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Interleukin-17 Receptor E and C-C Motif Chemokine Receptor 10 Identify Heterogeneous T Helper 17 Subsets in a Mouse Dry Eye Disease Model

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Dry eye disease (DED) features the inflammatory response of the ocular surface. Pro-inflammatory T helper 17 (Th17) cells are important for the pathogenesis of DED. In the present study a mouse DED model was used to discover two Th17 subsets in draining lymph nodes and conjunctivae based on the expression of IL-17 receptor E (IL-17RE) and CCR10: IL-17RE^{low}CCR10^{-} Th17 and IL-17RE^{high}CCR10^{+} Th17. IL-17RE^{high}CCR10^{+} Th17 expressed more retinoic acid–related orphan receptor gamma t but fewer T-box-expressed-in-T-cells than IL-17RE^{low}CCR10^{-} Th17. In addition, the former expressed higher IL-17A, IL-21, and IL-22 but fewer IFN-γ than the latter. Further analysis showed that IL-17RE^{high}CCR10^{+} Th17 did not express IFN-γ *in vivo*, whereas IL-17RE^{low}CCR10^{-} Th17 contained IFN-γ–expressing Th17/Th1 cells. Moreover, IL-17RE^{high}CCR10^{+} Th17 possessed more phosphorylated p38 mitogen-activated protein kinase (MAPK) and Jnk than IL-17RE^{low}CCR10^{-} Th17, suggesting higher activation of MAPK signaling in IL-17RE^{high}CCR10^{+} Th17. *In vitro* treatment with IL-17C effectively maintained IL-17A expression in Th17 cells through p38 MAPK rather than Jnk MAPK. Furthermore, the adoptive transfer of the two Th17 subpopulations indicated their equivalent pathogenicity in DED. Interestingly, IL-17RE^{high}CCR10^{+} Th17 cells were able to phenotypically polarize to IL-17RE^{low}CCR10^{-} Th17 cells *in vivo*. In conclusion, the current study revealed novel Th17 subsets with differential phenotypes, functions, and signaling status in DED, thus deepening the understanding of Th17 pathogenicity, and exhibited Th17 heterogeneity in DED. *(Am J Pathol 2022, 192: 332–343; https://doi.org/10.1016/j.ajpath.2021.10.021)*

Dry eye disease (DED) is a significant public health challenge featuring increased osmolarity of the tear film along with the inflammatory response of the ocular surface, which remarkably lowers quality of life and even incurs blindness.[1,2] Although DED can be grouped into various classes, cumulative studies have proposed that all DEDs are characterized by varying ocular surface inflammation.[3,4] CD4^{+} T helper 1 (Th1) cells and T helper 17 (Th17) cells are deeply involved in the physiopathology of DED. Th17 cells have been regarded as a key contributor to the pathogenesis of multiple inflammatory and autoimmune disorders owing to their production of IL-17A, IL-17F, IL-22, and granulocyte-macrophage colony-stimulating factor.[5,6] In DED, IL-17 is correlated with the compromise of the corneal epithelial barrier.[7] In addition, enhanced levels of Th17 cell–inducing factors such as IL-6, transforming growth factor-β, and IL-23 are found on the ocular surface of patients with DED.[7–9] Consistently, increased IL-17 in tears and augmented Th17 cell numbers were detected on the ocular surface in DED animal models.[7,10] Therefore, Th17 cells have been suggested as a crucial player in the development of DED.

However, Th17 cells are a heterogeneous population exhibiting a considerable degree of plasticity. Distinct Th17 subsets might play differential roles in the pathogenesis of DED. Recent evidence suggests the presence of an IL-10–producing Th17 population in conjunctivae and draining lymph nodes (LN) of DED mice.[11] Another study revealed the existence of IL-17^{+}IFN-γ^{+} Th17/Th1 cells in draining LN of aged mice with DED.[12] Nonetheless, details of the phenotypes

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and regulatory mechanisms underlying the function of these Th17 subpopulations have not been thoroughly investigated.

In the current study, two Th17 subsets in draining LN and conjunctivae were identified based on the expression of IL-17 receptor E (IL-17RE) and CCR10 in a mouse DED model. The functional heterogeneity of these subsets and differential activation of Th17-associated signal pathways were also characterized. Furthermore, the role of IL-17C, which is the ligand of IL-17RE, in the maintenance of IL-17A expression in Th17 cells was ascertained. In conclusion, the study identified novel Th17 subsets with phenotypical, functional, and signaling heterogeneity in DED.

Materials and Methods

Mice

The animal study was approved by China Three Gorges University Animal Care and Use Committee and conducted following the standards of China Three Gorges University for the Use of Animals in Ophthalmic and Visual Research. Eight-week–old female IL-17A-enhanced green fluorescent protein (EGFP) transgenic mice (*C57BL/6-Il17a*^{tm1Bcgen}) and wild-type C57BL/6 mice were purchased from Biocytogen Inc. (Beijing, China). B6.Rag1 KO mice (*C57BL/6-Rag1*^{em1Smoc}) were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China).

DED Model

To induce DED, mice were housed in an environmentally controlled room with a relative humidity of 13.1 ± 3.5%, an airflow of 2.2 ± 0.2 m/second, and a temperature of 22 ± 2°C. Each cage had an open grate on each side to allow for continuous airflow from a fan placed 6 inches in front of it for 16 hours per day. After housing for 10 days, subcutaneous scopolamine hydrobromide injections (0.5 mg/0.2 mL; Sigma-Aldrich, St. Louis, MO) were given three times a day for 5 days at 8:00 AM, noon, and 2:00 PM. Control mice were kept in a normal humidity environment (approximately 65%) without exposure to continuous airflow, and no scopolamine hydrobromide was administered. The development of DED was evaluated by adding 1 mL of 1% fluorescein (Thermo Fisher Scientific, Waltham, MA) to the lateral conjunctival sac after the mice were anesthetized by inhalation of 3% of isoflurane (Sigma-Aldrich). Three minutes later, punctate staining was measured under a slit-lamp biomicroscope with cobalt blue light. The left eye of each animal was scored as previously described[10] (Supplemental Figure S1A).

Enrichment of Cells from LN and Conjunctivae

Submandibular and draining LN were taken by using curved tissue forceps and ground on a 70-μm cell strainer (Corning, Corning, NY) to prepare single-cell suspensions. To isolate conjunctival cells, each set of experiments consisted of a pool of 30 eyes, and a total of six experimental sets were performed. Conjunctivae were collected by using curved tissue forceps and Vannas scissors, followed by incubation in RPMI 1640 (Thermo Fisher Scientific) containing 2 mg/mL collagenase IV (Sigma-Aldrich), 100 U/mL DNase I (Sigma-Aldrich), 5 mmol/L $CaCl_2$, and 10% fetal calf serum (Thermo Fisher Scientific) for 45 minutes at 37°C. Conjunctival cells were then passed through a 70-μm cell strainer (Corning) to prepare single-cell suspensions. Cells from 30 to 50 conjunctivae were pooled for analysis. The cell suspensions were washed with phosphate-buffered saline (PBS) once and subjected to flow cytometry analysis as described in the following section.

Flow Cytometry Analysis

PerCP-Cy5.5- or Pacific Blue–conjugated anti-mouse CD3 antibody (17A2), APC/Cy7–conjugated anti-mouse CD4 (RM4-5) antibody, and PE/Cyanine7–conjugated anti-mouse IFN-γ antibody (XMG1.2) were obtained from BioLegend (San Diego, CA). PerCP-eFluor 710–conjugated anti-mouse IL-22 (IL22JOP) and PE/Cyanine7–conjugated anti-mouse IL-21 (mhalx21) were obtained from Thermo Fisher Scientific. Rat anti-mouse IL-17RE antibody (944904) and phycoerythrin-conjugated rat anti-mouse CCR10 (248918) antibody were purchased from R&D Systems (Minneapolis, MN). For cell detection and sorting, 1×10^5/mL cells were incubated with 2 μg/mL of each antibody without the anti-mouse CCR10 antibody in PBS on ice for 20 minutes. Cells were then washed with PBS and incubated with 2 μg/mL of APC-conjugated goat anti-rat IgG (Poly4054, BioLegend) on ice for 20 minutes. Cells were washed again with PBS and incubated with 2 μg/mL of PE-conjugated rat anti-mouse CCR10 antibody on ice for 15 minutes. Approximately 1×10^5 single events were acquired for analyzing lymph node cells, and 10^6 single events were acquired for analyzing conjunctiva cells.

