Transforming Growth Factor β Signaling Controls Activities of Human Intestinal CD8+T Suppressor Cells

KEREN M. RABINOWITZ,[1] YUANYUAN WANG,[2] EDWARD Y. CHEN,[3] ZARA HOVHANNISYAN,[1] DAVID CHIANG,[1] M. CECILIA BERIN,[4] STEPHANIE DAHAN,[1] DAMIEN CHAUSSABEL,[2] AVI MA'AYAN,[3] and LLOYD MAYER[1]

[1]Immunology Institute, [3]Department of Pharmacology and Systems Therapeutics, Systems Biology Center New York, and [4]Division of Pediatric Allergy and Immunology, Mount Sinai School of Medicine, New York, New York; and [2]INSERM Unité 899, Baylor Institute for Immunology Research-ANRS Center for Human Vaccines, Dallas, Texas

BACKGROUND & AIMS: In healthy individuals, interactions between intestinal epithelial cells and lamina propria lymphocytes give rise to a population of CD8$^+$ T cells with suppressor functions (Ts cells). Disruption of Ts cell activities can lead to mucosal inflammation. We investigated what factors were required for expansion of the Ts cell population or loss of their activity in patients with Crohn's disease (CD). **METHODS:** We developed a method to generate Ts cell lines from freshly isolated lamina propria lymphocytes from patients with ulcerative colitis (UC), patients with CD, or healthy individuals (controls). Cells were stimulated with a monoclonal antibody against CD3, interleukin (IL)-7, and IL-15. After 14 days in culture, CD8$^+$T cells were purified and cultured with IL-7 and IL-15. The resulting Ts cells were analyzed for suppressor activity, expression of surface markers, and cytokine secretion profiles. RNA was isolated from the 3 groups of Ts cells and used in microarray analyses. **RESULTS:** Ts cells from patients with UC and controls suppressed proliferation of CD4$^+$ T cells; the suppression required cell contact. In contrast, Ts cells from patients with CD had a reduced capacity to suppress CD4$^+$ T-cell proliferation. The difference in suppressive ability was not associated with surface or intracytoplasmic markers or secretion of cytokines. Microarray analysis identified changes in expression of genes regulated by transforming growth factor (TGF)-β that were associated with the suppressive abilities of Ts cells. We found that TGF-β or supernatants from Ts cells of patients with CD reduced the suppressor activity of control Ts cells. **CONCLUSIONS: Ts cells isolated from patients with CD have a reduced ability to suppress proliferation of CD4$^+$T cells compared with control Ts cells. TGF-β signaling reduces the suppressor activity of Ts cells.**

Keywords: Regulatory T Cells; Treg Cells; Immune Regulation; Inflammatory Bowel Disease.

Regulatory T (Treg) cells or suppressor T (Ts) cells are known to be critically important T-cell subsets for homeostasis of the immune system and are primarily responsible for dampening immune responses. Dysfunction of any of these cell subsets may lead to a number of immune/inflammatory disorders, leading to autoimmunity, tumors, transplant rejection, and so on. Controlled inflammation in the gastrointestinal tract is maintained partially by regulatory lymphocytes. Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), reflects a loss of mucosal homeostasis. CD4$^+$-expressing FoxP3$^+$ CD25$^+$ Treg cells have been extensively studied in the context of IBD and shown to suppress proliferation of T cells and to protect from the induction of experimental colitis by CD4$^+$ CD45RB^{high}-expressing cells transferred into SCID mice.[1–3] Yet, CD4$^+$ Treg cells have also been shown to be up-regulated in the mucosa of both patients with UC and patients with CD and to have intact suppressor activity (suppressing the proliferation of effector CD4$^+$ CD25$^-$ T cells[4,5]). Therefore, there is a question as to whether CD4$^+$ Treg cells are the critical cell type mediating mucosal homeostasis.

CD8$^+$ Ts cells are present in the systemic and mucosal immune system. In fact, there may be many distinct subsets. A role for CD8$^+$ Ts cells has been implicated in several IBD animal models. CD8$^+$ Ts cells have in vivo suppressor activity and are able to prevent the development of colitis in a CD45RBhi CD4$^+$ T-cell transfer model and spontaneous ileitis in TNFΔARE mice.[6–8]

We have previously shown that human CD8$^+$ Ts cells are activated and expanded when cultured with isolated human intestinal epithelial cells (IECs) through a complex of the nonclassic class I molecule CD1d with glycoprotein gp180, a member of the carcinoembryonic antigen subfamily.[9–11] Defective expression of this complex in patients with IBD is associated with the inability of IBD IECs to activate CD8$^+$ Ts cells, specifically T cells that had biased expression of BV5.1. In this report, we show a more global defect in CD8$^+$ lamina propria (LP) Ts cells in patients with CD and investigate the nature of these differences compared with controls. Using a high-profiling approach, we have identified molecular differences between CD8$^+$ Ts lines that have high and low suppressor activity. We have correlated the expression of multiple

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Abbreviations used in this paper: CFSE, carboxyfluorescein succinimidyl ester; ChIP, chromatin immunoprecipitation; ENG, endoglin; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; LP, lamina propria; LPL, lamina propria lymphocyte; NL, normal; PBMC, peripheral blood mononuclear cell; TGF, transforming growth factor; Treg cell, regulatory T cell; Ts cell, suppressor T cell.

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genes associated with transforming growth factor (TGF)-β signaling with suppressor activity. Furthermore, we have shown that TGF-β is increased in tissue derived from patients with CD (compared with controls) and that the presence of TGF-β inhibits the suppressor activity of $CD8^+$ Ts cells.

