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Differentiation and Expansion of T Cells with Regulatory Function from Human Peripheral Lymphocytes by Stimulation in the Presence of TGF-β

Patricia E. Rao, Andria L. Petrone, and Paul D. Ponath

T cells with immunoregulatory function have been described in human and mouse systems. In both systems these cells can be differentiated either in the thymus or from peripheral T cells. To date, more progress has been made in the study of murine regulatory T cells, because it has been very difficult to isolate human regulatory T cells of sufficient purity and in sufficient numbers to permit detailed examinations of their biochemistry. We report in this study that human T cells with regulatory function can be differentiated in vitro from naive CD4⁺CD25⁺T cells by stimulation with anti-CD3 and anti-CD28 in the presence of TGF-β. Cells derived in this manner express a surface phenotype (CD25⁺, CD122⁺, HLA-DR⁺, glucocorticoid-induced TNF receptor-related gene (GITR)⁺, CD103⁺, CTLA-4⁺) described for human and mouse regulatory T cells and express protein and message for the transcription factor forkhead/winged helix transcription factor (FOXP3). They produce primarily TGF-β and IL-10, with lesser amounts of IFN-γ and IL-13, when stimulated through their TCRs and are capable of inhibiting cytokine production and proliferation by stimulated naive T cells. Unlike Th1 and Th2 cells, these TGF-β-derived regulatory T cells do not appear to be dependent on the protein kinase Cθ pathway of NF-κB activation for Ag-induced responses. The Journal of Immunology, 2005, 174: 1446–1455.

When initially described, regulatory T cells were identified from the CD4⁺CD25⁺ subset of murine peripheral lymphocytes (1–8). These cells were shown to be directly thymus derived (9–11) and capable of inhibiting proliferation in vitro of murine CD4⁺CD25⁺ T cells (12), and CD8⁺ T cells (13). Additionally, transfer of CD4⁺CD25⁺ cells in various disease models has demonstrated their suppressive ability in vivo (14–19). Association of regulatory function with a similar human population was also described several years later (20–24). Although obtaining populations of murine lymphocytes sufficiently enriched in a regulatory population to permit studying these cells has been possible, it has proved more difficult to obtain sufficiently pure human regulatory T cells. Human CD4⁺CD25⁺ T cells with regulatory function have been obtained from peripheral blood in sufficient purity for study by using only a subpopulation of CD4⁺CD25⁺ cells expressing high levels of CD25 (25, 26).

The possibility that regulatory cell populations might also be derived from naive peripheral lymphocytes, distinct from the thymus-derived CD4⁺CD25⁺ population, has also been recognized in the description of Tₘ₁ cells (27, 28), which are capable of inhibiting colitis in mice, and Th3 cells (29, 30), which are capable of mediating tolerance to orally administered Ags in mice. Additional evidence for the ability of peripheral lymphocytes to differentiate into regulatory T cells was recently presented in the murine system through cultivation of murine CD4⁺CD25⁺ T lymphocytes in vitro in the presence of TGF-β (31). Regulatory T cells differentiated under these conditions expressed forkhead/winged helix transcription factor (FOXP3), secreted TGF-β, suppressed proliferation of normal T cells in vitro, and inhibited Ag-specific responses of TCR transgenic animals in vivo.

We report in this study that human regulatory T cells can be differentiated in vitro by cultivation of naive human cord blood or PBL in the presence of exogenous IL-2, TGF-β, and IL-15. Our data strongly suggest that exposure of peripheral blood CD4⁺T cells to TGF-β can up-regulate the expression of FOXP3, which may have physiological significance during healing when TGF-β is highly expressed. Cells differentiated in this manner express a surface phenotype associated with human and murine regulatory T cells (CD4⁺, CD25⁺, CD69⁺, glucocorticoid-induced TNF receptor-related gene (GITR)⁺), express cytokines appropriate to regulatory cells, up-regulate the transcription factor FOXP3, and suppress division and cytokine production when cocultured with stimulated naive lymphocytes. These culture conditions permit the expansion of cells with a regulatory phenotype to numbers useful for larger biological studies or screening assays.

Materials and Methods

Culture medium

X-VIVO-15 (Cambrex Biosciences) supplemented with 10% FBS was used for the culture of T cells and PBL.

