Inhibition of Interleukin-17 Promotes Differentiation of CD25$^-$ Cells Into Stable T Regulatory Cells in Patients With Autoimmune Hepatitis

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BACKGROUND & AIMS: Patients with autoimmune hepatitis (AIH) have reduced numbers and function of CD4$^+$CD25^{high}FOXP3$^+$ T regulatory cells (Tregs). Tregs can be generated from CD25$^-$ (ngTreg) cells, which suppress the immune response less efficiently than Tregs. We investigated whether their differentiation into T-helper (Th)17 cells, an effector subset that has the same CD4$^+$ progenitors as Tregs, accounts for the reduced suppressive functions of ngTregs. We investigated whether blocking interleukin (IL)-17 increased the immunosuppressive activity of Tregs. **METHODS:** ngTregs were generated from 36 patients with AIH and 23 healthy subjects (controls). During Treg differentiation, expression of IL-17 was inhibited by physical removal of IL-17–secreting cells, exposure to recombinant transforming growth factor β or neutralizing antibodies against IL-6 and IL-1β (to promote differentiation of ngTregs vs Th17 cells), small inhibitory RNAs specific for the Th17 transcription factor RORC, or a combination of all these approaches. **RESULTS:** ngTregs from patients with AIH contained greater proportions of IL-17$^+$ and RORC$^+$ cells than Tregs from controls. All approaches to inhibit IL-17 increased expression of FOXP3 by ngTregs and their suppressive functions. Inhibition of IL-17 led to development of ngTregs that were phenotypically stable and did not acquire proinflammatory properties after exposure to IL-6 and IL-1β. **CONCLUSIONS: Blocking Th17 allows ngTregs to differentiate into functionally stable immune inhibitory cells; this approach might be developed for therapy of patients with AIH.**

Keywords: Immunotherapy; Liver Inflammation; Immune Regulation; T-Cell Development.

BASIC AND TRANSLATIONAL LIVER

Experimental and clinical evidence indicates that failure of immunoregulation is key to development of autoimmune disease. CD4$^+$CD25^{high}FOXP3$^+$ T regulatory cells (Tregs) are major players in maintaining immune homeostasis; defective Treg numbers and function result in autoimmune disorders in mice and humans.[1–5] Numerical and functional Treg impairment is documented in autoimmune hepatitis (AIH),[6–8] an inflammatory condition characterized by hypergammaglobulinemia, circulating autoantibodies, and interface hepatitis.[9,10] Treg impairment in AIH varies with disease stage, appearing worse at presentation than during remission, showing functional restoration potential.[6,7]

We reported that following exposure to anti-CD3/anti-CD28 and high interleukin (IL)-2 concentrations, Tregs derived from CD4$^+$CD25^{high} cells expand in both healthy subjects (HS) and patients with AIH.[11] Tregs expressing higher levels of FOXP3 and suppressing more efficiently than freshly isolated Tregs[11] can be expanded, but in limited numbers, hampering their immunotherapeutic use. To overcome this limitation, using a similar experimental approach, we generated Tregs from CD4$^+$CD25$^-$ cells, an effector subset also containing lymphocytes with regulatory function.[12] Differently from Tregs obtained from conventional CD4$^+$CD25^{high} cells, newly generated Tregs (ngTregs) have greater ability to expand and are more resistant to apoptosis,[11] features important for therapeutic application. However, despite their Treg phenotype (CD25^{high}, CD127^{low}, and FOXP3$^+$), ngTregs suppress less efficiently than Tregs expanded from CD4$^+$CD25^{high} cells.[11] During differentiation, ngTregs may display effector cell features (ie, secretion/production of proinflammatory cytokines), offsetting their suppressive function.[13] This may derive from incomplete transition to a terminal regulatory status of a proportion of cells producing IL-17.[14] IL-17–producing CD4 effector T-cells (Th17 cells) originate from the same progenitors as Tregs in the presence of IL-6, IL-21, and transforming growth factor (TGF)-β in mice and IL-6, IL-1β, IL-23, and TGF-β in humans.[15–20]

The aims of this study were to assess whether the reduced suppressor function of ngTregs derives from their ability to produce IL-17 and whether control of those ngTregs producing IL-17 has a favorable effect on ngTreg function.

