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 μg 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 μg of OVA in 40 μL of PBS (20 μL 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 μg of human recombinant CC10 (R&D Systems) was administered to mice intraperitoneally 2 hours before each OVA sensitization or 1.4 μg of CC10 in 40 μL of PBS was administered intranasally (20 μL 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 μg 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 μg of HDM antigen in 40 μL of PBS or an equivalent volume of PBS (control; 20 μL 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 μg/mL), anti–IFN-γ (20 μg/mL), and anti–IL-2 (10 μg/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 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2.

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 μmol/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 $IL\text{-}17A^+CD4^+$ 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 μg/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 μmol/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°for 30 minutes before stimulation with WKYMVM (50 nm, Tocris Bioscience). In some experiments 50 μmol/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 simulation 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 μmol/L of the antagonist WRW4 (see Fig E8). The percentage of inhibition was approximately 70% ± 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 μg/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 μg/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 μg/mL streptomycin (Gibco, Carlsbad, Calif) at 37°C with 5% CO_2 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-1β 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-IgG_1, 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-IgG_1, rat allophycocyanin-IgG_1, 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/10^6 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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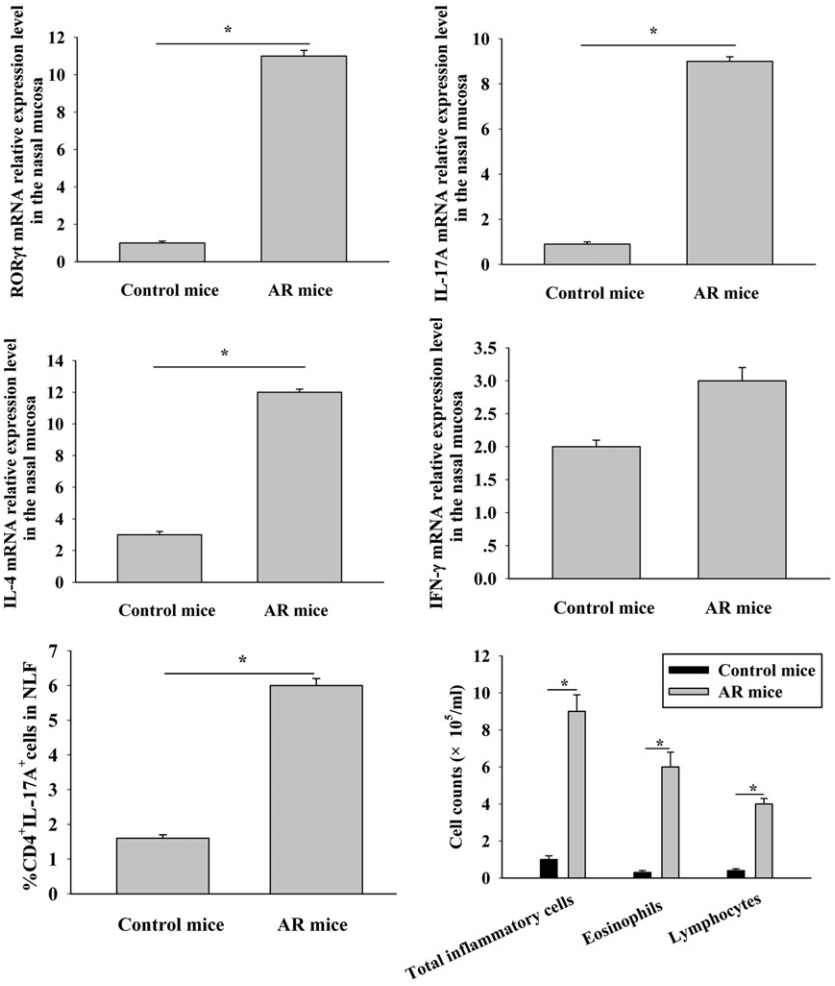