Cytokines

All cytokines used in this study were recombinant human proteins obtained from R&D Systems. For differentiation of naive T cells, the following concentrations were used: 65 U/ml IL-2 (stock activity, 1.3 × 10⁴ U/µg), 62 U/ml IL-12 (stock activity, 1.24 × 10⁵ U/µg), 145 U/ml IL-4 (stock activity, 2.9 × 10⁴ U/µg), 32 U/ml TGF-B1 (stock activity, 3.2 × 10⁵ U/µg), 42 U/ml IL-9 (stock activity, 8.4 × 10⁴ U/µg), and 4500 U/ml IL-15 (stock activity, 4.5 × 10⁴ U/µg). For PBL cultures, cytokine concentrations are indicated.

Abbreviations used in this paper: FOXP3, forkhead/winged helix transcription factor; GITR, glucocorticoid-induced TNF receptor-related gene; PKC, protein kinase C; TRITC, tetramethylrhodamine isothiocyanate; GATA3, GATA-binding protein-3; TBX21, T-box expressed in T cells.
Antibodies
The following Abs were obtained from BD Pharmingen: anti-CD3 (clone UCHT1), anti-human IL-4 (clone MP4-25D2), anti-human IFN-γ (clone B27), anti-human IL-12 (clone C8.6), anti-CD25-FTTC (clone M-A251), anti-CD22-PE (clone Mi-β2), anti-HLA-DR-PE (clone G46-6), anti-CD69-PE (clone FN50), and anti-CD152-biotin (clone BN13). Isotype control Abs were also obtained from BD Pharmingen. Anti-CD28 (clone ANC28.1/SD10) was obtained from Ancell. Anti-CD103-biotin and anti-GITR-biotin were obtained from Beckman Coulter and R&D Systems, respectively. To detect surface TGF-β, two Abs from R&D Systems were used: biotin-conjugated, goat anti-human TGF-β (catalog no. BAF240). The secondary reagent streptavidin-quantum Red (Sigma-Aldrich) was used for detection of biotinylated Abs. For immunofluorescence microscopy, goat anti-human FOXP3 was obtained from Abcam; HRP-conjugated anti-protein kinase Cθ (anti-PKCθ) was obtained from BD Transduction Laboratories; anti-μβTCR was obtained from BD Pharmingen; tetramethylrhodamine isothiocyanate (TRITC)-conjugated AffiniPure F(ab′)2 donkey anti-goat IgG(1,1L) and TRITC-conjugated AffiniPure Fl(λ)3 goat anti-HRP were obtained from Jackson ImmunoResearch Laboratories. For inhibition of TGF-β or IL-10, the following Abs from R&D Systems were used: neutralizing mouse anti-human IL-10, clone 23738; neutralizing mouse anti-human TGF-β1, clone 9016; and neutralizing anti-human TGF-β1-2, -3, clone 1D11.

Preparation of cells
PBL were prepared by Ficoll density gradient centrifugation (Amersham Biosciences). PBL were washed into culture medium for direct use in assays or were washed into sorting medium for separation of naive CD4+ T cells.

For T cell differentiation using adult peripheral naive CD4+ T cells, PBL were separated using an AutoMACS cell sorter (Miltenyi Biotech). First, CD4+ T cells were purified by negative selection using the CD4+ Mulsitix kit (Miltenyi Biotech) according to the manufacturer’s instructions. These CD4+ T cells were then further sorted by positive selection using CD45RA+ microbeads (Miltenyi Biotech). The CD4+ CD45RA- cells were washed into medium for use in cell culture. The purity of sortings was confirmed by FACS to be between 90 and 95%.

For T cell differentiation using human cord blood naive CD4+ T cells, frozen CD4+ CD45RA+ cells isolated using magnetic beads were obtained from Allcells. Cells were thawed and washed out of DMSO into culture medium for use in cell culture.