To measure IFN-γ expression, 1×10^5 sorted Th17 cells or Th17 subsets were cultured in RPMI 1640 supplemented with 10% fetal calf serum for 6 hours with 50 ng/mL phorbol-12-myristate-13-acetate plus 1 μg/mL ionomycin (both from Sigma-Aldrich). Two hours after stimulation, 5 μg/mL brefeldin A and 5 μg/mL monensin (both from Sigma-Aldrich) were added into the culture. At the end of stimulation, cells were first stained with antibodies against IL-17RE and CCR10 as described in the previous paragraph, followed by fixation in 2% paraformaldehyde for 15 minutes, permeabilization in 90% methanol on ice for 30 minutes, and incubation with 5 μg/mL of PE/Cyanine7–conjugated anti–IFN-γ antibody at room temperature for 1 hour. An LSR II flow cytometer was used for analysis, and a FACSAria sorter was used for sorting (BD Biosciences, Franklin Lakes, NJ).

Table 1 Primer Sequences

Gene	Sense	Antisense
RORC	5′-GTGGAGTTTGCCAAGCGGCTTT-3′	5′-CCTGCACATTCTGACTAGGACG-3′
TBX21	5′-CCACCTGTTGTGGTCCAAGTTC-3′	5′-CACAAACATCCTGTAATGGCTTG-3′
GATA3	5′-CCTCTGGAGGAGGAACGCTAAT-3′	5′-GTTTCGGGTCTGGATGCCTTCT-3′
FOXP3	5′-TACTTCAAGTTCCACAACATGGG-3′	5′-CACAAAGCACTTGTGCAGACTCAG-3′
IFNG	5′-GCGTCATTGAATCACACCTGA-3′	5′-TGTGGGTTGTTGACCTCAAACT-3′
IL17A	5′-CCTGGACTCTCCACCGCAAT-3′	5′-AGCTTTCCCTCCGCATTGAC-3′
IL21	5′-GCCTCCTGATTAGACTTCGTCAC-3′	5′-CAGGCAAAAGCTGCATGCTCAC-3′
IL22	5′-TTTCCTCGTCGGCTTGCTCTGT-3′	5′-CGTGTTCTTGGATGAAGCGTAGG-3′
CCR10	5′-CAGTCTTCGTGTGGCTGTTGTC-3′	5′-TCACAGTCTGCGTGAGGCTTTC-3′
ACTB	5′-CATTGCTGACAGGATGCAGAAGG-3′	5′-TGCTGGAAGGTGGACAGTGAGG-3′

Reverse Transcription and Real-Time PCR

RNAs were purified by using the Arcturus PicoPure RNA Isolation Kit (Thermo Fisher Scientific), and cDNAs were prepared using the BeyoRT III First Strand cDNA Synthesis Kit (Bieyotime Biotech, Beijing, China) according to the manufacturers' manuals. The SYBR Green Quantitative RT-PCR Kit (Sigma-Aldrich) was used for quantitative PCR on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) following the standard amplification procedures. The amounts of target transcripts were normalized to β-actin transcript levels and computed by using the $2^{-\Delta\Delta Ct}$ formula. Primer sequences are provided in Table 1.

Western Blot

Cellular proteins were extracted by using the RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitors (Sigma-Aldrich). The Phospho-NF-κB p65 (Ser536) monoclonal antibody (93H1), NF-κB p65 monoclonal antibody (D14E12), Phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182) antibody, (D3F9) p38 MAPK monoclonal antibody (D13E1), Phospho-JNK (Thr183/Tyr185) monoclonal antibody (G9), JNK monoclonal antibody (2C6), and glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (D16H11) were purchased from Cell Signaling Technology (Danvers, MA).

Cell Culture and Treatment

To test the effect of IL-17C, a 96-well half-area culture plate (Corning) was precoated with 5 μg/mL agonistic CD3 antibody (17A2, eBioscience, San Diego, CA) overnight. Then, 1×10^5 sorted Th17 cells were seeded into each well in the presence of 2 μg/mL agonistic CD28 antibody (37.51, eBioscience). PBS or 20 ng/mL recombinant mouse IL-17C (2306-ML-025/CF, R&D Systems) was added into the culture from the beginning. After 24-hour culture, the intensity of IL-17A-EGFP was measured by flow cytometry. In some experiments, 1×10^5 sorted IL-17RE^{high}CCR10$^+$ Th17 cells were pretreated with 1 μmol/L SB202190 (1264/10, R&D Systems) or 10 μmol/L SP600125 (1496/10, R&D Systems) for 1 hour, followed by IL-17C treatment as described above.

Adoptive Transfer

CD4$^+$IL-17A(GFP)$^+$IL-17RE^{low}CCR10$^-$ Th17 and CD4$^+$IL-17A(GFP)$^+$IL-17RE^{high}CCR10$^+$ Th17 cells were sorted from draining lymph node cells of DED mice by using flow cytometry. Each subpopulation (4×10^4 cells) was infused into naive B6.Rag1 KO mice via retro-orbital injection. The recipients were then placed in the DED-inducing environment as described above for 10 days without scopolamine hydrobromide administration. DED severity was assessed by using 1% fluorescein as described above. Conjunctivae were collected for analyzing exogenous Th17 cells.

Statistical Analysis

Each experiment was independently performed two or three times. Each sample contained cells pooled from five to 20 mice. The data are shown as means ± SD. The *t*-test or one-way ANOVA with Tukey post hoc test was for statistical analysis. $P < 0.05$ was regarded as significant.

Results

Th17 Cells Are Increased in the Draining LN and Conjunctivae in DED

DED was induced in IL-17A-EGFP transgenic mice (Supplemental Figure S1A), and cells were isolated from the draining LN and conjunctivae. As shown in Figure 1A, CD3$^+$CD4$^+$ T cells were gated among single cells, and IL-17A-EGFP$^+$ Th17 cells were then distinguished within CD3$^+$CD4$^+$ T cells. Compared with control healthy mice, the proportion of CD4$^+$ T cells was increased in conjunctivae but not in LN of mice with DED (Figure 1B). The number of Th17 cells were very low in healthy mice, accounting for about 1.5% of CD4$^+$ T cells in either conjunctivae or LN (Figure 1, A and C). However, the

