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IFN-γ–Expressing Th17 Cells Are Required for Development of Severe Ocular Surface Autoimmunity

Yihe Chen, Sunil K. Chauhan, Chunyi Shao, Masahiro Omoto, Takenori Inomata, and Reza Dana

Th17 cells are critical effectors mediating the ocular surface autoimmunity in dry eye disease (DED). Increased IFN-γ has also been implicated in DED; however, it remains unclear to what extent Th1 cells contribute to DED pathogenesis. In this study, we investigated the cellular source of IFN-γ and assessed its contribution to corneal epitheliopathy in DED mice. We discovered a significant IL-17A+IFN-γ+ (Th17/1) population and determined that these cells are derived from Th17 precursors. Adoptive transfer of Th17/1, but not Th1, cells confers the disease to naive recipients as effectively as do Th17 cells alone. DED-induced IL-12 and IL-23 are required for in vivo transition of pathogenic Th17 cells to IFN-γ producers. Furthermore, using IFN-γ–deficient Th17 cells, we demonstrate the disease-amplifying role of Th17-derived IFN-γ in DED pathogenesis. These results clearly demonstrate that Th17 cells mediate ocular surface autoimmunity through both IL-17A and IFN-γ.

Materials and Methods

Animals

Female 6- to 8-wk-old wild-type (WT) C57BL/6 mice (Charles River Laboratories), B6.Rag1 KO mice, and B6.IFN-γ KO mice (The Jackson Laboratory) were used for this study. All animal experiments were approved by the Schepens Eye Research Institute Animal Care and Use Committee and adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

DED induction

DED was induced in mice as previously described (9). In brief, mice were placed in a controlled-environment chamber with a relative humidity <20%, airflow of 15 l/min, and a constant temperature of 21–23°C for 14 consecutive days. Thereafter, mice were transferred to the standard nondesiccated vivarium, where mice were maintained for an additional 4 mo. Corneal epithelial disease was evaluated using fluorescein (Sigma-Aldrich) staining and scored using the National Eye Institute (Bethesda, MD) grading system.

Histology

The whole eyeball was excised and fixed in 10% formalin for fixation. After dehydration, the specimens were embedded in methacrylate, cross-sectioned, and stained with H&E. The morphology of the cornea and the conjunctiva was observed under a microscope (Nikon Eclipse E800) with a ×40 objective.

Flow cytometry analysis

Conjunctiva tissues were first digested in RPMI 1640 (Invitrogen) with 2 mg/ml DNase and 2 mg/ml collagenase (Roche) at 37°C. The following

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The online version of this article contains supplemental material.

Abbreviations used in this article: DED, dry eye disease; KO, knockout; Th17/1, IL-17A+IFN-γ+; WT, wild-type.

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Abs were used for flow cytometry analysis: FITC-conjugated anti-CD3, FITC-conjugated anti-CD4, PerCP-Cy5.5– or allophycocyanin-conjugated anti–IFN-γ (BioLegend), and PE-Cy7– or PE-conjugated anti–IL-17A (eBioscience). For intracellular IL-17A staining, cells were stimu-
lated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) for 6 h at 37°C and 5% CO2 in the presence of GolgiStop (4 µl/ml cell culture; BD Biosciences) to inhibit cytokine secretion. Stained cells were examined with an LSR II flow cytometer (BD Biosciences), and the results were analyzed using FlowJo software (Tree Star).