FIG E1. Enhanced T_H17 and T_H2 responses in the nose in mice with dust mite–induced AR. The relative mRNA expression of retinoic acid–related orphan receptor γt *(RORγt)*, IL-17A, IL-4, and IFN-γ in the nasal mucosa and the percentage of T_H17 cells and numbers of inflammatory cells in NLF from mice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

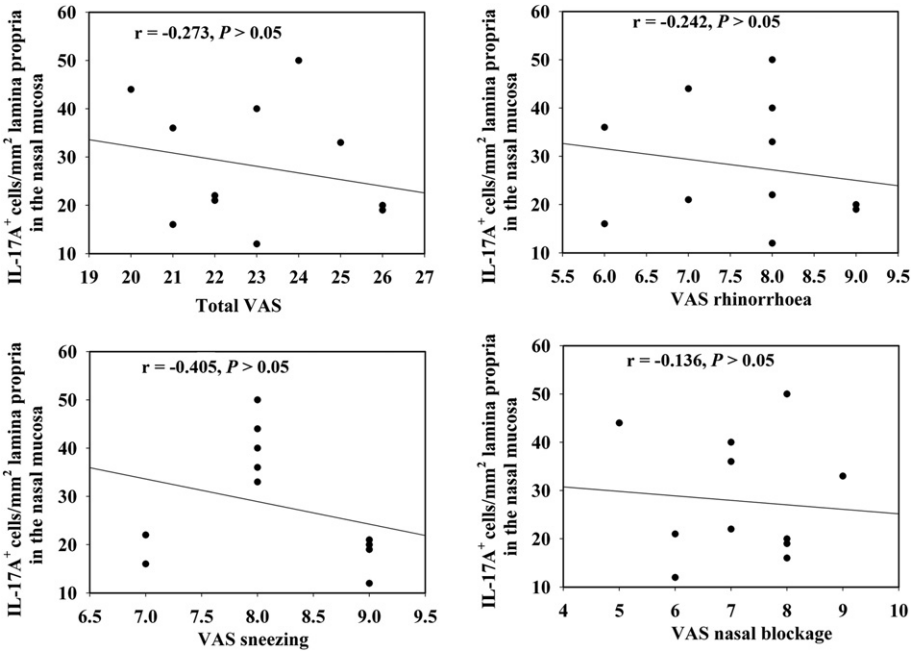

FIG E2. No significant correlation between IL-17A expression in the nasal mucosa and VAS symptom scores in 12 patients with AR.