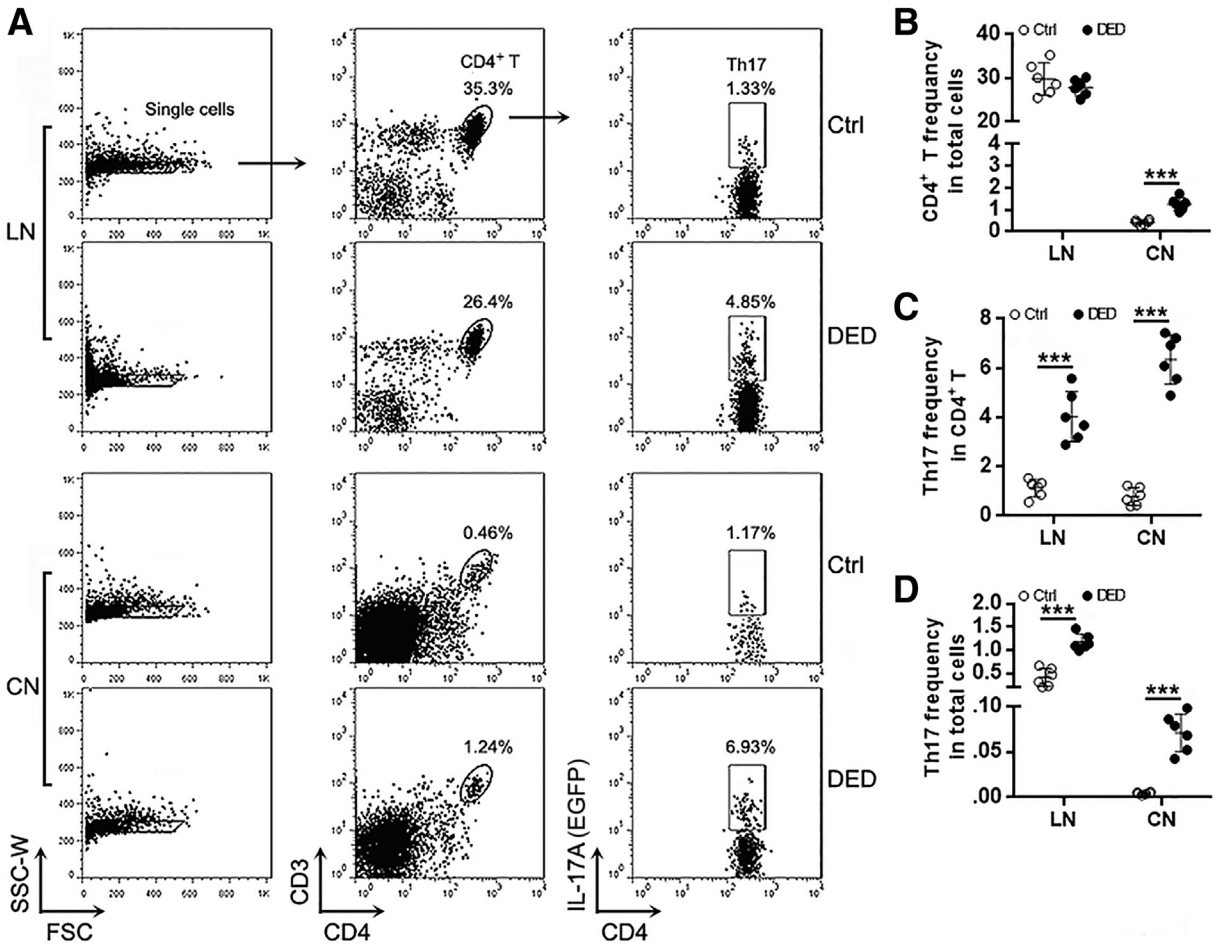

Figure 1 Presence of T helper 17 (Th17) cells in draining lymph nodes (LN) and conjunctivae (CN). **A:** Representative dot plots indicating Th17 cells. **B:** The frequency of $CD3^+CD4^+$ T cells in total isolated cells. **C:** Frequencies of Th17 cells in $CD3^+CD4^+$ T cells. **D:** Frequencies of Th17 cells in total isolated cells. $N = 6$ samples. $***P < 0.001$. Ctrl, control healthy mice; DED, mice with dry eye disease; FSC, forward scatter; SSC-W, side scatter pulse width.

proportion of Th17 cells in $CD4^+$ T cells was profoundly increased in both conjunctivae and LN in mice with DED, accounting for about 4% and 6% of $CD4^+$ T cells, respectively (Figure 1, A and C). The proportion of Th17 cells in total isolated cells was also increased after DED induction (Figure 1D). This change was also observed in wild-type mice (Supplemental Figure S1, B and C).

IL-17RE and CCR10 Label Th17 Subsets in DED

As shown in Supplemental Figure S2, in draining LN and conjunctivae of healthy mice, IL-17RE expression was positive but low on Th17 cells, whereas CCR10 was almost negative. After DED induction, IL-17RE was remarkably up-regulated, whereas CCR10 was moderately up-regulated on some Th17 cells both in LN and conjunctivae (Figure 2, A-C). Therefore, in DED, Th17 cells could be divided into two subsets: IL-$17RE^{low}CCR10^-$ Th17 and IL-$17RE^{high}CCR10^+$ Th17, respectively. Each subset accounted for about 50% of Th17 cells. Interestingly, each subset in LN expressed higher IL-17A than its counterpart in conjunctivae, whereas IL-$17RE^{high}CCR10^+$ Th17 expressed higher IL-17A than IL-$17RE^{low}CCR10^-$ Th17 in both in LN and conjunctiva (Figure 2, D and E).

The expression of master regulators key to the differentiation of helper T cells was then assessed. As shown in Figure 3A, in LN, IL-$17RE^{high}CCR10^+$ Th17 expressed more retinoic acid–related orphan receptor gamma t (RORγt) but fewer T-box-expressed-in-T-cells (T-bet) than IL-$17RE^{low}CCR10^-$ Th17. They expressed low and equivalent GATA Binding Protein 3 (GATA3) and Forkhead box protein P3 (FOXP3). A similar expression pattern was seen in conjunctivae (Figure 3B). Analysis of cytokine mRNA levels revealed that IL-$17RE^{high}CCR10^+$ Th17 expressed higher IL-17A, IL-21, and IL-22 but fewer IFN-γ than IL-$17RE^{low}CCR10^-$ Th17 in LN (Figure 3C). This was also the case in conjunctivae except that IL-22 was equivalently expressed in the two Th17 subsets (Figure 3D). The changes in the expression of RORγt, T-bet, IL-21, and IL-22 were validated by Western blot (Figure 3E) or flow cytometry (Figure 3F) analysis, respectively.

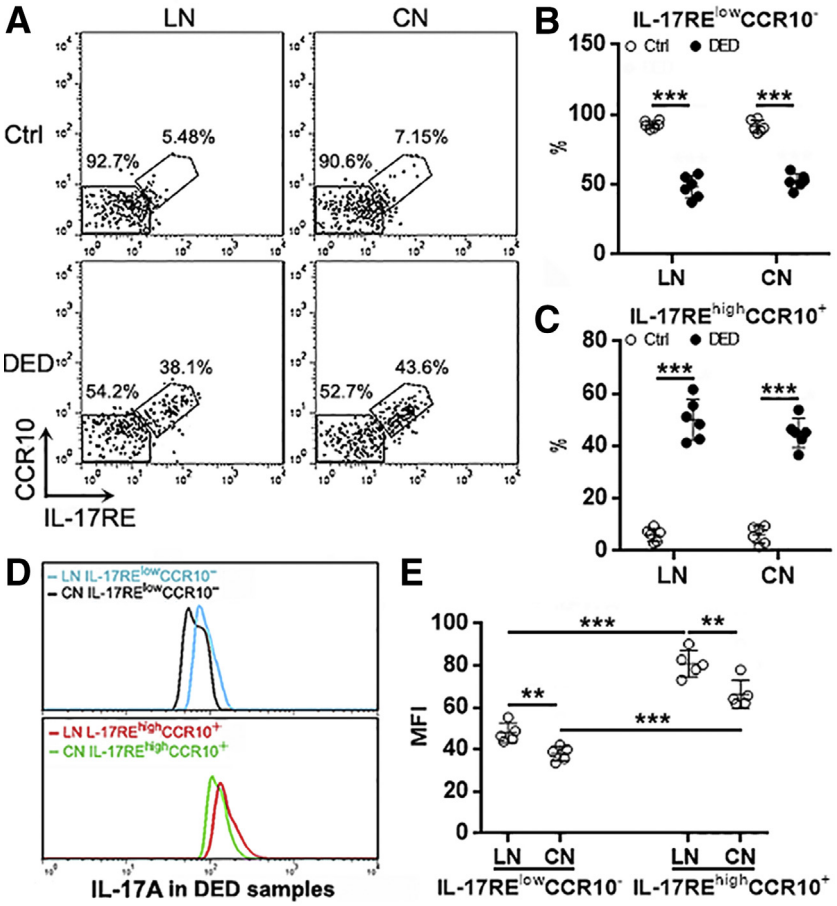

Figure 2 Expression of IL-17RE and CCR10 on T helper 17 (Th17) cells. **A:** Representative dot plots indicating the staining of IL-17RE and CCR10 on the surface of Th17 cells. **B** and **C:** Frequencies of IL-17RE^{low}CCR10^{-} Th17 (**B**) and IL-17RE^{high}CCR10^{+} Th17 (**C**) in total Th17 cells. **D:** Representative histograms indicating the intensity of IL-17A-EGFP in each Th17 subset isolated from draining lymph nodes (LN) and conjunctivae (CN) after dry eye disease (DED) induction. **E:** Statistics for (**D**). $N = 5$ to 6 samples. $^{**}P < 0.01$; $^{***}P < 0.001$. Ctrl, control healthy mice; MFI, mean fluorescence intensity.

