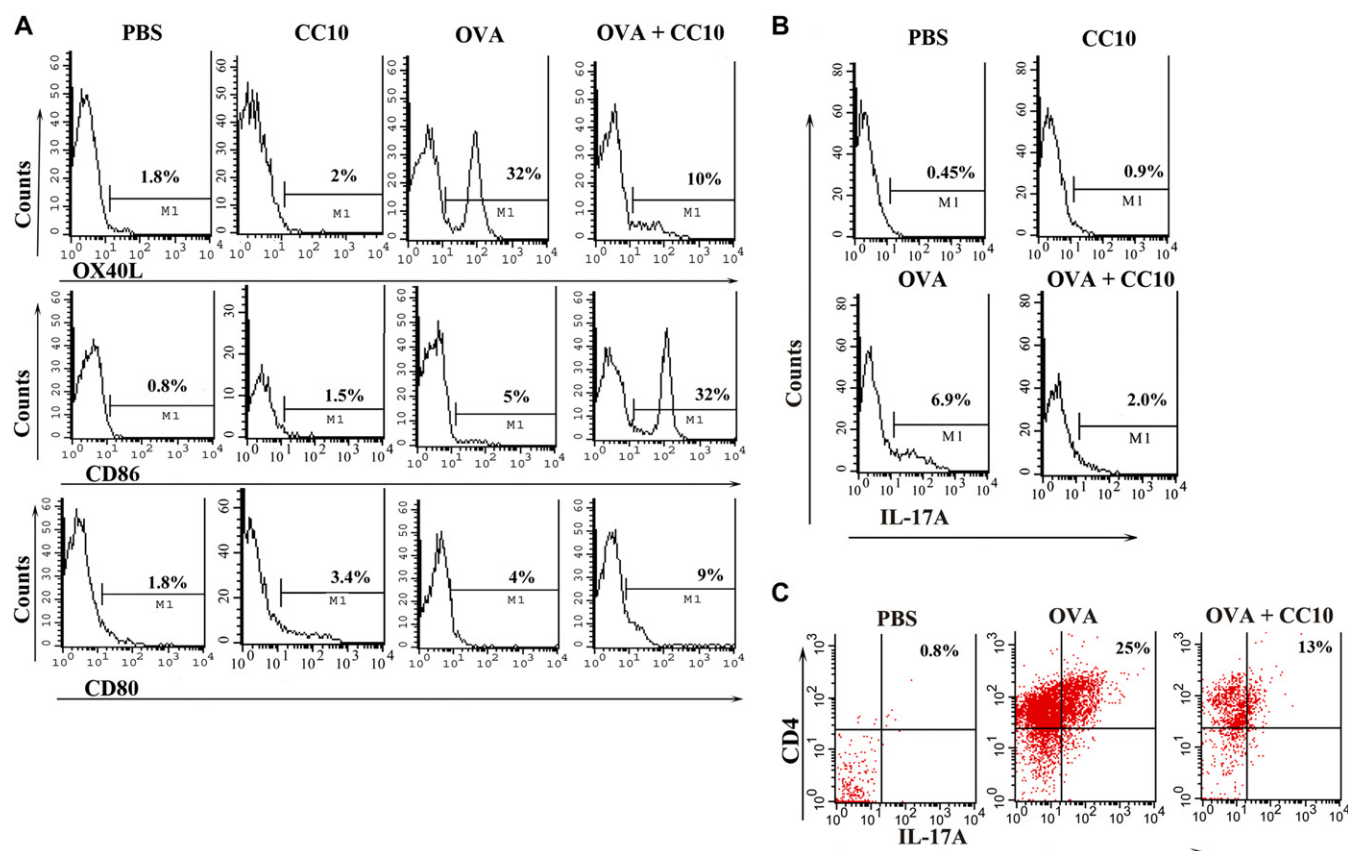


FIG E6. CC10 affects TH17 responses through modulation of the function of CD11c⁺ DCs. **A**, Representative results of flow cytometric experiments of surface marker expression on CD11c⁺ DCs. **B**, Representative results of flow cytometric experiments of TH17 cells differentiated from naive CD4⁺ T cells cocultured with CD11c⁺ DCs. **C**, A representative result of flow cytometric experiments of TH17 cells in NLF from DC-transferred mice.