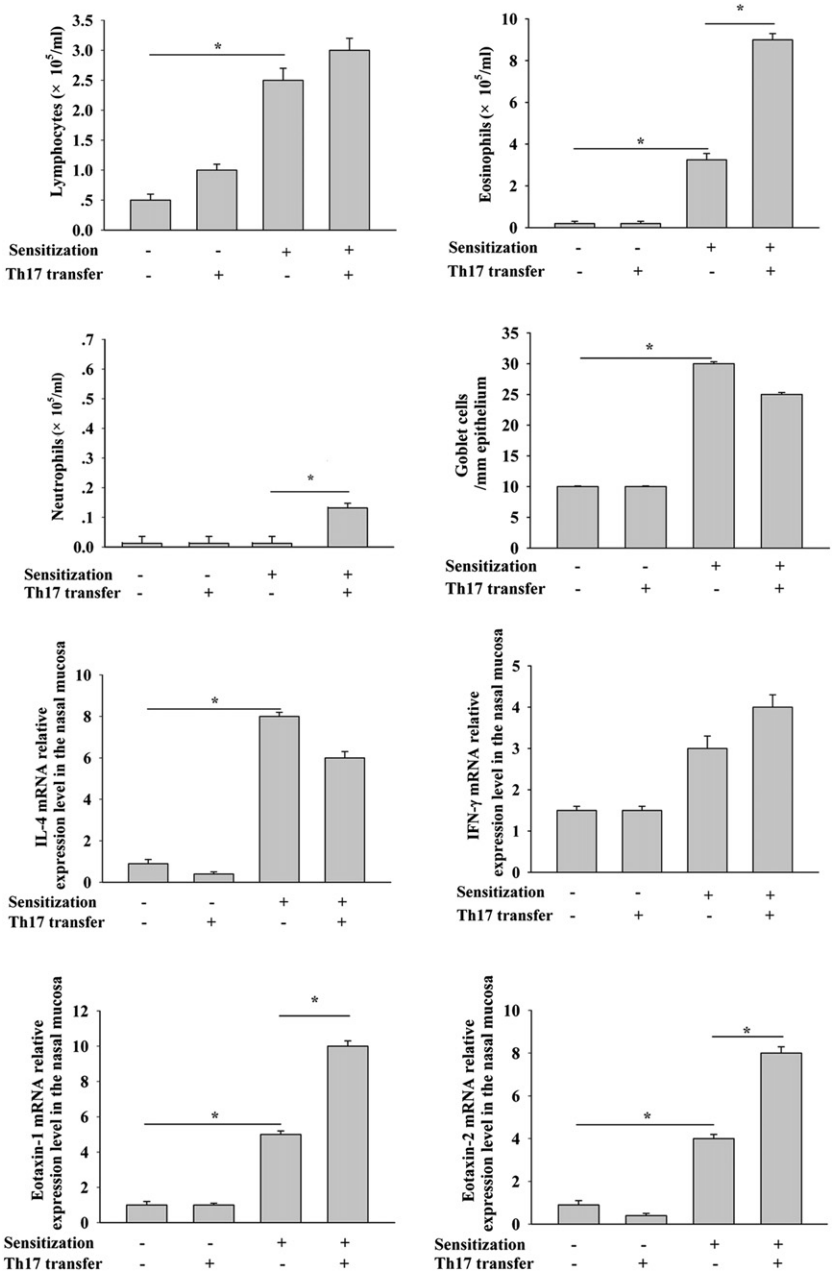

FIG E3. Antigen-specific T_H17 cells enhance antigen-induced eosinophil and neutrophil recruitment into the upper airways of sensitized mice. OVA- or PBS-sensitized mice were transferred with OVA-specific T_H17 cells or PBS controls. The number of inflammatory cells in the NLF and the goblet cell hyperplasia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