T cell adoptive transfer

Draining lymph node cells from DED mice were harvested and their CD4+ T cells were enriched via negative selection with a CD4+ T cell isolation kit (Miltenyi Biotec). Thereafter, Th17 (IL-17A+IFN-γ+), Th17/1 (IL-17A+IFN-γ+), and Th1 (IL-17A+IFN-γ−) subsets were further sorted using IL-17A and IFN-γ cytokine secretion assay kits (Miltenyi Biotec) and a BD FACSaria sorter (BD Biosciences). Each of these subsets (2 × 106 cells) was subsequently injected i.v. into naive B6.Rag1 KO mice, which were then placed in the controlled-environment chamber for 5 d. In the Ab treatment studies, sorted Th17 cells were injected i.v. into naive B6.Rag1 KO mice, which were then placed in the controlled-environment chamber for 5 d. These recipient mice were injected i.p. with 200 µg of anti–IL-12 (R&D Systems), 100 µg of anti–IL-23R (R&D Systems), 100 µg of anti–IL-12p40 Abs (R&D Systems), or 100 µg of isotype IgG (R&D Systems) 1 d before and 1 d after the cell transfer. Disease severity was evaluated using corneal fluorescein staining described above.

Real-time PCR

Conjunctivae from mice were harvested, frozen in TRizol reagent (Invi-
trogen), and stored at −80°C until use. Total RNA was isolated with an RNaseasy Micro Kit (Qiagen) according to the manufacturer’s recommen-
dations and reverse transcribed using a SuperScript III kit (Invitrogen). Real-time PCR was performed using TaqMan Universal PCR Master mix and predesigned primers for IFN-γ (Mm01168134_m1) and GAPDH (Mm99999915_g1) (Applied Biosystems) in a LightCycler 480 II system (Roche Applied Science). The GAPDH gene was used as an endogenous control for each reaction. The results of quantitative PCR were analyzed by the comparative CT method in which the target change = 2ΔΔCT. The results were normalized by the C T value of GAPDH, and the mean C T of relative mRNA level in the control IgG group was used as the calibrator.

ELISA

For protein extraction, draining lymph nodes were harvested and stored in cold sterile PBS containing protease inhibitors (Sigma-Aldrich) at −80°C until used. The samples were homogenized on ice and centrifuged. The supernatant was assayed using commercial ELISA kits for levels of the total protein (Thermo Scientific), IL-12, and IL-23 (eBioscience).

Statistical analyses

A Mann–Whitney U test was used with the software Prism 5, and differ-
ences were considered significant at p < 0.05.

Results

Presence of Th17/1 cells in severe DED

Acute and chronic DED was induced using our well-established murine models, and disease severity peaked at acute day 14 (Fig. 1A). The ocular surface showed characteristic change in corneal epithelial thickness and decreased numbers and atrophy of conjunctival goblet cells at day 14 (Fig. 1B). We further investigated IFN-γ single producer (Th1), IL-17A single producer (Th17), and IL-17A and IFN-γ producer (Th17/1) at multiple time points by flow cytometry (Fig. 1C). In the eye-draining lymph nodes, Th17 cells occurred during the initial disease induction phase and continuously in-
creased as the disease exacerbated. Following the emergence of Th17 cells, we observed double-positive Th17/1 cells as the disease severity exacerbated. When the disease entered a chronic stage (~4 mo) the Th17 frequency decreased, but it still persisted at high levels. These Th17 cells have been previously identified as memory Th17 cells, which principally mediate the chronicity of DED (9). During this stage when DED is characterized as persisting low-grade inflammation on the ocular surface (9), the Th17 frequency diminished (Fig. 1D). When these chronic DED mice were

rechallenged with desiccating stress, Th17/1 cells increased again, along with exacerbated disease (Supplemental Fig. 1). The same ki-netic patterns of Th17 and Th17/1 cells were observed at the inflamed ocular surface (conjunctiva) (Fig. 1E). In contrast, Th1 cell frequen-
cies remained unchanged throughout the course of DED (Fig. 1C). Th17/1 cells isolated from DED are capable of inducing ocular surface inflammation