IL-17RE^{low}CCR10^{-} Th17 Contains Th17/Th1 Cells

The aforementioned data suggest that IL-17RE^{high}CCR10^{+} Th17 were more like genuine Th17 cells, whereas IL-17RE^{low}CCR10^{-} Th17 might be Th17/Th1 cells. To test this hypothesis, intracellular IFN-γ in freshly isolated IL-17RE^{high}CCR10^{+} Th17 and IL-17RE^{low}CCR10^{-} Th17 was measured. As shown in Figure 4, A–C, in both LN and conjunctivae, very few IL-17RE^{high}CCR10^{+} Th17 expressed IFN-γ, whereas more than 10% of IL-17RE^{low}CCR10^{-} Th17 expressed IFN-γ. These cells were then stimulated with phorbol-12-myristate-13-acetate and ionomycin before IFN-γ measurement. Interestingly, 30% to 40% of IL-17RE^{low}CCR10^{-} Th17 expressed IFN-γ and only about 10% of IL-17RE^{high}CCR10^{+} Th17 expressed IFN-γ, respectively (Figure 4, D–F). Therefore, although both Th17 subsets were able to produce IFN-γ, IL-IL-17RE^{high}CCR10^{+} Th17 did not express IFN-γ *in vivo*, whereas IL-17RE^{low}CCR10^{-} Th17 contained IFN-γ–expressing Th17/Th1 cells.

IL-17RE^{high}CCR10^{+} Th17 Has Higher p38 MAPK and Jnk MAPK Signaling than IL-17RE^{low}CCR10^{-} Th17

IL-17C/IL-17RE axis has been reported to activate MAPK and NF-κB signaling.[13] The activating phosphorylation of NF-κB p65, p38 MAPK, and Jnk MAPK was analyzed in the two Th17 subsets. As shown in Figure 5, A and B, no significant difference in the level of phosphorylated NF-κB p65 was seen between the two Th17 subsets. However, more phosphorylated p38 MAPK and Jnk MAPK were found in IL-17RE^{high}CCR10^{+} Th17 relative to IL-17RE^{low}CCR10^{-} Th17 (Figure 5, A, C, and D).

IL-17C Sustains IL-17A Expression *in Vitro*

To test the role of IL-17RE in Th17 function, IL-17A-EGFP^{+} Th17 cells were cultured in the presence or absence of agonistic antibodies and IL-17C. About 95% of freshly sorted Th17 were IL-17A-EGFP^{+} (Supplemental Figure S3). After 24-hour culture either with or without the agonistic antibodies, the frequency of IL-17A-EGFP^{+} cells was remarkably decreased to about 40% when IL-17C was absent (Figure 6). However, when IL-17C was present in the culture, the frequency of IL-17A-EGFP^{+} cells remained 55% to 60%, respectively (Figure 6). CCR10 expression was not profoundly altered after the treatment (Supplemental Figure S4). Hence, IL-17C sustained IL-17A expression in Th17 cells.

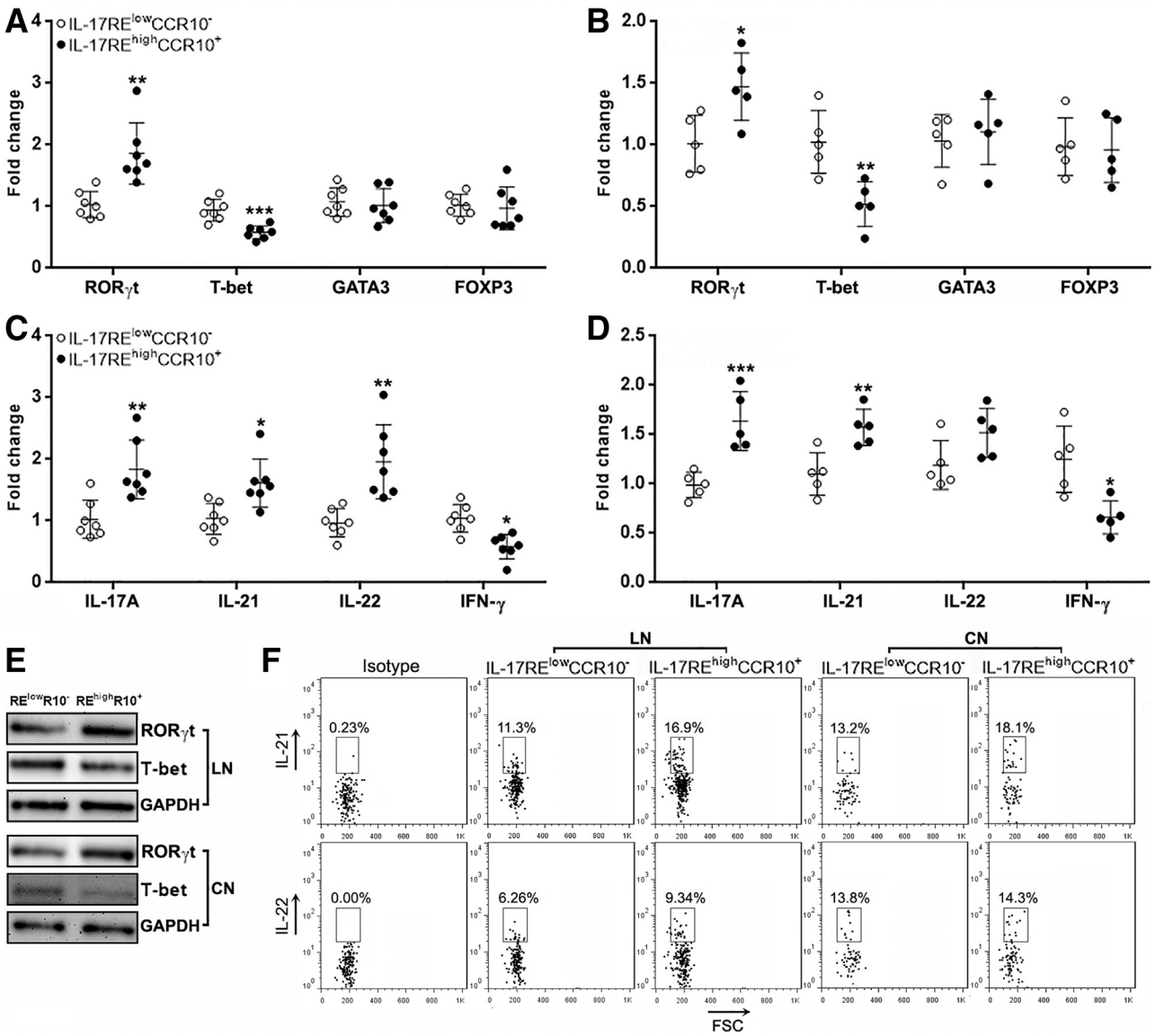

Figure 3 Transcripts of master regulators and cytokines in T helper 17 (Th17) subsets isolated from mice with dry eye disease. **A** and **B:** Fold changes of transcripts of indicated master regulators in Th17 subsets isolated from lymph nodes (LN) (**A**) and conjunctivae (CN) (**B**). **C** and **D:** Fold changes of transcripts of indicated cytokines in Th17 subsets isolated from LN (**C**) and CN (**D**). **E:** Western blot images of retinoic acid—related orphan receptor gamma t (RORγt) and T-box-expressed-in-T-cells (T-bet). $RE^{low}R10^{-}$: IL-$17RE^{low}CCR10^{-}$ Th17. $RE^{high}R10^{+}$: IL-$17RE^{high}CCR10^{+}$ Th17. **F:** Dot plots showing IL-21 and IL-22 expression in Th17 subsets isolated from LN and CN. $N = 5$ to 6 samples (**A-D**). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. FOXP3, Forkhead box protein P3; FSC, forward scatter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA3, GATA Binding Protein 3.