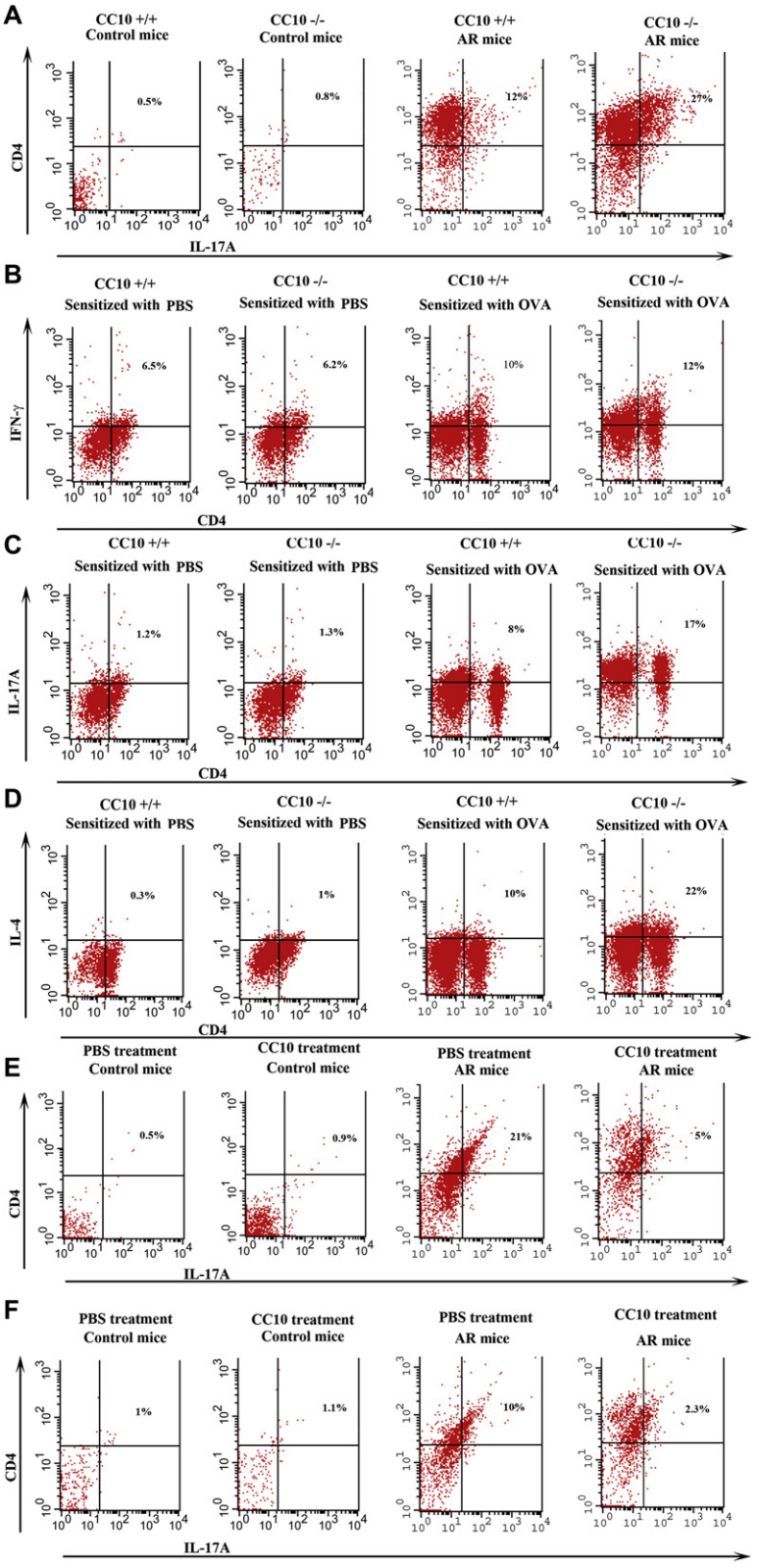

FIG E4. CC10 inhibits T_H17 responses in the setting of AR. **A,** A representative result of flow cytometric experiments of T_H17 cells in NLF from mice. **B-D,** Representative results of flow cytometric experiments of splenic T_H1 (Fig E4, *B*), T_H17 (Fig E4, *C*), and T_H2 (Fig E4, *D*) cells in 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.

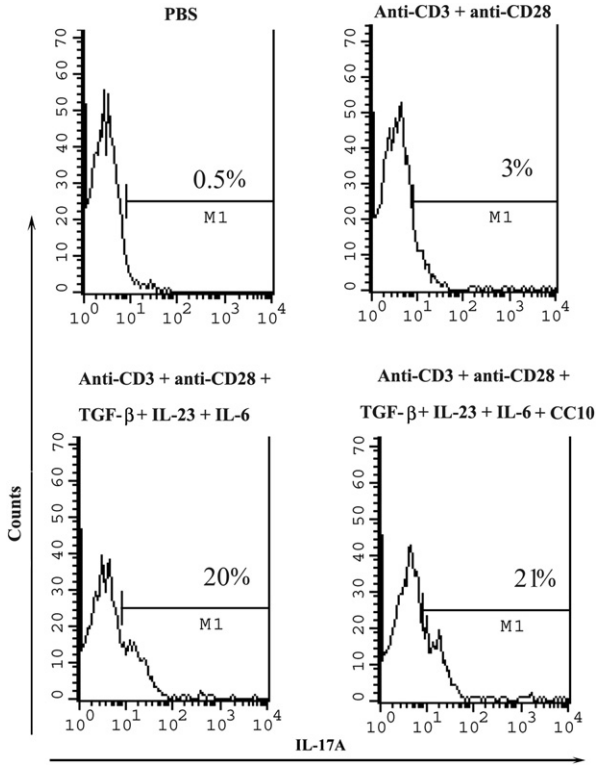

FIG E5. CC10 does not affect cytokine-induced *in vitro* T_H17 cell differentiation. Representative results of flow cytometric experiments of T_H17 cells differentiated from naive $CD4^+$ T cells in the presence of polarizing cytokines with or without CC10 treatment.