The pathogenicity of Th17 cells in DED has been demonstrated previously by blocking IL-17A in vivo (5, 7, 9). In the present study, we adoptively transferred Th subsets from DED mice into T cell–deficient naive Rag1 KO mice to determine their function. Th1, Th17, and Th17/1 cells were isolated (Supplemental Fig. 2) at day 14 after the development of severe DED, and they were then i.v. transferred to Rag1 KO recipients, which were subse-
sequently challenged with desiccating environmental stress for 5 d. Rag1 KO mice without adoptive transfer served as controls and showed no disease after 5 d of desiccating stress (mean disease score, 1.38 out of 15 points), confirming the central role of T cells in inducing corneal epitheliopathy in DED (Fig. 2A, 2B). Simi-
larly, mice adoptively transferred with single-positive Th1 cells developed no disease as well. In contrast, both Th17 and Th17/1 recipients exhibited significantly higher disease scores than did control or Th1 recipients. There were no significant differences in the disease severity between Th17 and Th17/1 recipients (Fig. 2A, 2B). We also found increased T cell infiltration in the conjunctiva of Th17 and Th17/1 recipients, but not in the control or Th1 re-
cipients (Fig. 2C). No T cells were detected in the draining lymph node as well in Th1 recipients (Supplemental Fig. 3), suggesting that Th17 cells isolated from DED were not disease-specific ef-
tectors and unable to migrate to target tissues.

Pathogenic Th17 cells are plastic and convert into IFN-γ producers via support of IL-12 and IL-23

In vitro–polarized Th17 cells have been shown to give rise to IFN-
γ-producing cells after adoptive transfer in a murine model of colitis (12, 13). To investigate whether in vivo spontaneously gen-
erated pathogenic Th17 cells from DED have the similar plastic capacity developing into double-positive Th17/1 cells, we adopt-
ively transferred IL-17A* single-positive Th17 cells to Rag1 KO mice and induced DED. After 5 d we analyzed the draining lymph nodes for IFN-γ-producing T cells by flow cytometry. We found that ~50% of Th17 cells converted into IL-17A/IFN-γ double producers (Th17/1) after being adoptively transferred to the Rag1 KO mice (Fig. 3A, upper panel, 3B). Meanwhile, the other group of naive Rag1 KO mice receiving Th17 cells was housed in the normal environment without DED induction. After 5 d a milder ocular surface epitheliopathy was developed (Fig. 3C) along with only ~20% of Th17 cells becoming IFN-γ–producing cells (Fig. 3A, lower panel, 3B). Additionally, a substantial part of transferred Th17 cells lost the expression of IL-17A in both with desiccating stress (~30%) and without desiccating stress (~65%) groups, suggesting that subpopulations of purified DED Th17 cells might have different capacity for stable IL-17A expression and additional IFN-γ acquisition. We further examined IL-12 and IL-23 cytokine levels in the draining lymph nodes. DED-induced recipients exhibited a significant 2-fold upregulation of both IL-12 and IL-23 mRNA than did those without being subjected to desiccating stress (Fig. 3D). Protein levels of these two cytokines in DED-induced recipients were also consistently increased (Fig. 3E).

IL-12 and IL-23 are required for Th17 conversion to IFN-γ producers

We next delineated whether the DED-induced IL-12 and IL-23 are responsible for the acquisition of IFN-γ in DED-pathogenic
Th17 cells. It has been reported that both IL-12 and IL-23 are essential for enhanced IFN-γ production by in vitro–polarized Th17 cells (12, 14, 15), and Th17 cells from IL-23p19–deficient IL-17A reporter mice completely lack IL-17A/IFN-γ double-positive or IFN-γ single-positive cells (16). To determine the roles of IL-12 and IL-23 in IFN-γ secretion in vivo–induced, disease-specific Th17 cells, we isolated Th17 cells from DED mice and adoptively transferred them into Rag1 KO mice. The recipient mice were