P38 MAPK Maintains IL-17A Expression in IL-$17RE^{high}CCR10^{+}$ Th17

To evaluate the roles of the p38 MAPK and Jnk MAPK in the maintenance of Th17 identity and function, IL-$17RE^{high}CCR10^{+}$ Th17 cells, in which p38 MAPK and Jnk MAPK were highly activated, were sorted. These cells were pretreated with the p38 MAPK inhibitor SB202190 or Jnk inhibitor SP600125, followed by activation and IL-17C treatment. Then, the expression of T-bet, RORγt, IFN-γ, and IL-17A was measured by using Western blot or flow cytometry. As indicated in Figure 7, A and B, no changes in T-bet and RORγt were found after treatment with either SB202190 or SP600125. In addition, IFN-γ expression was not influenced by either SB202190 or SP600125 (Figure 7, C and D). However, SB202190 down-regulated IL-17A expression while SP600125 could not (Figure 7, E—G). Therefore, it appears that p38 MAPK is important for IL-17A expression but dispensable for the expression of T-bet, RORγt, and IFN-γ. Jnk MAPK does not affect these molecules. Therefore, the key signaling essential for T-bet expression needs to be explored in the future.

The Two Th17 Subpopulations Equivalently Induce DED

To evaluate the pathogenicity of the two Th17 subpopulations, IL-$17(GFP)^{+}$IL-$17RE^{low}CCR10^{-}$ and IL-$17(GFP)^{+}$IL-$17RE^{high}CCR10^{+}$ Th17 cells were sorted from

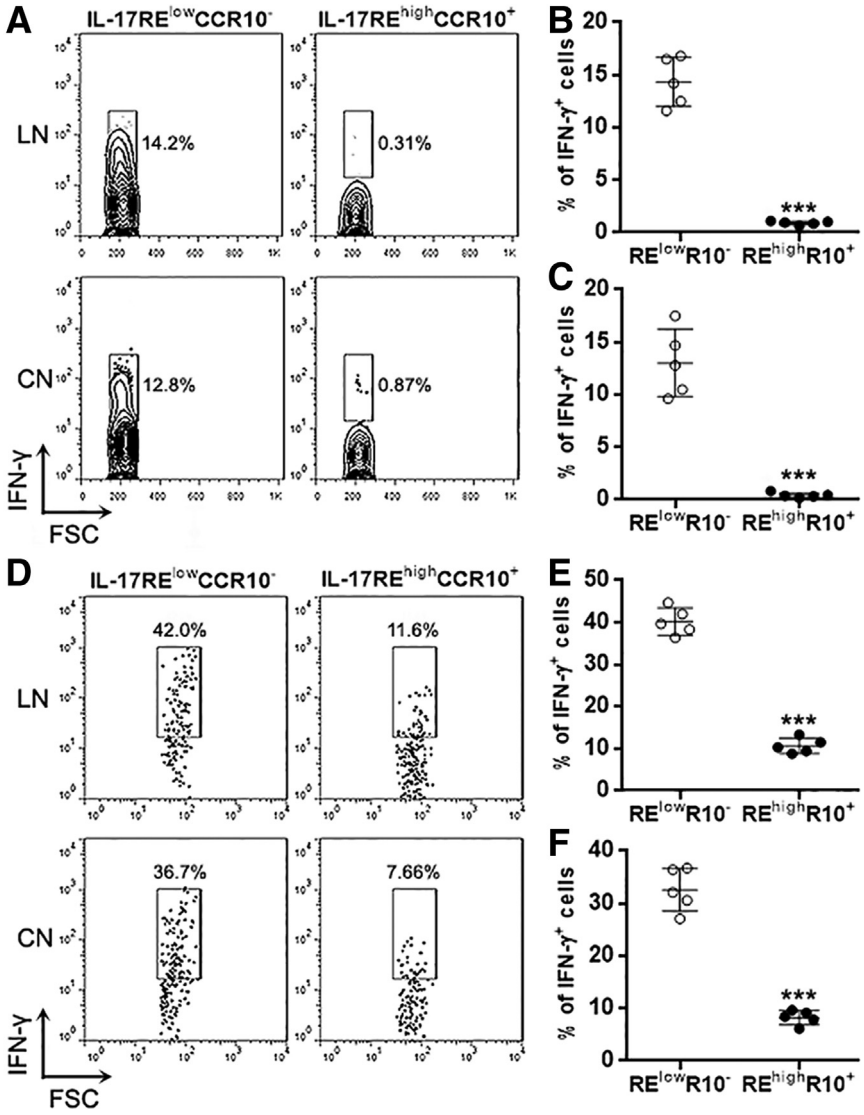

Figure 4 IFN-γ expression in T helper 17 (Th17) subsets isolated from mice with dry eye disease. **A:** Representative contour plots indicating the intracellular staining of IFN-γ in freshly isolated Th17 subsets. **B** and **C:** Frequencies of IFN-γ—producing cells in each Th17 subset isolated from draining lymph node (LN) (**B**) and conjunctivae (CN) (**C**). $RE^{low}R10^{-}$: IL-$17RE^{low}CCR10^{-}$ Th17. $RE^{high}R10^{+}$: IL-$17RE^{high}CCR10^{+}$ Th17. **D:** Representative dot plots indicating the intracellular staining of IFN-γ in Th17 subsets after 6 hours' stimulation with phorbol-12-myristate-13-acetate and ionomycin. **E** and **F:** Frequencies of IFN-γ—producing cells in each stimulated Th17 subset isolated from LN (**E**) and CN (**F**). $N = 5$ mice. $^{***}P < 0.001$. FSC, forward scatter.

draining LN of DED mice by flow cytometry, and then separately transferred into naive B6.Rag1 KO mice via retro-orbital injection, respectively. The recipients were placed in the DED-inducing environment for 10 days. Corneal fluorescein staining revealed comparable DED severity in B6.Rag1 KO mice receiving IL-$17(GFP)^{+}$IL-$17RE^{low}CCR10^{-}$ or IL-$17(GFP)^{+}$IL-$17RE^{high}CCR10^{+}$ Th17 cells (Figure 8A and Supplemental Figure S5). Comparable infiltrating T cells were found in conjunctivae of IL-$17RE^{low}CCR10^{-}$ Th17 and IL-$17RE^{high}CCR10^{+}$ Th17 recipients (Figure 8, B and C). The phenotype of these T cells was then analyzed. Interestingly, around 20% of exogenous IL-$17RE^{high}CCR10^{+}$ Th17 cells became IL-$17RE^{low}CCR10^{-}$ Th17 cells in conjunctivae, whereas no exogenous IL-$17RE^{low}CCR10^{-}$ Th17 cells changed their phenotype (Figure 8, D and E). Exogenous IL-$17RE^{low}CCR10^{-}$ Th17 cells still expressed higher IFN-γ but lower IL-17A than exogenous IL-$17RE^{high}CCR10^{+}$ Th17 cells (Figure 8, F and G). Therefore, the two Th17 subpopulations had the equivalent capability to induce DED, whereas IL-$17RE^{high}CCR10^{+}$ Th17 cells can polarize to IL-$17RE^{low}CCR10^{-}$ Th17 cells *in vivo*.