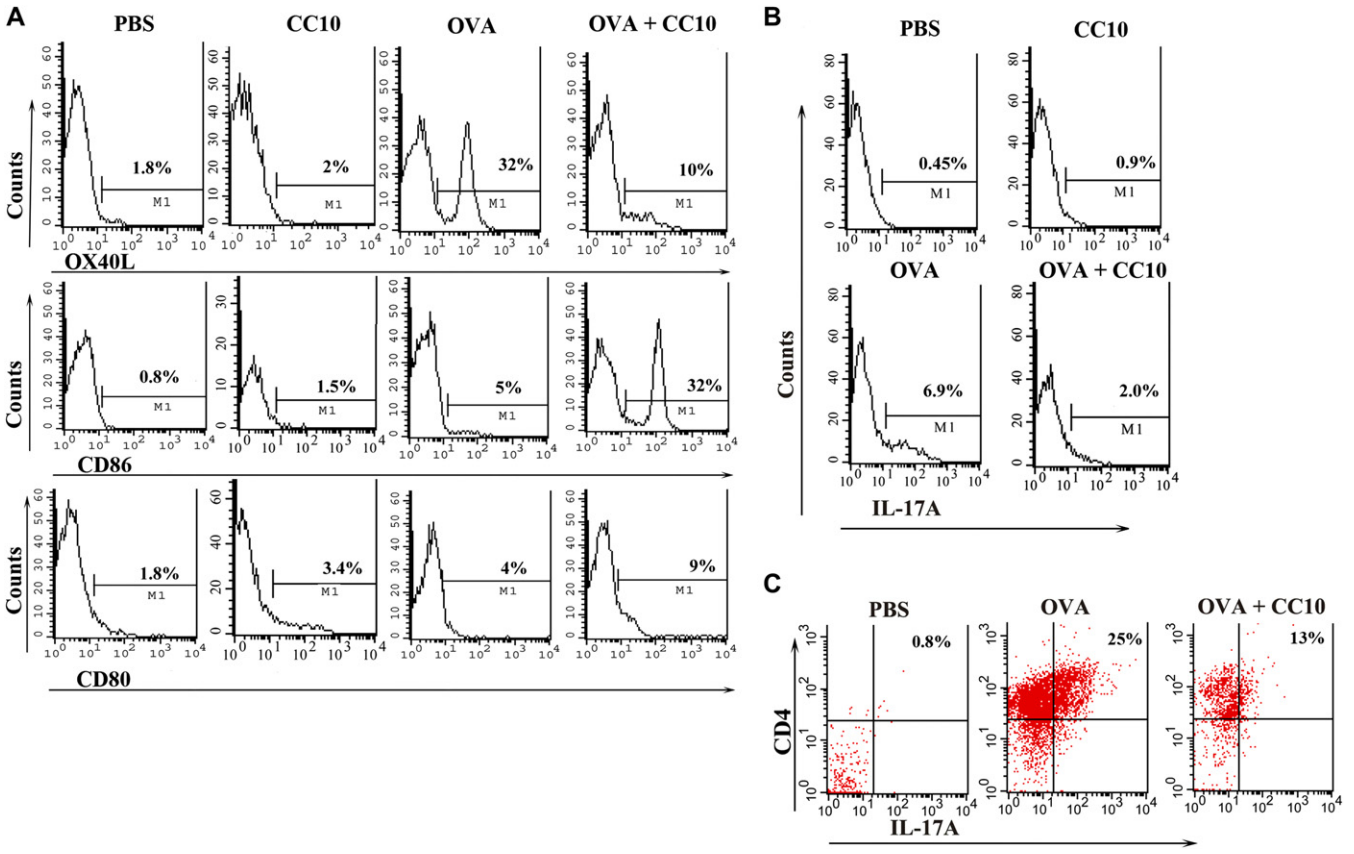

FIG E6. CC10 affects T_H17 responses through modulation of the function of $CD11c^+$ DCs. **A,** Representative results of flow cytometric experiments of surface marker expression on $CD11c^+$ DCs. **B,** Representative results of flow cytometric experiments of T_H17 cells differentiated from naive $CD4^+$ T cells cocultured with $CD11c^+$ DCs. **C,** A representative result of flow cytometric experiments of T_H17 cells in NLF from DC-transferred mice.