T cell differentiation
CD4+ CD45RA- T cells from either cord blood or adult peripheral blood were stimulated in the presence of differentiative cytokines. Twelve-well Nunc plates were coated with 5 μM anti-CD3 and 10 or 5 μM/ml anti-CD28. Briefly, wells of a tissue culture plate were coated with 0.5 ml of PBS containing the Ab mixture and incubated overnight at 4°C or for 2 h at 37°C. Wells were then washed twice with 1 ml of PBS before plating cells at 0.5–10 × 10^6 cells/well in 2 ml of culture medium. Cytokines and neutralizing anti-CD3 Abs were added to the appropriate cultures. All cultures contained a final IL-2 concentration of 65 U/ml. Final culture concentrations for each cell type were as follows: for Th1: IL-12, 62 U/ml; and anti-IL-4, 200 ng/ml; for Th2: IL-4, 145 U/ml; anti-IFN-γ, 20 μg/ml; and anti-IL-12, 10 μg/ml; and for regulatory T cells: TGF-β1, 32 U/ml; IL-9, 42 U/ml; IL-15, 4500 U/ml; anti-IFN-γ, 20 μg/ml; and anti-IL-12, 10 μg/ml. Cultures were incubated for 2–3 days at 37°C in 5% CO₂. On days 2–3, cells were removed from the stimulation plate and split into larger culture vessels, feeding with fresh media and cytokines. Cells were expanded and fed as needed for 7–10 days. In some cases a second round of stimulation and selection was performed as described above.

Real-time quantitative PCR assays
To isolate total RNA from differentiated cells, cells were differentiated as described above, and 2–4 × 10^6 cells were harvested at the indicated time points. Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. To measure up-regulation of transcription factor message in activated PBL, 4 × 10^6 PBL were obtained with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) in the presence of 124 U/ml IL-12, 290 U/ml IL-4, or 160 U/ml TGF-β1. After 48 h of culture, total RNA was extracted using the RNeasy kit according to the manufacturer’s instructions. For both assays, cDNA was made from 5 μg of total RNA using the cDNA Archive kit (Applied Biosystems). Real-Time PCR was performed using an ABI 7600 Sequence Detection System (Applied Biosystems). Primers and probes for T-box expressed in T cells (TBX21), GATA-binding protein-3 (GATA3), and FOXP3 were obtained as assays on demand from Applied Biosystems. For each analysis, transcription of the gene of interest was compared with transcription of the housekeeping gene, β-actin, which was amplified in parallel.

Cytokine assays
After 7–10 days of culture, the differentiated cells were assayed for cytokine production. Cells were washed out of culture medium into serum-free X-VIVO 15. Cells (0.5 ml) at 1 × 10^6 cells/ml were plated into 48-well plates coated with anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml). Cells were incubated for 72 h, and supernatants were collected and frozen. Supernatant samples were sent to Pierce Endogen for multiplexed Searchlight ELISA analysis of the following cytokines: IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, TNF-α, and TGF-β. Total TGF-β, after acidification, was measured in these assays.

Flow cytometry
For flow cytometric analysis, 10^6 cells were stained with 100 μl of appropriately diluted Abs for 30 min on ice and washed twice with FACS buffer (PBS with 2% FBS and 0.1% sodium azide). For biotinylated Abs, cells were additionally stained with 100 μl of a 1/10 dilution of streptavidin-Quantum Red for 30 min on ice. Cells were washed twice with FACS buffer, resuspended in 200 μl of buffer, and analyzed on a FACScan (BD Biosciences). Intracellular staining of cells was performed after cell surface labeling and involved fixation and permeabilization of cells using the Fix and Perm kit (Caltag Laboratories) according to the manufacturer’s instructions.

In vitro suppression of naive cell activation
Differentiated cells were tested for their ability to suppress the proliferation and cytokine production of naive T cells to polyclonal activation. Naive autologous CD4+ cord blood cells or adult peripheral blood cells were labeled with the Vybrant CFSE cell tracer kit according to the manufacturer’s instructions (Molecular Probes). The CFSE-labeled cells (1 × 10^6 cells/ml) were stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) in the presence of either 10^4 differentiated cells in a final culture volume of 200 μl. In some experiments TGF-β-derived cells were prevented from directly contacting the CFSE-labeled cells by addition to the upper chamber of 6.5-mm Transwells with 0.4-μm pore size membranes (Costar) and stimulated by the addition of CD3/CD28 Dynabeads (Dynal Biotech), added directly to the upper well. Cultures were incubated for 4–5 days at 37°C in 5% CO₂. After culture, supernatants were collected for cytokine analysis, and cells were stained with anti-CD25-PE and anti-CD4-biotin, followed by secondary staining of streptavidin-Quantum Red. Cells were resuspended in buffer and analyzed by flow cytometry using a FACScan. Equal numbers of live CFSE-labeled cells were acquired for analysis.