Materials and Methods

Patients and Tissues

Surgical specimens from patients undergoing bowel resection for cancer or IBD at Mount Sinai Medical Center were used as the source for lamina propria lymphocytes (LPLs). "Normals" (NLs) consisted of patients undergoing bowel resection for colon cancer, tubulovillous adenoma, or diverticulitis. Within this group, cells were always isolated from normal tissue >10 cm from the tumor (except diverticulitis) and the samples in this group were derived from noninflamed tissues. UC and CD patient samples were all isolated from areas containing moderate to severe inflammation. Patients with UC and patients with CD shared common medications (corticosteroids, infliximab, azathioprine, mesalamine). This study was approved by the Mount Sinai Institutional Review Board.

Cell Purification

LPLs were isolated according to an established protocol.[12] Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood on a Ficoll-Hypaque density gradient (Amersham Biosciences, Piscataway, NJ) according to standard procedures.

Lines of $CD8^+$ Ts Cells

Whole LPLs were stimulated with the soluble humanized non–FcR-binding αCD3 monoclonal antibody visilizumab (100 ng/mL; PDL, Biopharma Inc, Incline Village, NV). Five days later, cells were treated with interleukin (IL)-7 (10 ng/mL), IL-15 (20 ng/mL) (R&D Systems, Minneapolis, MN), and feeder cells (irradiated allogeneic PBMCs, 3000 rads at a 1:1 ratio). Fourteen days later, $CD8^+$ T cells were positively selected using a $CD8^+$ T-cell selection kit ($CD8^+$ Selection Kit; Stemcell Technologies, Vancouver, British Columbia, Canada). Lines were tested and were shown to be >96% CD8αβ. Cells were maintained in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamine, amphotericin B), IL-7, and IL-15 and expanded for 3 to 6 months.

Tissue Explant Cultures

Explant cultures from surgical specimens were performed following a modification as previously described.[13] Briefly, intestinal mucosa (2-cm segments) was washed in phosphate-buffered saline, standardized by weight, and cultured in 24-well culture plates in serum-free RPMI 1640 medium supplemented with penicillin, streptomycin, glutamine, amphotericin B, a protease inhibitor cocktail (Thermo Scientific, Rockford, IL), and a phosphatase inhibitor cocktail (Thermo Scientific). The volume of the media was adjusted to the tissue weight. After 24 hours, supernatants were collected and stored at −20°C.

In Vitro Suppression Assay

We studied the effect of $CD8^+$ Ts lines on CD3/CD28-stimulated PBMCs. PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA) and either unstimulated or stimulated with αCD3/CD28 beads (Invitrogen) at a 1:1 ratio. The $CD8^+$ T cells were washed and added to PBMCs at a ratio of 1:1 to 1:16 (1 $CD8^+$ Ts line to 16 PBMC). Proliferation of $CD4^+$ T cells was analyzed by flow cytometry. Percent suppression was calculated based on the following equation: 100 × (1 − [% of Proliferating Cells − Experiment]/[% of Proliferating Cells – Control]) = % Suppression.

Recombinant human TGF-β (BioLegend, San Diego, CA) was added to the suppressor assay as noted. Tissue supernatants were added to the suppressor assay in a 1:100 dilution. αTGF-β neutralizing antibody was used (10 μg/mL; Abcam, Cambridge, MA). Transwell cultures (6.5-mm diameter inserts, 3.0-mm pore size; Corning, Lowell, MA) were used to physically separate the responders from the suppressor cells. A total of 1×10^6 cells were placed in each chamber of the Transwell. αCD3/CD28 beads were placed in both chambers, and thus both responder and suppressor cells were activated.

RNA Extraction and Processing for Microarray Analysis

RNA was isolated from 72-hour CD3/CD28-stimulated $CD8^+$ Ts cells using the RNeasy Micro Kit (Qiagen, Valencia, CA). A total of 200 ng of total RNA was amplified using the Illumina TotalPrep-96 RNA Amplification Kit (Applied Biosystems/Invitrogen, Grand Island, NY). A total of 750 ng of amplified antisense complementary RNA targets was hybridized to Illumina Human HT-12 V4 BeadChip Arrays (Illumina, San Diego, CA) and incubated at 58°C for 17 hours. The arrays were washed, stained, and then scanned on an Illumina Beadstation 500 (Illumina). Signal intensity was captured for each probe on the array using BeadArray Reader software (Illumina).

Microarray Data Analysis

After background subtraction, intensity values were scaled to the median average intensity of the entire sample set using the average normalization function available in GenomeStudio V2009.1 software (Illumina). GeneSpring GX version 7.3.1 (Agilent Technologies, Santa Clara, CA) was used for all downstream analyses. Intensity values were floored to 10, and the intensity of each probe in each sample was normalized to the median intensity of this probe across all samples.

Scoring Scheme to Determine Statistical Enrichment for Protein Kinases and Transcription Factors Given an Input List of Differentially Expressed and Background Networks

Given an input list of differentially expressed genes, the enrichment *P* values for transcription factors listed in a database developed from chromatin immunoprecipitation (ChIP)-ChIP and ChIP-seq studies[14] were analyzed using the Fisher exact test. The top 10 transcription factors were used as seed nodes to construct a protein-protein interaction network, utilizing a merged database of protein-protein interactions, using the shortest path algorithm.[15] Comparisons were made by statistical enrichment for protein kinases using the Fisher exact test and a database of kinase-substrate interactions.[16]

Statistical Analysis

All statistical analyses (other than the microarray analysis) were performed with Prism software (GraphPad, La Jolla, CA). Statistical significance was determined by one-way analysis

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