Patients and Methods

Patients and Controls

Thirty-six patients with anti-nuclear antibody (ANA)-positive and/or anti–smooth muscle antibody (SMA)-positive

Abbreviations used in this paper: AIH, autoimmune hepatitis; ANA, anti-nuclear antibody; HS, healthy subjects; IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; ngTreg, newly generated T regulatory cell; PBMC, peripheral blood mononuclear cell; rIL-2, recombinant interleukin-2; rTGF-β, recombinant transforming growth factor β; siRNA, small interfering RNA; SMA, anti–smooth muscle antibody; TGF, transforming growth factor; Th, T-helper; Treg, T regulatory cell.

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AIH (AIH type 1; 23 female; median age, 13.9 years; range, 8.6–17.9 years) were studied, all having histologic evidence of interface hepatitis at diagnosis. Patients were studied during immunosuppressive treatment, because pretreatment Tregs are inefficient at suppressing effector functions of their targets.[7] Thirty patients were in remission (normal aspartate aminotransferase levels), four were in relapse, and 2 had recently started immunosuppressive treatment (aspartate aminotransferase levels, 59 and 69 IU/L; normal value, <50 IU/L). At the time of study, median aspartate aminotransferase level was 30 IU/L (range, 18–79 IU/L), bilirubin level was 8 μmol/L (range, 4–29 μmol/L; normal value, <20 μmol/L), immunoglobulin G level was 13.3 g/L (range, 6.6–28.6 g/L; normal value, 6.5–17 g/L), ANA titer was 1/40 (range, 1/10–1/320), and SMA titer was 1/160 (range, 1/20–1/2560). Patients were receiving prednisolone (2.5–5 mg daily) and azathioprine (1–2 mg/kg/day). Controls were 23 HS (19 female; median age, 31 years; range, 25–41 years), recruited from hospital staff, because for ethical reasons blood could not be obtained from healthy children. To test whether age disparity may account for differences, patients were divided into 2 subgroups (≤14 and >14 years old). Informed consent was obtained from each subject. The study was approved by the local ethics committee.

Cell Separation

Peripheral blood mononuclear cells (PBMCs) were obtained as previously described.[6] Cell viability, determined by Trypan blue exclusion, exceeded 98%.

Characterization of Cytokine Profile

The percentage of cytokine-producing ngTregs was determined by intracellular staining.[6,7] Cells were stained with fluorescein isothiocyanate– or APC-Cy7–conjugated anti-CD4, APC-conjugated or PE-Cy7–conjugated anti-CD25 monoclonal antibodies (BD Bioscience, Oxford, England), fluorescein isothiocyanate– or PE-conjugated anti–IL-17A (eBioscience, San Diego, CA), PE-conjugated monoclonal antibodies to interferon (IFN)-γ (IQ Products, Groningen, The Netherlands), IL-10 (BD Bioscience), IL-1β and IL-6 (eBioscience), and PerCP-conjugated anti-human LAP (TGF-β1) (R&D Systems, Abingdon, England). Flow cytometry was performed on a BD FACSCanto II (Immunocytochemistry Systems, BD Bioscience), acquiring a minimum of 1×10^4 gated events/sample; FACSDiva software was used for analysis.

Cell Purification

$CD4^+CD25^-$ and $CD4^+CD25^{high}$ cells were purified from PBMCs using immunomagnetic beads (Dynal Invitrogen, Oslo, Norway).[8] Their purity exceeded 90% and 95%, respectively.