**FIGURE 1.** Increased IFN-γ+ Th17 cells (Th17/1) in DED. Frequencies of Th1, Th17, and Th17/1 cells were examined by flow cytometry throughout the course of DED (normal, acute, and chronic stages). (A) DED severity was evaluated by corneal fluorescein staining scores at different stages (n = 20 eyes per time point). *p < 0.05 as compared with day 0. (B) Pathological changes of corneal (upper panel) and conjunctival epithelium (lower panel) in different stages of DED. Ocular surface in DED by H&E staining is characterized by change in epithelial thickness in the cornea and loss of goblet cells (marked as G) in the conjunctiva. (C) Representative flow cytometry plots show cell frequencies gated on CD4+ cells. (D) Kinetic changes of Th17 (blue line) and Th17/1 (red line) cells in eye-draining lymph nodes (DLN) during the course of disease are summarized as mean ± SEM from one representative experiment out of three performed (n = 4 mice per time point per group). (E) Kinetic changes of the Th17 (blue line) and Th17/1 (red line) cells in conjunctivae (CONJ) are summarized as mean ± SEM from all three experiments with four to six eye tissues pooled together as one flow cytometry sample (n = 4 samples per time point per group). *p < 0.05 as compared with day 0.

**FIGURE 2.** Both Th17 and Th17/1, but not Th1, cells isolated from DED are pathogenic. Severe DED was induced for 14 d in WT mice, and then Th1, Th17, and Th17/1 cells were isolated from draining lymph nodes. Cells (1 × 10⁶) from each subset were i.v. injected into Rag1 KO mice. Immediately after the adoptive transfer, these Rag1 KO mice were subjected to desiccating stress for 5 d. (A) Clinical disease severity was evaluated by corneal fluorescein staining and representative images show baseline (day 0) and 5 d after adoptive transfer. (B) DED scores in each group at day 5 are summarized as mean ± SEM in bar graphs (n = 6–8 eyes per group). *p < 0.05 as compared with no adoptive transfer or Th1 adoptive transfer group. (C) Representative flow cytometry dot plots from three separate experiments (four to six eye tissues pooled together for each group) show increased infiltration of transferred T cells in the conjunctivae (CONJ) in mice with adoptive transfer of Th17 and Th17/1 cells. AT, adoptive transfer.
challenged with desiccating environmental stress for 5 d and treated with anti–IL-12, anti–IL-23R, anti–IL-12p40 Abs, or control IgG 1 d before and 1 d after the transfer of Th17 cells. All three Ab treatments led to a substantial reduction of IFN-γ+ T cells, especially Th17/1 cells. Anti–IL-23R and anti–IL-12p40 treatments resulted in the most significant reduction of Th17/1 cells (Fig. 4A). Correspondingly, the development of ocular surface inflammation was significantly delayed and disease severity was dramatically decreased in all treated groups (Fig. 4B). Furthermore, diminished disease correlated with significantly lower ocular surface IFN-γ levels in Ab-treated Th17 recipients (Fig. 4C).

IFN-γ secreted by Th17 cells contributes to DED severity

Th17 plasticity plays important functions in autoimmune disease pathogenesis, and it has even been considered indispensable for efficient disease induction in several forms of autoimmunity (13, 16). To examine whether Th17-derived IFN-γ is required for the development of DED, we induced DED in IFN-γ KO mice and then isolated their Th17 cells. Th17 cells from IFN-γ KO DED showed comparable expression levels of IL-17A as did Th17 cells from WT DED (Fig. 5A). Subsequently, these cells were adoptively transferred to Rag1 KO mice. Compared to recipients of WT DED Th17 cells, those receiving IFN-γ KO DED Th17 cells developed significantly milder disease (Fig. 5B, 5C). Correspondingly, T cells recovered from draining lymph nodes of IFN-γ KO Th17 recipients showed no IFN-γ expression (Fig. 5D). These data demonstrated that Th17 plasticity with the capability of producing IFN-γ is required for their enhanced pathogenicity in DED.

Discussion

The present study shows that in addition to the well-known pathogenic cytokine IL-17A, IFN-γ derived from Th17 cells also amply contributes to ocular surface epitheliopathy and autoimmune inflammation in DED. Previously, we have reported that activated NK cells are the major source of IFN-γ during the early acute phase of DED; however, it has remained unclear whether a Th1 response contributes to DED progression via its secreted IFN-γ as well. In this study, we investigated the pathogenicity of different Th subsets in DED and found that Th1 cells isolated from DED cannot induce disease due to their inability to migrate to the ocular surface. In contrast, both IL-17A+ single-positive Th17 cells and double-positive Th17/1 cells migrate to...
the ocular surface where they contribute to DED pathology. Furthermore, the conversion of Th17 cells to Th17/1 cells requires microenvironmental IL-12 and IL-23, and the IFN-γ produced by Th17 cells is essential and indispensable for the severe ocular surface immune response, suggesting that Th17 cells drive DED via both IL-17A and IFN-γ.