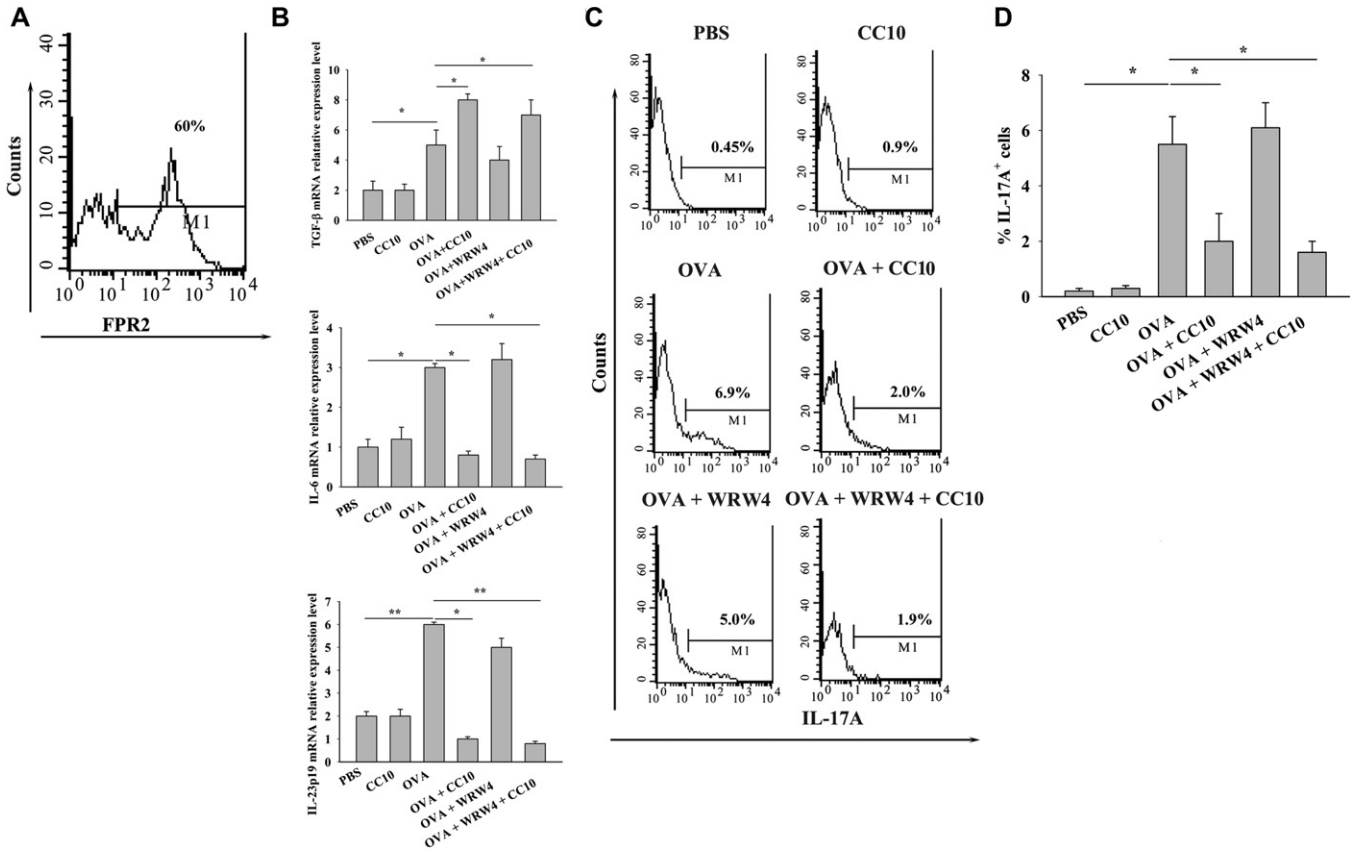

FIG E7. FPR2 is not involved in the regulatory effect of CC10. **A,** A representative result of flow cytometric experiments of the expression of FPR2 on $CD11c^+$ DCs (n = 4-5 mice). **B,** mRNA levels of cytokines in $CD11c^+$ DCs. **P* < .05 and ***P* < .01 (n = 4-5). **C** and **D,** Representative 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).

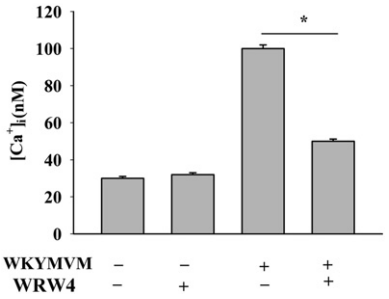

FIG E8. WRW4 inhibits WKYMVM-induced cytosolic calcium increase in murine CD11c$^+$ DCs. Murine CD11c$^+$ DCs were stimulated with WKYMVM in the presence or absence of WRW4. The $[Ca^{2+}]_i$ value was determined fluorometrically by using fura-2/AM. The peak $[Ca^{2+}]_i$ value was monitored. *$P < .05$ (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′	59	230
	[a]5′-CAAATTTCCCTTCCCATCTAATAA-3′		
Human RORC	[s]5′-GCTGTGATCTTGCCCAGAACC-3′	60	83
	[a]5′-CTGCCCATCATTGCTGTTAATCC-3′		
Human IL-17A	[s]5′-CAAGACTGAACACCGACTAAG-3′	59	231
	[a]5′-TCTCCAAAGGAAGCCTGA-3′		