Immunofluorescence microscopy
Differentiated cells were counted and set at 1 × 10^6 cells/ml. One hundred microliters of cell suspension was loaded into the funnel of a cytopin, and the cells were spun onto microscope slides. Slides were air-dried overnight, fixed in acetone for 5 min, then air-dried for 1 h. Slides were blocked with 10% FBS in PBS for 15 min in a humid chamber, then stained with anti-FOXP3, HRP-conjugated anti-PKCθ, or appropriate isotype-matched negative control Ab for 2 h in a humid chamber. Slides were washed for 10 min in PBS with 0.2% gelatin. For detection of FOXP3 Ab, the slides were then stained with TRITC-conjugated donkey anti-goat IgG (1/200) diluted in donkey serum for 30 min in a humid chamber in the dark. For detection of PKCθ, slides were stained with TRITC-conjugated goat anti-HRP (1/200 in blocking buffer). Slides stained for PKCθ were also stained with FITC-conjugated anti-αβTCR (BD Pharmingen). Slides were again washed for 10 min in PBS with 0.2% gelatin. A single drop of Vectashield was placed over the cells, and a coverslip was applied. The slides were then read under the immunofluorescence microscope.

Inhibition of PKCθ
To analyze the effects of PKCθ inhibitors on the proliferative response of the differentiated cells, 96-well, flat-bottom plates (Nunc) were coated with 50 μl of anti-CD3 (5 μg/ml) and anti-CD28 (10 μg/ml) diluted in PBS, incubated overnight, then washed with PBS. Cells were plated at 2 × 10^5 cells/well in a final volume of 200 μl in the presence or the absence of PKCθ inhibitors. Rottlerin, the novel PKCθ inhibitor, was purchased from Calbiochem and used at final concentrations of 1, 5, and 50 μM.
inhibitor peptide (NH$_2$-RQIKIWFQNRRMKWKKMDQNMFRN5FEMNP-COOH) was purchased from New England Peptide and used at final concentrations of 2, 20, and 40 μM. After 3 days of culture, cells were pulsed with 0.5 μCi/well [3H]thymidine and harvested 16 h later.

## Results

### Addition of TGF-β to stimulated PBL up-regulates FOXP3 message

Human PBL were stimulated by CD3 and CD28 bound to the tissue culture plate for 48 or 72 h. Quantitative PCR performed on mRNA from these samples indicated that with no additional selective pressures, transcription factors associated with the differentiation of Th1 (TBX21), Th2 (GATA3), and regulatory T cells (FOXP3) were readily detected, although GATA3 was relatively more prevalent than the other two transcription factors. When cultures were established with stimulating Abs and the addition of exogenous IL-4, IL-12, or TGF-β, increases in one transcription factor message relative to the others was observed (Fig. 1a). Addition of IL-4 resulted in a relative increase in GATA3 (~240% of the expression levels in PBL stimulated under control conditions), IL-12 resulted in a 235% relative increase in TBX21, and addition of TGF-β resulted in a 190% relative increase in FOXP3. Addition of other cytokines associated with T cell differentiation, such as IFN-γ or IL-13, or cytokines previously reported to augment in vitro differentiation of regulatory cells or survival of differentiated cells (IL-7, IL-1α, or IL-9) did not result in a similar relative increase in the transcription factors associated with T cell differentiation (Fig. 1b). Only addition of IL-10 produced a skewing of transcription factor usage in the population. Addition of IL-10 resulted in relative suppression of TBX21 and FOXP3 messages below the level normally seen in stimulated PBL, but levels of GATA3 remained at the control levels.

**FIGURE 1.** Enhancement of transcription factor usage by PBL stimulated in the presence of exogenous cytokines. PBL were stimulated by incubation with CD3 and CD28 Abs in the absence or the presence of cytokines for 72 h, and total RNA was prepared. Quantitative PCR was performed on all samples, and the number of copies of message for FOXP3, GATA3, or TBX21 present in samples derived from cells stimulated with cytokines was expressed relative to the number of copies found in PBL stimulated in the absence of cytokines, which was arbitrarily set at 100. Comparison of transcription factor message induction in the presence of IL-4, IL-12, and TGF-β (a) is shown. The effects of addition of other cytokines on induction of messages for the three transcription factors are also shown in representative experiments (b).