Discussion

The presence of Th17 cells, including IL-10—producing Th17 and IL-17^{+}IFN-γ^{+} Th17/Th1 cells, has been described in humans and mice with DED in earlier reports.[10–12] Importantly, a previous DED study discovered a significantly pathogenic IFN-γ^{+} Th17/Th1 population derived from Th17 precursors. Adoptive transfer of these Th17/Th1 cells can induce DED to naive animals as effectively as Th17 cells alone. Furthermore, IL-12 and IL-23 are essential for the transition of pathogenic Th17 cells to Th17/Th1 cells.[14] In the chronic stage, both Th17 and

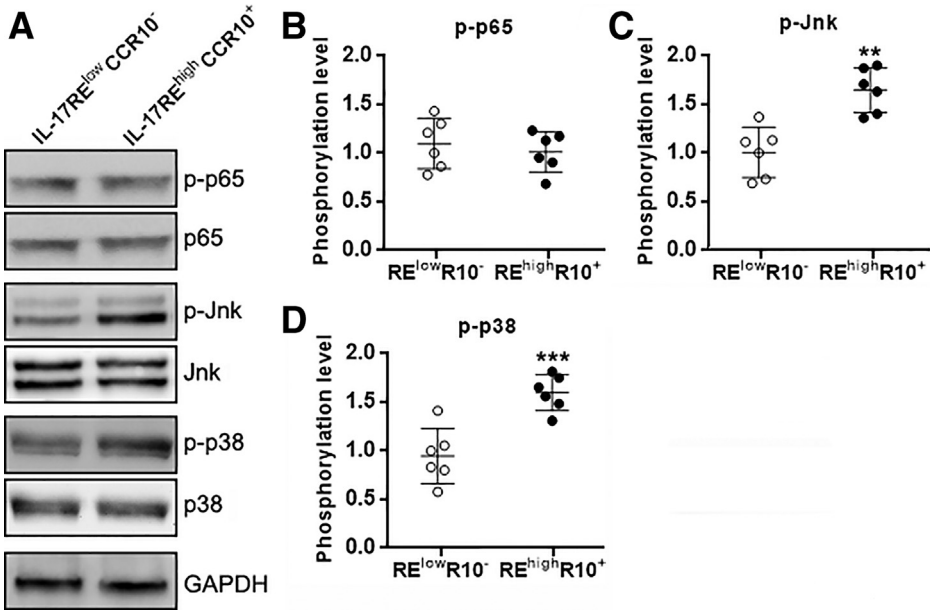

Figure 5 Activation status of mitogen-activated protein kinase (MAPK) and NF-κB pathways in T helper 17 (Th17) subsets isolated from mice with dry eye disease. **A:** Representative Western blot images showing the phosphorylation of indicated signaling proteins. $RE^{low}R10^{-}$: IL-$17RE^{low}CCR10^{-}$ Th17. $RE^{high}R10^{+}$: IL-$17RE^{high}CCR10^{+}$ Th17. **B**–**C:** Statistics of the phosphorylation levels of NF-κB p65 (**B**), Jnk MAPK (**C**), and p38 MAPK (**D**). $N = 6$ samples. $^{**}P < 0.01$; $^{***}P < 0.001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Th17/1 subsets develop into memory Th17 cells that mediate the chronic inflammation in DED. Th17/1 cells produce IL-17A, IFN-γ, and granulocyte-macrophage colony-stimulating factor to mediate the apoptosis of corneal epithelial cells, enhance lymphangiogenesis, and promote the maturation of immature antigen-presenting cells, thus contributing to ocular surface inflammation.[15] These studies exhibit Th17 heterogeneity/plasticity in DED. However, whether heterogeneous Th17 subpopulations in DED can be identified by cell surface markers for future clinical investigations is unclear. The current study found two Th17 subsets cells in terms of the expression of IL-17RE and CCR10: IL-$17RE^{low}CCR10^{-}$ Th17 and IL-$17RE^{high}CCR10^{+}$ Th17. IL-17RE is a receptor discovered in 2006 on epithelial cells of the lung, kidney, stomach, intestine, and testis of mice.[16] IL-17RE is also expressed on Th17 cells and is strongly promoted upon stimulation with IL-6, transforming growth factor-β, IL-1, IL-23, and IL-17C.[17] The data indicated that IL-17RE was negative or weakly expressed on IL-17A-GFP^{+} Th17 cells in healthy mice but increased in mice with DED, suggesting that the pro-inflammatory milieu in

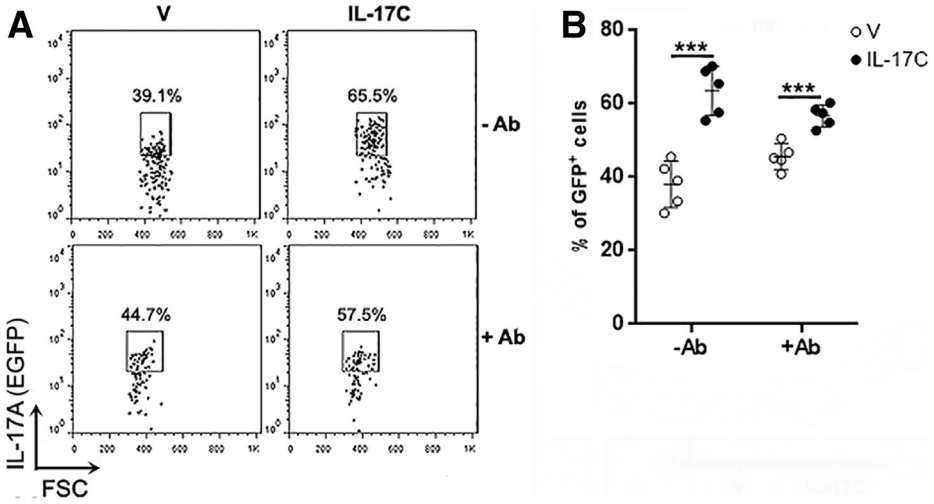

Figure 6 IL-17A expression after *in vitro* culture of T helper 17 cells. **A:** Representative dot plots indicating IL-17A-$EGFP^{+}$ cells after 24-hour culture of LN T helper 17 cells. **B:** Statistics of the frequencies of IL-17A-$EGFP^{+}$ cells after culture. $N = 5$ samples. $^{***}P < 0.001$. −Ab, without agonistic antibodies; +Ab, with agonistic antibodies; FSC, forward scatter; V, with vehicle (phosphate-buffered saline).