Human CCL20	[s]5′-GCTACTCCACCTCTGCGGCG-3′	64	112
	[a]5′-CAGCTGCCGTGTGAAGCCCA-3′		
Human GADPH	[s] 5′- ACCCAGAAGACTGTGGATGG-3′	61	201
	[a]5′-TTCTAGACGGCAGGTCAGGT-3′		
Mouse IL-4	[s] 5′-TCTCGAATGTACCAGGAGCC-3′	59	143
	[a]5′-TGTTCTTCGTTGCTGTGAGG-3		
Mouse IFN-γ	[s] 5′-GCGTCATTGAATCACACCTG-3′	59	129
	[a] 5′-TGAGCTCATTGAATGCTTGG-3′		
Mouse IL-23p19	[s] 5′-ATCACCCCCGGGAGACCCAA-3′	59	138
	[a]5′-CTGCTCCGTGGGCAAAGACCC-3′		
Mouse IL-23p40	[s]5′-AGGCTACCCCTGAACCAGGCAG-3′	59	158
	[a]5′-GGGCATCGGGAGTCCAGTCCA-3′		
Mouse IL-17A	[s]5′-GCGCTGTGTCAATGCGGAGG-3′	58	169
	[a]5′-CTGGCGGACAATCGAGGCCA-3′		
Mouse RORγt	[s]5′-TGACCGAACCAGCCGCAACC-3′	59	262
	[a]5′-AGGTGAGGCGCCCAGTGGTA-3′		
Mouse eotaxin-1	[s]5′- TGTCTCCCTCCACCATGCA-3′	55	136
	[a]5′-GATCTTCTTACTGGTCATGATAAAGCA-3′		
Mouse eotaxin-2	[s]5′- TCTCCTGGTAGCCTGCCCGT-3′	55	280
	[a]5′- GGTTCTCACTGCCTTGGCCCC-3′		
Mouse GADPH	[s]5′-CGGATTTGGCCGTATTGGG-3′	59	215
	[a]5′-CTCGCTCCTGGAAGATGG-3′		

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