RORγ-Expressing Th17 Cells Induce Murine Chronic Intestinal Inflammation via Redundant Effects of IL-17A and IL-17F

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Background and Aims: **IL-17–producing CD4+ T-helper cells (Th17) contribute to chronic autoimmune inflammation in the brain, and levels of Th17-derived cytokines increase in patients with colitis, suggesting a role in pathogenesis. We analyzed the roles of Th17 cells and the transcription factor retinoic acid receptor-related organ receptor (ROR)γ, which regulates Th17 differentiation, in chronic intestinal inflammation.** ***Methods:*** **Using an adoptive transfer model of colitis, we compared the colitogenic potential of wild-type, interleukin-17A (IL-17A)–, IL-17F–, IL-22–, and RORγ-deficient CD4+CD25− T cells in RAG1-null mice.** ***Results:*** **Adoptive transfer of IL-17A–, IL-17F–, or IL-22–deficient T lymphocytes into RAG1-null mice caused severe colitis that was indistinguishable from that caused by wild-type cells. In contrast, transfer of RORγ-null T cells failed to increase mucosal IL-17 cytokine levels and did not induce colitis. Treatment with IL-17A was able to restore colitis after transfer of RORγ-null T cells, indicating a crucial role for Th17 cells in pathogenesis. Treatment of RAG1 mice that received IL-17F–null (but not wild-type) T cells with a neutralizing anti–IL-17A antibody significantly suppressed disease, indicating redundant biological effects of IL-17A and IL-17F.** ***Conclusions:*** **We have identified a crucial role of RORγ-expressing Th17 cells in chronic intestinal inflammation. RORγ controls IL-17A and IL-17F production, and these cytokines have a redundant but highly pathogenic role in gut inflammation. Reagents that target RORγ or a combination of anti–IL-17A and anti–IL-17F might be developed as therapeutics for chronic colitis.**

Inflammatory bowel diseases (IBDs: Crohn's disease, ulcerative colitis) are characterized by chronic relapsing inflammations of the gastrointestinal tract that are not caused by specific pathogens. Although the precise etiology of IBDs remains unclear, recent studies have suggested a key pathogenic role of barrier dysfunction with consecutive immune dysregulation and enhanced mucosal T-cell responses.[1] While Crohn's disease has been associated with a T-helper 1 (Th1) T-cell cytokine profile, T cells in ulcerative colitis produce some Th2-type cytokines such as interleukin-5 (IL-5) and IL-13.[2]

Previous concepts on the role of T-helper cells in chronic inflammatory and autoimmune diseases have been challenged by the description of a novel T-cell subset characterized by the production of IL-17.[3] These Th17 cells have been described as producers of a distinct cytokine pattern, especially IL-17A, IL-17F, IL-22, and, to a lesser extent, tumor necrosis factor and IL-6.[4] The differentiation program of Th17 cells in vitro is controlled by synergistic effects of transforming growth factor β (TGFβ) and IL-6–mediated IL-21 expression, whereas stabilization of the Th17 phenotype has been shown to be achieved via IL-23 receptor (IL-23R) signaling.[5,6] Importantly, retinoic acid receptor-related organ receptor (ROR)γt has been recently identified as the master transcription factor guiding Th17 differentiation, while at the same time synergizing with other transcription factors such as signal transducer and activator of transcription 3, RORα, and interferon regulatory factor 4.[7–10] Interestingly, Th17 cells are closely linked to Foxp3+ T-regulatory cells, as it has been shown that both cell types require TGFβ for in vitro differentiation and that Foxp3 controls Th17 differentiation by direct interaction with RORγt.[11]

Single nucleotide polymorphisms in the *IL23r* gene modulate the susceptibility to IBDs.[12] In addition, Th17-derived cytokines are augmented in IBD patients, indicating a potential role of these cells in disease pathogenesis.[13,14] However, the role of Th17 cells in chronic mucosal inflammation is still incompletely understood.

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; MPO, myeloperoxidase; ROR, retinoic acid receptor-related orphan receptor; TGF, transforming growth factor; Th, T helper.

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Therefore, we analyzed in this study the role of Th17 cells in the pathogenesis of chronic intestinal inflammation in vivo.

Materials and Methods

Mice

IL-17A$^{-/-}$, IL-22$^{-/-}$, and IL-17F$^{-/-}$ mice were provided by Regeneron Pharmaceuticals (Tarrytown, NY) and Merck Serono (Geneva, Switzerland), respectively. All mice were on a C57BL/6 background. Wild-type littermates were used as controls. RAG1$^{-/-}$ mice, aged 6 to 12 weeks, were obtained from the animal facility of the University of Mainz. All mice were kept under specific pathogen-free conditions, and all experiments were performed in agreement with local regulations.