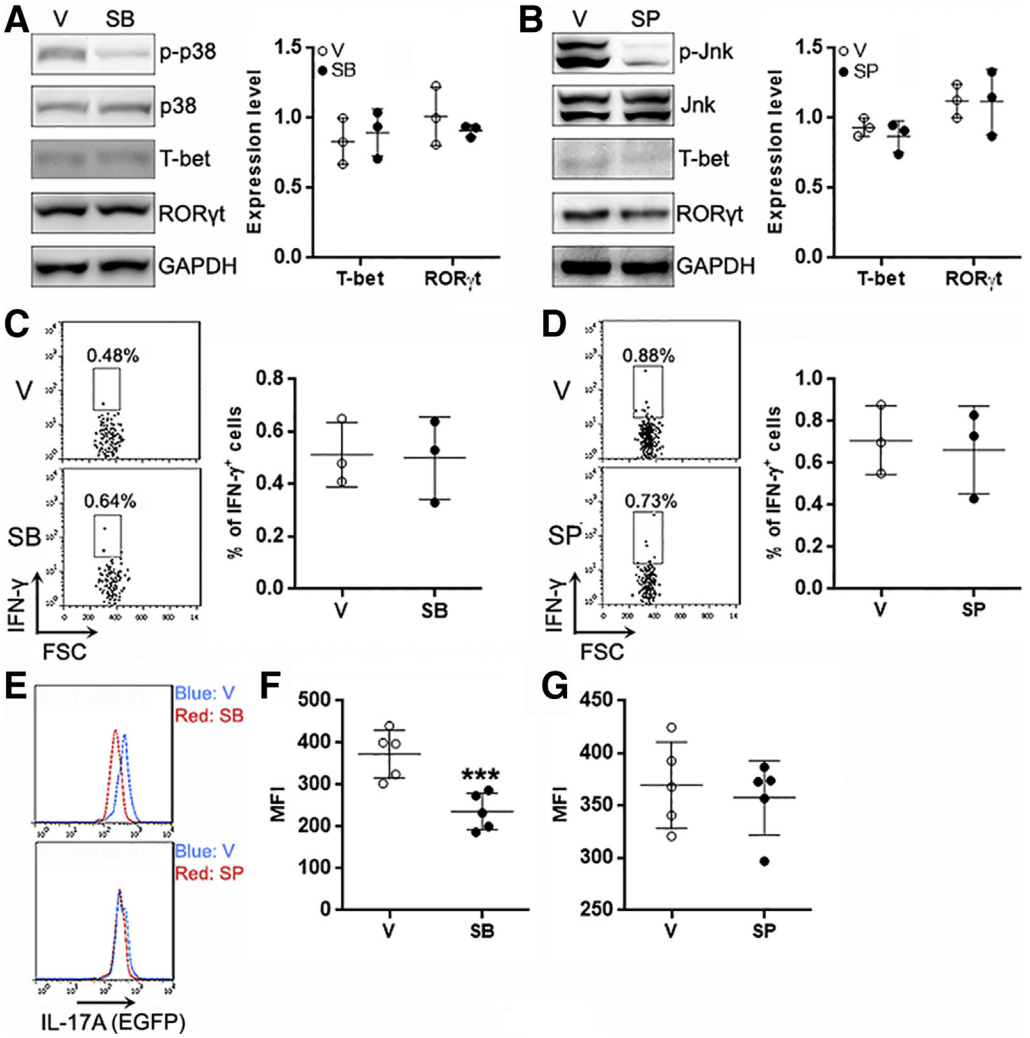

Figure 7 The effect of p38 mitogen-activated protein kinase (MAPK) and Jnk MAPK on IL-17RE^{high}CCR10^{+} T helper 17 (Th17) cells. **A:** Expression of T-box-expressed-in-T-cells (T-bet), retinoic acid–related orphan receptor gamma t (RORγt), and phosphorylated p38 MAPK in IL-17RE^{high}CCR10^{+} Th17 cells after SB202190 (SB) treatment. **Left panel:** Western blot images. **Right panel:** Statistics of the relative expression levels of T-bet and RORγt. **B:** Expression of T-bet, RORγt, and phosphorylated Jnk MAPK in IL-17RE^{high}CCR10^{+} Th17 cells after SP600125 (SP) treatment. **Left panel:** Western blot images. **Right panel:** Statistics. (**C** and **D**) Expression of IFN-γ after SB treatment (**C**) or SP treatment (**D**). **Left panel:** Dot plots. **Right panel:** Statistics. **E**–**G:** IL-17A (GFP) expression in IL-17RE^{high}CCR10^{+} Th17 cells. Histograms are shown in (**E**). Statistics of the mean fluorescence intensity (MFI) of IL-17A (GFP) are shown in (**F**) and (**G**). $N = 3$ or 5 samples. $***P < 0.001$. FSC, forward scatter; V, dimethyl sulfoxide.

draining LN and conjunctivae induced IL-17RE expression during DED progression. The presence of IL-6, transforming growth factor-β, IL-1, IL-23, and IL-17C in draining LN and conjunctivae will be measured in future studies, with the goal of identifying the factors crucial for the up-regulation of IL-17RE.

The expression of CCR10 on Th17 cells was unexpected. Prior studies indicate that CCR10 is expressed on T helper 22 (Th22) cells, but not Th17 cells.[18] Th17 cells are induced by IL-6 and transforming growth factor-β, whereas Th22 cells are generated by IL-6, IL-1β, and TNF-α.[19,20] Interestingly, Th22 cells have been proposed as more pathogenic Th17 cells producing not only IL-17A and IL-17F but also IL-22 and granulocyte-macrophage colony-stimulating factor.[20] Therefore, the temporal and/or spatial differences in the levels of pro-inflammatory mediators in draining LN and conjunctivae might generate both CCR10^{+} Th22 cells and CCR10^{-} Th17 cells. It is also possible that Th17 cells and Th22 cells reciprocally differentiate into one another when they are educated by various cytokines. Therefore, the differential CCR10 expression on IL-17A-GFP^{+} CD4^{+} T cells might reflect the unclear Th17 plasticity. In the future, transcriptome sequencing or other molecular tools will be used to further dissect IL-17A-GFP^{+} CD4^{+} T cells and clarify the Th17 plasticity in the current experimental setting.

IL-17RE^{high}CCR10^{+} Th17 expressed higher Th17-related cytokines and RORγt but lower IFN-γ and T-bet than IL-17RE^{low}CCR10^{-} Th17. This suggests that IL-17RE^{high}CCR10^{+} Th17 are more like bona fide Th17 cells, whereas IL-17RE^{low}CCR10^{-} Th17 cells contain Th17/Th1