Optimal growth and differentiation of naive human lymphocytes with TGF-β

Because activation of human PBL in the presence of TGF-β appeared to induce increased levels of FOXP3 message, similar to the induction of TBX21 and GATA3 induced by IL-12 and IL-4, respectively, naive human lymphocytes were stimulated in the presence of TGF-β to determine whether regulatory T cells could be differentiated under these conditions. Human CD4$^+$CD45RA$^-$ (naive) cord blood or PBL, isolated by magnetic bead separations and 90–99% pure, respectively, were stimulated by plate-bound CD3 and CD28 in the presence of IL-12 and anti-IL-4 (Th1 conditions); IL-4, anti-IL-12, and anti-IFN-γ (Th2 conditions); or TGF-β (+IL-9), anti-IL-4, anti-IFN-γ, and anti-IL-12. IL-2 was added to all cultures.

Lymphocytes differentiated in the presence of TGF-β divided more slowly than cells differentiated under Th1 or Th2 conditions. In developing the culture conditions for TGF-β-derived cells, we investigated the effects of various additional cytokines on the cells. IL-15 was absolutely required for expansion of these cells. As shown in Fig. 2, addition of IL-15 to Th1 or Th2 cell cultures improved cell expansion marginally in the final days of culture after stimulation. However, TGF-β-derived cells failed to expand beyond 2- to 3-fold from their initial numbers without addition of IL-15, and viable cell numbers dropped steadily after day 3 (data not shown). Addition of IL-15 in the absence of IL-2 supported expansion of these cells, but significantly less well than when IL-2 was added ($p = 0.03$, by Student’s t test). The combination of IL-9 and IL-15 provided an additional, although statistically nonsignificant, boost to cell growth, but the best expansion of TGF-β cells was observed when IL-15 and IL-2 or IL-15, IL-9, and IL-2 were combined. Under these conditions, TGF-β-derived cells expanded to 20-fold their starting numbers.
Cells were stimulated and allowed to expand and differentiate under these conditions for 7–12 days. Once they returned to a resting state, cells were restimulated on plate-bound CD3 and CD28 in serum-free medium to assess cytokine production. Supernatants collected at 72 h were analyzed for cytokine production. As expected, cells differentiated under Th1 conditions (Fig. 3) produced IFN-γ, and those differentiated under Th2 conditions produced primarily IL-13 and IL-10, with lesser and variable amounts of IL-5 and TGF-β. Cells differentiated in the presence of TGF-β produced primarily TGF-β, with lesser and variable amounts of IFN-γ, IL-13, and IL-10. All cell types produced TNF-α; notably, IL-2 was produced at some level by all cell types. Greater variability in production of the less abundant cytokines was seen when cultures were derived from adult naive lymphocytes rather than cord blood naive lymphocytes (data not shown). Restimulation of differentiated cells with plate-bound CD3 alone or plate-bound CD3 and CD28 in the presence of exogenous IL-2 and in the presence or the absence of exogenous IL-2 (data not shown). Restimulation of TGF-β-derived cells by CD3 and CD28 without addition of the differentiative cytokine/Ab mixture produced cells that when stimulated still produced the cytokine profile associated with regulatory T cells (data not shown).
Phenotype of differentiated cells

After one round of stimulation and differentiation, Th1, Th2, or TGF-β-differentiated cells were stained with a variety of Abs recognizing surface molecules associated with lymphocyte activation and/or regulatory T cells (days 7–12). The results of this phenotyping are shown in Fig. 4. At the peak of activation, all cell types expressed high levels of CD25, CD69, and CD122 (data not shown). Resting, differentiated Th1 and Th2 cells down-regulated the expression of CD25, CD122, and CD69; however, the expression of these markers, although diminished from their expression at the peak of stimulation, remained well above background levels on the cells differentiated in the presence of TGF-β. As shown in Fig. 4, addition of IL-15 to cultures of TGF-β-inhibited cells stained for CD122 indicated staining of cells grown in IL-15. Staining with a polyclonal Ab indicates staining of cells grown in the absence of IL-15, whereas the filled shape indicates staining of cells grown in IL-15. Staining with a polyclonal goat anti-human TGF-β Ab is shown (a). Differentiated Th1, Th2, and TGF-β-derived cells were stained with Wright’s stain and examined microscopically (×200; b) or were spun onto microscope slides, dried, fixed, and stained as described for the expression of FOXP3 using a goat-anti-human FOXP3 Ab. Detection of this Ab was made using TRITC-conjugated donkey anti-goat IgG and fluorescent microscopy at ×400 magnification (c).