In the past several years, the critical role of IL-17A+ Th cells in ocular surface autoimmunity in DED has been reported (5, 7, 8). Alternatively, although increased IFN-γ has been consistently reported in DED (1–3–5), its precise role in DED immunopathogenesis has remained incompletely understood. The major hurdle in the cellular source of IFN-γ during disease progression because NK and Th1 cell numbers are not significantly increased in DED (4, 6, 11). Recently, the classic notion that diverse Th cell subsets are terminally differentiated has been revised; owing to their plasticity, Th17 cells can acquire IFN-γ expression in vitro (12).

For the first time in our experience, we discovered double-positive Th17/1 cells emerging from Th17 cells upon disease induction. Our data suggest that plastic Th17 cells serve as a major source of IFN-γ during DED.

**FIGURE 4.** IL-12 and IL-23 mediate the conversion of Th17 cells into IFN-γ–secreting cells. DED-specific Th17 cells were isolated and adoptively transferred to Rag1 KO mice, which were then subjected to desiccating stress for 5 d. These mice were injected with anti–IL-12, anti–IL-23R, anti–IL-12p40 Abs or control IgG 1 d before and 1 d after the transfer of Th17 cells. (A) Eye-draining lymph nodes were collected and analyzed for IFN-γ–expressing Th17 cells. Representative flow cytometry graphs gated on CD4+ are shown on the left, and the IFN-γ+IL-17A- and IFN-γ+IL-17A+ cell frequencies are summarized as mean on the right bar graphs. Anti–IL-12, anti–IL-23R, and anti–IL-12p40 Ab-treated groups exhibited significantly decreased IFN-γ+IL-17A+ cells (24.5 ± 0.8, 13.6 ± 6.0, and 9.0 ± 1.9%, respectively) as compared with control IgG group (41.2 ± 5.9%, p < 0.05). Furthermore, both anti–IL-23R and anti–IL-12p40–treated groups showed even lower IFN-γ+IL-17A+ cell frequencies than did the anti–IL-12–treated group (p < 0.05). No significant differences between anti–IL-23R and anti–IL-12p40–treated groups. (B) Clinical disease severity was evaluated by corneal fluorescein staining with the representative images exhibited. (C) IFN-γ mRNA expression in the conjunctivae was quantified by real-time RT-PCR, and data are shown as relative changes to control IgG–treated mice. n = 6–8 eyes per group. *p < 0.05 as compared with control IgG group. AT, adoptive transfer.
progression and demonstrate that increased IFN-γ expression is part of the pathogenic Th17 response in severe DED, and thus reconcile existing data on the role of IFN-γ in DED (1, 3–5). When the disease enters its chronic stage characterized by persistent, low-grade inflammation, double-positive Th17/1 cells diminish and memory Th17 cells represent the predominant pathogenic effectors (8). However, upon rechallenge with desiccating environmental stress, Th17/1 cells increase again in these chronic DED and correlate with exacerbated disease (Supplemental Fig. 1). Similarly, in a bacterial infection model, vaccination-induced memory Th17 cells became Th1-like cells after infection with the bacteria (17). In vivo transfer of pathogenic Th17 cells proves their ability to secrete IFN-γ and induce immune damages in the target tissues, suggesting that the critical pathogenic role of Th17 in ocular surface autoimmunity correlates with both IL-17A and IFN-γ. Th17/1 cells, upon adoptive transfer, are able to infiltrate the ocular surface and induce DED comparable to Th17 cells. In contrast, adoptive transfer of Th1 cells into immunodeficient mice showed no ocular infiltration of T cells nor induced DED, demonstrating that these single-positive Th1 cells cannot specifically migrate to the ocular surface to induce DED. Migration of Th17 cells and, most likely, also Th17/1 cells to the ocular surface is mediated through their expression of CCR6 (6, 18) and the enriched CCL20 environment at the ocular surface in DED (6).