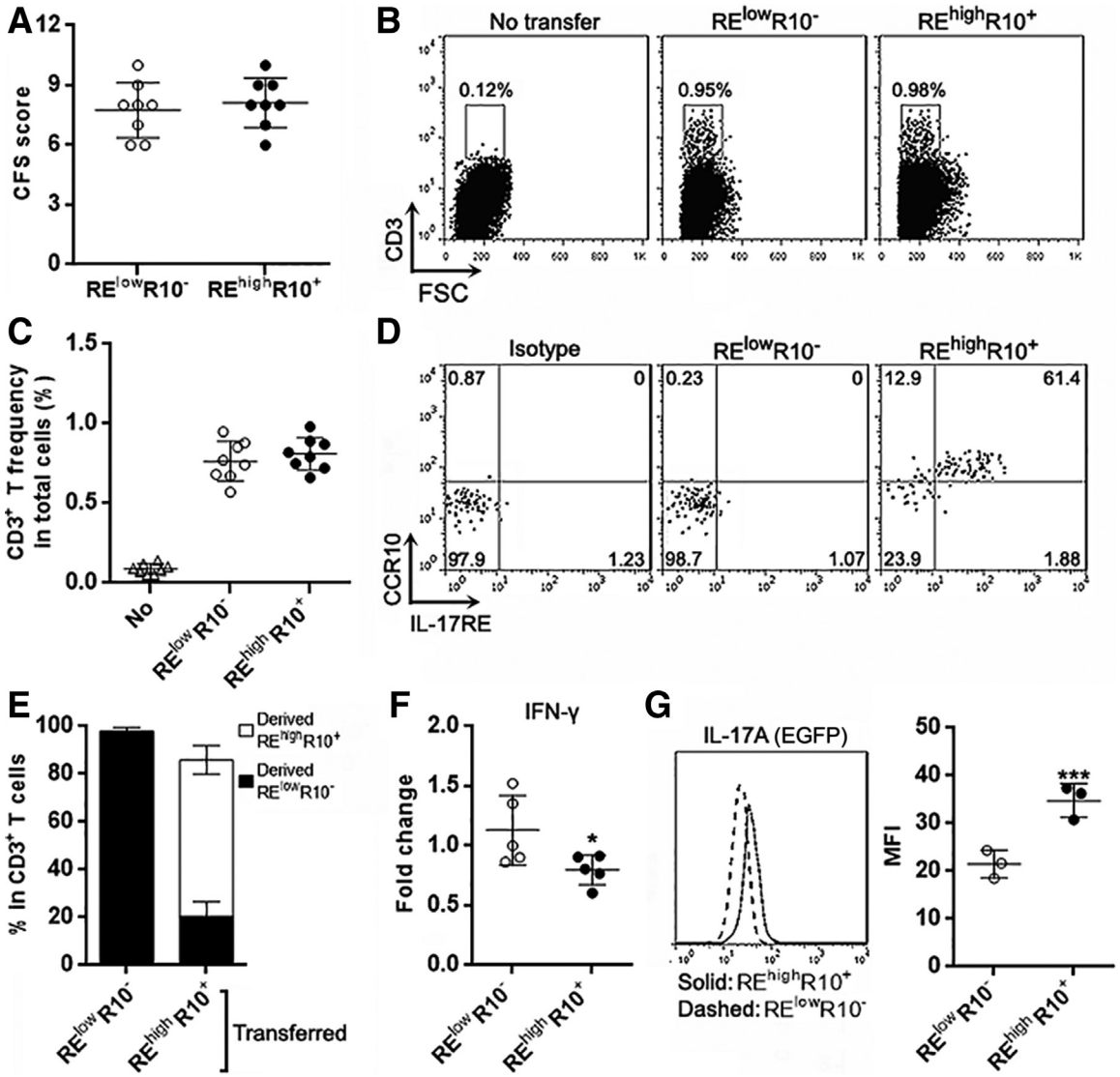

Figure 8 Pathogenicity of the two T helper 17 (Th17) subpopulations. **A:** Corneal fluorescein staining (CFS) scores after adoptive transfer and dry eye disease induction. $RE^{low}R10^{-}$: IL-$17RE^{low}CCR10^{-}$ Th17 recipients. $RE^{high}R10^{+}$: IL-$17RE^{high}CCR10^{+}$ Th17 recipients. **B** and **C:** The proportion of infiltrating $CD3^{+}$ T cells in conjunctivae. Representative dot plots are shown in (**B**), and statistics are shown in (**C**). **D** and **E:** Phenotype change of exogenous Th17 subpopulations. Representative dot plots are shown in (**D**), and statistics are shown in (**E**). Isotype: isotype control. **F:** Transcript levels of IFN-γ in exogenous IL-$17RE^{low}CCR10^{-}$ Th17 and IL-$17RE^{high}CCR10^{+}$ Th17 cells. **G:** IL-17A (GFP) expression in exogenous IL-$17RE^{low}CCR10^{-}$ Th17 and IL-$17RE^{high}CCR10^{+}$ Th17 cells. **Left panel:** Representative histograms. **Right panel:** Statistics. $N = 3$ to 8 samples per group. $^{*}P < 0.05$; $^{***}P < 0.001$. FSC, forward scatter; MFI, mean fluorescence intensity.

cells, supporting a previous study identifying IL-17^{+}IFN-γ^{+} Th17/Th1 cells in draining LN of aged mice with DED.[12] This finding also indicates the complicated Th17 plasticity in DED. Th17/Th1 cells, alias Th1-like Th17 cells, co-express RORγt and T-bet and produce both IL-17 and IFN-γ. Recent reports have shown that Th17/Th1 cells play active roles in the pathogenesis of autoimmune diseases such as inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis.[21] It is thus likely that Th17/Th1 cells also cause damages to the eyes during the development of DED. Nevertheless, not all IL-$17RE^{low}CCR10^{-}$ Th17 cells expressed IFN-γ even under the stimulation of phorbol-12-myristate-13-acetate and ionomycin, suggesting that Th17/Th1 cells are only a part of IL-$17RE^{low}CCR10^{-}$ Th17 cells and other unidentified Th17 subsets might exist in IL-$17RE^{low}CCR10^{-}$ Th17 cells. Hence, further investigations are still needed to distinguish each Th17 subset in LN and conjunctivae.

Consistent with the high expression of IL-17RE, higher activation of Jnk and p38 MAPK was found in IL-$17RE^{high}CCR10^{+}$ Th17 relative to IL-$17RE^{low}CCR10^{-}$ Th17. As stated above, IL-17RE and IL-17A form a receptor complex to engage with IL-17C. The IL-17C/IL-17RE axis activates the MAPK and NF-κB pathways to induce the expression of IL-17A, IL-17F, and IL-22 in Th17 cells.[13] Although no significant enhancement of NF-κB p65 phosphorylation was seen, the promoted phosphorylation of Jnk

and p38 MAPK likely represents the effect of IL-17C on IL-17RE^{high}CCR10^{+} Th17. Moreover, *in vitro,* IL-17C treatment notably maintained IL-17A expression in IL-17RE^{high}CCR10^{+} Th17, further confirming the significant role of the IL-17C/IL-17RE axis in sustaining Th17 identity. However, this poses a question of whether IL-17C is expressed in draining LN or conjunctivae. Under a steady condition, IL-17C is expressed by epithelial cells and not by hematopoietic cells.[13] In a colitis model, IL-17C expression was found in mesenteric LN.[22] IL-17C might also be expressed in draining LN during DED. In addition, to our knowledge, no previous evidence has shown IL-17C expression in conjunctivae either in normal or DED mice. To answer this question, we plan to measure *IL17C* mRNA levels in draining LN and conjunctivae before confirming the protein expression with flow cytometry or immunohistochemistry.

It should be noted that scopolamine was used in this DED model. Systemic administration of scopolamine attenuates Th17 activity while promoting Th2 and regulatory T-cell reactions.[23] Although there is no previous evidence showing the impact of scopolamine on the expression of IL-17RE and CCR10, a better DED model should be adopted in future studies on Th17 cells to avoid the potential influence of scopolamine. It is reported that exposure to desiccating environmental stress triggers greater Th17 cell activity and regulatory T-cell dysfunction.[23] Therefore, a desiccating environment—induced DED model would benefit Th17 research.

According to the current data, adoptive transfer of DED mouse—derived Th17 cells to T cell—deficient mice induced DED in the recipients within 10 days even without administration of scopolamine hydrobromide. In contrast, the passively induced DED (ie, DED induced by desiccation and scopolamine hydrobromide) needed 15 days to become evident in our settings. This suggests that pathogenic Th17 cells drove DED development faster than desiccation. Indeed, previous studies have shown that CD4^{+} T cells from DED mice were sufficient to mediate DED in nude mice within just 3 days after adoptive transfer.[24,25] Another study showed that transferred Th17 cells triggered DED in a desiccating environment within 5 days, faster than the desiccating environment alone that took 14 days to induce DED.[14] Therefore, pathogenic CD4^{+} T cells including Th17 cells are very efficient in inducing DED, implying their fundamental contribution to DED development.

Administration of cyclosporine A is an ophthalmic therapeutic for DED through blocking T-cell activation and the subsequent release of inflammatory cytokines.[26] IL-17 production by Th17 cells and Th17 differentiation can be inhibited by cyclosporine A.[27,28] Furthermore, cyclosporine A can suppress the transition of IL-17A^{+}IFNγ^{-} Th17 cells toward IL-17A^{+}IFNγ^{+} Th17 cells in allogeneic stem cell transplantation.[29] Because IL-17RE^{high}CCR10^{+} Th17 cells and IL-17RE^{low}CCR10^{-} Th17 cells in the current experimental settings had equivalent DED-inducing ability, it seems plausible that cyclosporine A would effectively suppress the adoptively transferred DED regardless of which subpopulation is transferred. Therefore, the efficacy of cyclosporine A might be influenced by its permeability into the conjunctiva and the tear clearance rate but not by the predominance of Th17 subpopulations.

Taken together, this study shows the presence of heterogeneous Th17 subsets in draining LN and conjunctivae of mice with DED. The current study deepens the understanding of Th17 pathogenicity and suggests Th17 plasticity in DED development. With the recognition of differential functions of Th17 subsets and the underlying regulatory mechanisms, the pathogenesis of DED can be clarified and novel treatments can be designed.

Author Contributions

P.W. designed the research, supervised the study, and wrote the manuscript; J.W. conducted most experiments and analyzed data; J.G. carried out *in vitro* culture experiments; Q.Y. performed Western blot;. L.W. and Y.J. performed RNA extraction, cDNA preparation, and RT-qPCR.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2021.10.021*.

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