Isolation and Culture of Primary Cells

CD4$^+$CD25$^-$ T cells were isolated from murine splenocytes by MACS separation (CD4$^+$ T Cell Isolation Kit, CD25-PE Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) to a purity degree >95%, as evaluated by fluorescence-activated cell sorter (FACS) analysis. RPMI 1640 (Gibco–Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Perbio Science, Bonn, Germany) and penicillin/streptomycin (Biochrom, Berlin, Germany) was used for all cell isolation procedures.

Adoptive Transfer Colitis

Freshly isolated CD4$^+$CD25$^-$ T cells (5×10^5) from different mouse strains (v.s.) were administered intraperitoneally into RAG$^{-/-}$ mice in 200 μL of phosphate-buffered saline (PBS; Gibco–Invitrogen). The COLOVIEW high-resolution mouse video endoscopic system (Karl Storz, Tuttlingen, Germany) was used as previously described.[15] In some experiments, IL-17A was neutralized by intraperitoneal administration of 100 μg of a monoclonal antibody (clone 50104, R&D Systems, Wiesbaden, Germany) every other day.[16]

Recombinant murine IL-17A (ImmunoTools, Friesoythe, Germany) was injected intraperitoneally 3 times a week at a dose of 0.5 μg per mouse.

Isolation of Lamina Propria Mononuclear Cells

Murine lamina propria mononuclear cells were isolated as previously described.[17,18] In brief, colonic tissue was mechanically dissected, and intestinal epithelial cells were removed by incubation in EDTA. Remaining tissue was digested in collagenase D (Roche Diagnostics, Mannheim, Germany), DNase I (Sigma–Aldrich, Munich, Germany), and dispase II (Roche Diagnostics). Digested tissue was passed through a 40-μm cell strainer, and the remaining cellular content was separated from debris using a 40/80 Percoll gradient (Sigma–Aldrich).

Flow Cytometry Analysis

With the use of specific antibodies, cells were stained for CD4, CD25, CD62L, and CD69 (BD Biosciences, Heidelberg, Germany) expression, according to the manufacturer's instructions. Intracellular labeling for IL-17 (clone TC11–18H10.1, BioLegend, San Diego, CA) and interferonγ (IFNγ; clone XMG1.2, Caltag–Invitrogen, Karlsruhe, Germany) was performed with phycoerythrin- or fluorescein isothiocyanate–conjugated rat antimouse antibodies. Cells were stimulated with phorbol myristate acetate (50 ng/mL; Calbiochem Merck, Darmstadt, Germany), ionomycin (500 ng/mL; Sigma–Aldrich), and brefeldin A (5 μg/mL; Sigma–Aldrich) before staining, and Fix and Perm Cell Permeabilization reagents (Caltag–Invitrogen) were used according to the manufacturer's instructions after staining of surface antigens. Cells were analyzed by means of a FACSCalibur Flow Cytometer (BD Biosciences).

Cytokine Quantification

Supernatants of reisolated CD4$^+$ T cells cultured for 48 hours and stimulated with plate-bound anti-CD3 (10 μg/mL, clone 145-2C11) and soluble anti-CD28 (2 μg/mL, clone 37.51) antibodies were analyzed for cytokines by FlowCytomix assays according to the manufacturer's protocol (Bender MedSystems, Vienna, Austria). IL-17A and IL-17F were measured using enzyme-linked immunosorbent assay kits (ELISA Kits; eBioscience, San Diego, CA).

βGalactosidase Assay

The βGalactosidase Reporter Gene Assay Kit (Roche Applied Science, Mannheim, Germany) was used according to the manufacturer's protocol.

Immunohistochemistry

Immunofluorescence of cryosections was performed using the TSA Cy3 system (PerkinElmer, Waltham, MA) and a fluorescence microscope (Olympus, Hamburg, Germany) using primary antibodies specific for CD4 (Santa Cruz Biotechnology, Heidelberg, Germany), CD11c, and myeloperoxidase (MPO, BD Biosciences). Before examination, the nuclei were counterstained with Hoechst 3342 (Invitrogen Molecular Probes, Karlsruhe, Germany).

Statistical Analysis

Data were analyzed by the unpaired Student *t* test using Microsoft Excel (Microsoft, Redmond, WA) or an analysis of variance with post hoc Tukey HSD tests, as indicated, using SPSS software (SPSS Inc, Chicago, IL).

Results

T Cell–Dependent Experimental Colitis Is Not Affected by IL-17A Deficiency

To address the role of Th17 cells in the pathogenesis of colitis, we took advantage of an adoptive transfer