We also found that the in vivo conversion of DED-Th17 cells to IFN-γ-producing cells was associated with the increased microenvironmental IL-12 and IL-23 levels. It has been reported that in vitro treatment of polarized Th17 cells with IL-12 or IL-23 promotes their IFN-γ secretion (12, 19), which is dependent on the transcription factors STAT4 and T-bet (12). In contrast to existing studies on in vitro–generated nonspecific Th17 cells (12–15), we adoptively transferred disease-specific Th17 cells into naive recipients and then evaluated the effect of IL-12 and IL-23 blockade on their acquisition of IFN-γ. DED Th17 recipients treated with anti–IL-12, anti–IL-23R, or anti–IL-12p40 exhibited significantly less disease along with decreased frequencies of either Th17/1 or IL-17A+IFN-γ+ T cells, as well as decreased IFN-γ levels at the ocular surface. These findings demonstrate that both IL-12 and IL-23 are essential to Th17 plasticity and pathogenicity in vivo.

Furthermore, adoptive transfer of DED-Th17 cells from IFN-γ KO mice to T cell–deficient recipients causes DED corneal epitheliopathy. Although in the recipients there are other IFN-γ–producing cells than T cells, such as NK cells, they do not develop DED without T cells transfer (Fig. 2), and thus the disease observed is due to the pathogenic roles of the transferred IFN-γ KO Th17 cells. However, the damage is less severe than that caused by WT DED Th17 cells, demonstrating an important contribution of Th17-derived IFN-γ to DED pathogenesis. It has been reported that Th17 plasticity is indispensable for development of experimental autoimmune encephalomyelitis (16), colitis (13), and diabetes (19). In contrast, one study has shown that stable Th17 cells, which are derived from IL-12 KO mice, are capable of inducing the same severe experimental autoimmune encephalomyelitis as do plastic Th17 cells (20). Our results demonstrate that Th17 plasticity enhances Th17 pathogenicity and is required for a severe ocular surface immunoinflammation. Therefore, the specific

![FIGURE 5. IFN-γ KO Th17 cells leads to less severe disease. DED was induced in WT and IFN-γ KO mice, and then Th17 cells were isolated and adoptively transferred into Rag1 KO mice, which were then subjected to desiccating stress for 5 d. (A) IL-17A expression levels were assessed in Th17 cells from draining lymph nodes of WT and IFN-γ KO DED mice using flow cytometry. Sorted WT Th17 and IFN-γ KO Th17 cells from DED expressed comparable levels of IL-17A. (B) Representative corneal fluorescein staining images of mice receiving WT Th17 cells or IFN-γ KO Th17. (C) Mice receiving IFN-γ KO Th17 cells showed significantly reduced disease severity than did those receiving WT Th17 cells. *p < 0.05. (D) The expressions of IFN-γ and IL-17A by the transferred Th17 cells were analyzed by flow cytometry, and representative plots are shown. n = 6–8 eyes in each group.](http://www.jimmunol.org/)

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roles of Th17-derived IFN-γ and IL-17A in autoimmunity may be disease- and context-dependent.

In summary, our data demonstrate an important role of IFN-γ–secreting Th17 cells, but not conventional Th1 cells, in amplifying ocular surface autoimmunity. Thus, we reveal a previously undefined mechanism of Th17-derived IFN-γ in DED, highlighting the importance of blocking both Th17-derived IL-17A and IFN-γ in designing clinical strategies that target DED. Because IFN-γ secretion by Th17 cells in DED is driven by IL-12 and IL-23, blocking IL-12p40 (shared subunit of IL-12 and IL-23) may prove to be an effective strategy to reduce Th17 cell pathogenicity in DED, the most common ocular pathology.

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