Transcription factor usage by differentiated cells

After one round of stimulation/differentiation as described above, total RNA was isolated from resting populations of Th1, Th2, or TGF-β-derived cells. Relative usage of the TBX21, GATA3, and FOXP3 transcription factors was determined by quantitative PCR. As shown in Fig. 5, the differentiated Th1 and Th2 cells preferentially expressed TBX21 and GATA3 message, respectively, whereas TGF-β-derived cells preferentially expressed FOXP3 message. The level of FOXP3 relative to 10,000 copies of β-actin detected using these techniques from human CD4+CD25+ cells freshly isolated from PBL was 1597 ± 293.

Inhibition of naive cell stimulation by addition of TGF-β-differentiated cells

Cells derived in the presence of TGF-β resembled regulatory T cells both phenotypically and in their cytokine profile. We next examined the cells for regulatory function in vitro. Th1, Th2, or TGF-β-differentiated cells were added in increasing proportions to
autologous, CFSE-labeled, cord blood CD4⁺ CD45RA⁺ lymphocytes, or autologous CD4⁺ CD45RA⁺ PBL stimulated with plate-bound CD3 or CD3⁺ CD28. The effects of the addition of these cells were examined on both cytokine production and proliferation of CFSE-labeled cells.

Addition of differentiated Th1 cells to CFSE-labeled cells at ratios of Th1 cells to naive cells between 1:2 (50%) to 1:10 (10%) resulted in a general increase in the level of production of most cytokines measured (Fig. 6). In three independent experiments, addition of Th1 cells produced mean increases in levels of IL-10, TNF-α, and IFN-γ up to five times the levels produced by stimulated CFSE-labeled cells alone. Addition of Th2 cells at the same ratios strongly suppressed the production of IL-2 and IFN-γ, but increased the mean production of Th2 cytokines IL-4 and IL-10 to greater than five times the levels produced by CFSE-labeled cells alone. Addition of TGF-β-derived cells at the same ratios resulted in suppression of all cytokines except TGF-β. Only TGF-β showed a mean increase in expression of 1.6-fold above control levels; all other cytokines were suppressed. Experiments performed using the addition of differentiated cell types to alter cytokine production by allogeneic CD4⁺ CD45RA⁺ PBL yielded similar results, although the TGF-β-derived cells suppressed cytokine production by stimulated allogeneic cells by only 50% (data not shown).

Labeling naive cells with CFSE before activation and culturing them with unlabeled, differentiated cells permitted monitoring the division of the naive cells specifically, even in the presence of activating differentiated cells. The results of the addition of differentiated cells on the division of naive cells are shown in Fig. 7. In three independent experiments, addition of Th1 cells, even at a ratio of 1:2, resulted in normal proliferation of the naive cells (97 ± 10% of control proliferation). Addition of Th2 cells also resulted in no suppression of proliferation (79 ± 21% of control proliferation). Addition of Th2 cells also resulted in no suppression of proliferation (79 ± 21% of control proliferation) at a ratio of 1:2. Addition of TGF-β-derived cells, however, resulted in significant suppression of PBL proliferation at a ratio of 1:2 to 47 ± 33% of control levels (range, 27–85%; p = 0.01, by Student’s t test). TGF-β-derived cells that were restimulated in a second round in the absence of the differentiative cytokine and Ab mixture retained the ability to suppress proliferation.

FIGURE 5. Relative levels of message for TBX21, GATA3, or FOXP3 in differentiated T cells. Total RNA was extracted from differentiated Th1, Th2, or TGF-β-derived cells, and the relative number of copies of message for the transcription factors TBX21, GATA3, and FOXP3 was determined by real-time quantitative PCR for each cell type. Copy numbers were normalized such that the most prevalent message in a cell type was assigned a value of 100. A representative result of five experiments is shown.