Th17 Depletion

Freshly isolated $CD4^+CD25^-$ cells were cultured at 5×10^5 cells/well with anti-CD3/anti-CD28 T-cell expander (1 bead/cell) (Dynal Invitrogen), and recombinant IL-2 (rIL-2) (Eurocetus, Amsterdam, The Netherlands) was added at 300 U/mL the first week and each time the exhausted medium was replaced. Two-week expanded $CD4^+CD25^-$ cells were depleted of IL-17–secreting cells (Supplementary Figure 1) according to Streeck et al[21] (see Supplementary Materials and Methods). Following depletion, the frequency of IL-17–secreting cells within ngTregs was always <0.5%. IL-17–depleted cells were cultured for 3 additional weeks with 300 U/mL rIL-2. The frequency of cells positive for IL-17, RORC, a Th17 transcription factor,[22] and FOXP3 was monitored weekly. After a 3-week culture, ngTregs were purified from expanded $CD4^+CD25^-$ cells by CD4-negative selection followed by CD25-positive selection (Dynal Invitrogen)[8] (purity >95%) and their ability to suppress was assessed in a proliferation assay (see the following text). The frequency of $IL\text{-}17^+$, $RORC^+$, and $FOXP3^+$ cells during the 3-week culture, as well as the suppressor function of ngTregs isolated at the end of the third week, were also determined after stimulating Th17-depleted ngTregs with IL-6 (50 ng/mL) and IL-1β (10 ng/mL).

Phenotype and ability to suppress ngTregs obtained from $CD4^+CD25^-$ cells after treatment with neutralizing antibodies and/or cytokines and *RORC* gene knockdown were also evaluated (see the following text).

Characterization of ngTregs and Th17 Cell Phenotype

The phenotype of ngTregs was determined by flow cytometry using monoclonal antibodies to the surface markers CD25, CD62L, CD45RO, and CD127 (eBioscience) and to the intracellular molecules Granzyme B (BD Bioscience), a cytotoxic enzyme linked to Treg immunosuppressive function,[23] FOXP3 (clone PCH101; eBioscience), and CTLA-4 (BD Bioscience). Frequencies of IFN-γ, IL-10, IL-17A, IL-6, and IL-1β–producing ngTregs were determined as described previously. Expression of CD196 (CCR6) and IL-23 receptor, markers of Th17 cells,[24,25] and RORC was assessed in IL-17–producing CD4 cells using PerCP-conjugated anti-human CCR6 (BD Bioscience), anti-human IL-23 receptor (R&D Systems), and PE-conjugated anti-human RORC (eBioscience) monoclonal antibodies. Cells were analyzed by flow cytometry after gating on CD4 lymphocytes.

Inhibition of Proliferation

Following purification, ngTregs were added at a 1:8 ratio[6,7] to autologous $CD4^+CD25^-$ cells ($5–10 \times 10^4$/well) after 48-hour resting in T-cell expander- and IL-2–free medium. Experiments were performed in duplicate. After a 5-day culture, cells were pulsed with 0.25 μCi/well ^{3}H-thymidine and harvested 18 hours later. Incorporated thymidine was measured by a beta counter (Canberra Packard Ltd, Pangbourne, England). Percentage inhibition was calculated using the following formula: 1 – (Counts per Minute in the Presence of ngTregs/Counts per Minute in the Absence of ngTregs) × 100. Transwell assays were performed to determine whether ngTregs suppress through a direct cell-to-cell contact mechanism (see Supplementary Materials and Methods).

De Novo Generation of Tregs in the Absence or Presence of Neutralizing Antibodies and/or Cytokines

In preliminary experiments to determine the best maneuvers for inhibiting Th17 cell differentiation and boosting Treg function, exposure to rapamycin, retinoic acid, IL-17 neutralizing antibodies, and RORC–specific small interfering RNA (siRNA) were tested. Because the former 2 procedures proved ineffective (see Supplementary Materials and Methods), the latter were chosen.

$CD4^+CD25^-$ cells were cultured in the presence of T-cell expander and rIL-2 with or without recombinant TGF-β (5 ng/mL) and antibodies to IL-17A, IL-6, IL-1β, and IL-23 (R&D Systems), cytokines important for the development and differentiation of human Th17 cells, used at 10 μg/mL either individually or in combination. Following 4-week culture, ngTregs were purified (Supplementary Figure 1). True Treg (ie,