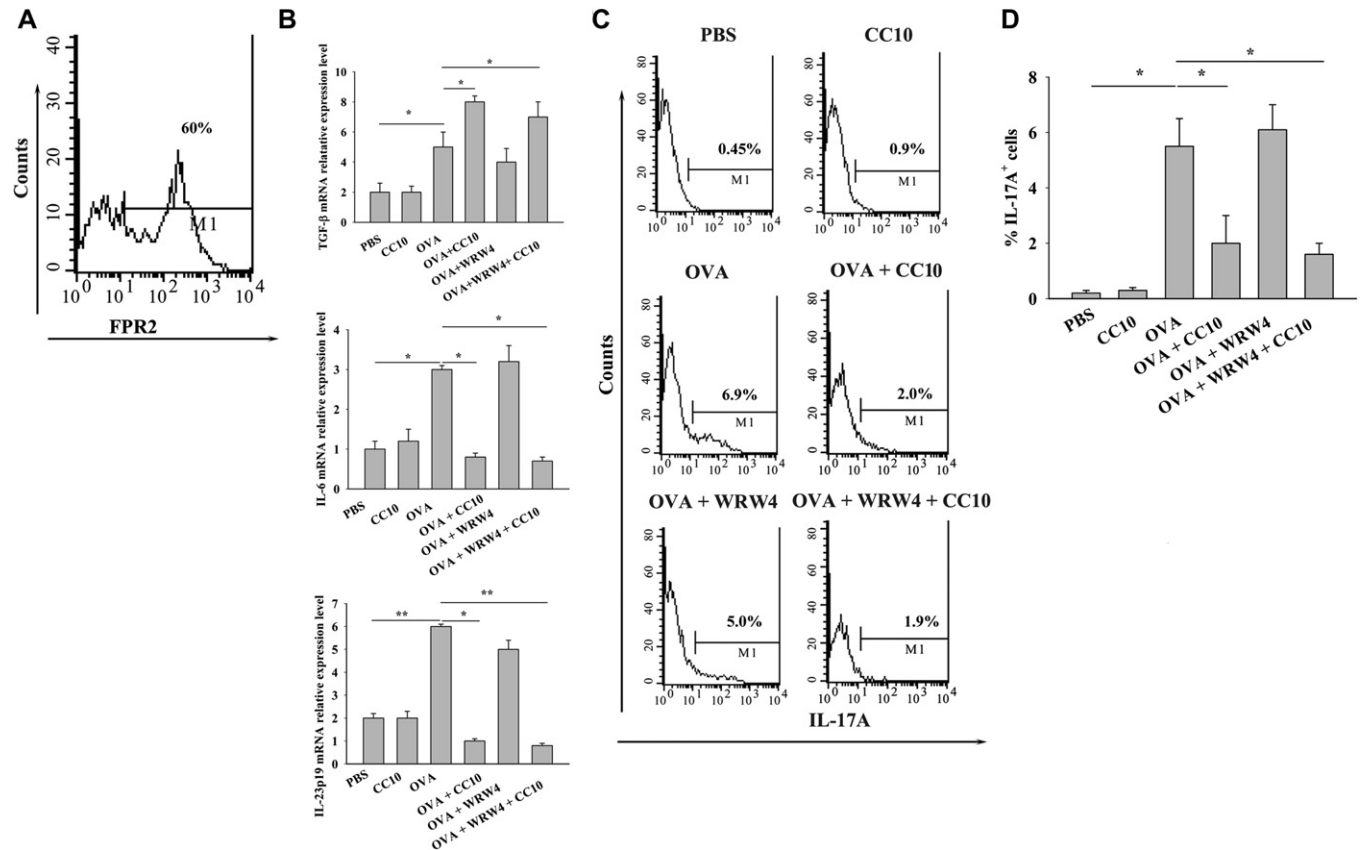


FIG E7. FPR2 is not involved in the regulatory effect of CC10. **A**, A representative result of flow cytometric experiments of the expression of FPR2 on CD11c⁺ DCs (n = 4-5 mice). **B**, mRNA levels of cytokines in CD11c⁺ DCs. * $P < .05$ and ** $P < .01$ (n = 4-5). **C** and **D**, Representative results of flow cytometric experiments (Fig E7, C) and quantification of the percentage of T_H17 cells differentiated from naive CD4⁺ T cells cocultured with CD11c⁺ DCs (Fig E7, D). * $P < .05$ (n = 4-5).

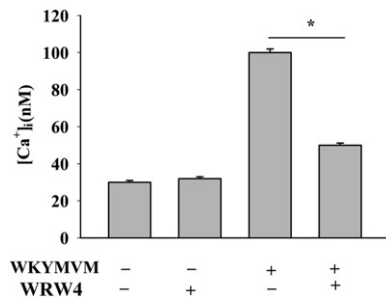


FIG E8. WRW4 inhibits WKYMVM-induced cytosolic calcium increase in murine CD11c⁺ DCs. Murine CD11c⁺ DCs were stimulated with WKYMVM in the presence or absence of WRW4. The [Ca²⁺]_i value was determined fluorometrically by using fura-2/AM. The peak [Ca²⁺]_i value was monitored. **P* < .05 (*n* = 3).

TABLE E1. Anti-mouse antibodies used in flow cytometry

Antibody	Species	Isotype	Concentration
OX40L	Rat	IgG ₁	0.5 µg/mL
CD80	Armenian hamster	IgG	0.1 µg/mL
CD86	Rat	IgG _{2a}	0.2 µg/mL
IFN-γ	Rat	IgG ₁	0.25 µg/mL
IL-4	Rat	IgG ₁	0.2 µg/mL
IL-17A	Rat	IgG _{2a}	0.25 µg/mL
CD4	Rat	IgG _{2a}	0.5 µg/mL

TABLE E2. Primer sequences for real-time PCR

Primer	Sequences	Annealing temperature (°C)	Expect product size (bp)
Human IL-23p19	[s]5'-GAGCAGCAACCCTGAGTCCCTA-3' [a]5'-CAAATTTCCCTTCCCATCTAATAA-3'	59	230
Human RORC	[s]5'-GCTGTGATCTTGCCCAGAACC-3' [a]5'-CTGCCCATCATTGCTGTTAATCC-3'	60	83
Human IL-17A	[s]5'-CAAGACTGAACACCGACTAAG-3' [a]5'-TCTCCAAAGGAAGCCTGA-3'	59	231
Human CCL20	[s]5'-GCTACTCCACCTCTGCGGCG-3' [a]5'-CAGCTGCCGTGTGAAGCCCA-3'	64	112
Human GAPDH	[s] 5'- ACCCAGAAGACTGTGGATGG-3' [a]5'-TTCTAGACGGCAGGTCAGGT-3'	61	201
Mouse IL-4	[s] 5'-TCTCGAATGTACCAGGAGCC-3' [a]5'-TGTTCTTCGTTGCTGTGAGG-3	59	143
Mouse IFN- γ	[s] 5'-GCGTCATTGAATCACACCTG-3' [a] 5'-TGAGCTCATTGAATGCTTGG-3'	59	129
Mouse IL-23p19	[s] 5'-ATCACCCCGGGAGACCCAA-3' [a]5'-CTGCTCCGTGGGCAAAGACCC-3'	59	138
Mouse IL-23p40	[s]5'-AGGCTACCCCTGAACCAGGCAG-3' [a]5'-GGGCATCGGGAGTCCAGTCCA-3'	59	158
Mouse IL-17A	[s]5'-GCGCTGTGTCAATGCGGAGG-3' [a]5'-CTGGCGGACAATCGAGGCCA-3'	58	169
Mouse ROR γ t	[s]5'-TGACCGAACCAGCCGCAACC-3' [a]5'-AGGTGAGGCGCCCAGTG GTA-3'	59	262
Mouse eotaxin-1	[s]5'- TGTCTCCCTCCACCATGCA-3' [a]5'-GATCTTCTTACTGGTCATGATAAAGCA-3'	55	136
Mouse eotaxin-2	[s]5'- TCTCCTGGTAGCCTGCCCGT-3' [a]5'- GGTTCTCACTGCCTTGCCCCC-3'	55	280
Mouse GAPDH	[s]5'-CGGATTTGGCCGTATTGGG-3' [a]5'-CTCGCTCCTGGAAGATGG-3'	59	215

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *ROR*, retinoic acid-related orphan receptor.