FIGURE 6. Effect of the addition of Th1, Th2, or TGF-β-derived cells on the cytokines produced by CD3⁺ CD28-stimulated naive cells. Differentiated Th1 (a), Th2 (b), or TGF-β-derived (c) cells were added to cultures of PBL stimulated with CD3⁺ CD28 at the percentages indicated (percentages represent the number of differentiated cells as a percentage of the total number of naive cells in the culture). Supernatants were removed from cultures after 5 days and assayed for cytokines. Cytokines produced by activated naive cells with no differentiated cells added were taken to represent 100%. In the experiment shown, production of IL-13, TNF-α, and IFN-γ exceeded 10-fold the control levels in cultures to which Th1 cells were added. The data shown represent one of three experiments performed using either cells differentiated from cord blood or adult peripheral blood naive cells.
of naive cells (data not shown). In some experiments blocking anti-IL-10 or anti-TGF-β Abs were included in cultures to which TGF-β-derived cells had been added to determine whether blockade of either of these cytokines would affect the inhibitory ability of the TGF-β-derived cells. Anti-IL-10 had no effect on the ability of TGF-β-derived cells to inhibit naive cell proliferation. Anti-TGF-β resulted in a slight reduction in the inhibition seen with addition of TGF-β-derived cells, but it also enhanced the proliferation of naive cells to which no TGF-β-derived cells were added. Blockade of IL-10 or TGF-β did not inhibit the suppression produced by addition of TGF-β-derived cells.

To determine whether the TGF-β-derived cells mediated their suppressive effects through cell-cell contact, TGF-β-derived cells were added to CFSE-labeled naive cells at a 1:2 ratio (50%) either in the same well or separated from the naive cells in a 0.4-μm pore size Transwell. Under these conditions, naive cells were suppressed by only ~50% compared with direct addition of the suppressive cells (Fig. 7g). No additional blockade of the remaining suppressive activity of TGF-β-derived cells was obtained by addition of anti-IL-10 or anti-TGF-β.

Cells in these experiments were also stained for CD25 expression. Addition of TGF-β-derived cells at a ratio of 1:2 suppressed CD25 up-regulation by activated naive cells from 79% of cells expressing CD25 with a mean channel fluorescence of 161 to only 34% of cells expressing CD25 with a mean channel fluorescence of 87.

**TGF-β-derived cells are less dependent on PKCθ**

Recently, using knockout mice, Berg-Brown et al. (32) demonstrated that the PKCθ pathway of NF-κB activation is important primarily for the development of effector Th cell responses. Based on these findings, we examined the expression of PKCθ by Th1, Th2, and TGF-β-derived cells using anti-PKCθ Ab and immunohistochemistry. As shown in Fig. 8, PKCθ was readily detectable in the cytoplasm of Th1 and Th2 cells. In contrast, PKCθ expression in TGF-β-derived cells was barely detectable.

We therefore examined the role of PKCθ in the proliferation of Th1, Th2, and TGF-β-derived cells using the inhibitor Rottlerin. As shown in Fig. 8, Rottlerin, within ranges established to specifically inhibit PKCθ functions in T cells (33, 34), inhibited the proliferation of Th1 and Th2 cells, but had minimal effects on TGF-β-derived cell proliferation driven by CD3 and CD28. At high concentrations, the proliferation of all cell types was inhibited, consistent with the known ability of Rottlerin to inhibit many cellular enzymes at high concentration (35). To further demonstrate that these effects were due to specific inhibition of PKCθ, a peptide that specifically blocks the interaction of PKCθ and its scaffolding proteins was synthesized, incorporating an antennapedia helical domain to permit cell entry (36), and its effect on cell proliferation was examined. This more specific inhibitor of PKCθ also inhibited the proliferation of Th1 and Th2 cells to 40–50% of control levels, but spared TGF-β-derived cells at concentrations between 12 and 50 μM (Fig. 8b).

**Discussion**

These data demonstrate that human naive peripheral CD4+ cells, stimulated through their TCR in the presence of TGF-β with exogenous IL-2 and IL-15, can expand and differentiate into cells expressing a surface phenotype consistent with thymus-derived regulatory cells. The TGF-β-derived cells up-regulate message for FOXP3, and the translated protein is present in the nucleus of the cells. Like thymus-derived CD4+CD25+ regulatory cells, TGF-β-derived cells are capable of suppressing proliferation and cytokine production of normal, naive cells in vitro. Recently, Chen et al. (31) demonstrated that activation of murine CD4+CD25+ cells through the TCR by Ag or anti-CD3 Ab stimulation in the presence of TGF-β and exogenous IL-2 resulted in differentiation of CD4+CD25+ cells expressing FOXP3 and capable of suppressing immune responses in vitro and in vivo.

In vitro differentiation of T cells with regulatory function has been reported using numerous methods. Coculture of T cells with dendritic cells or other APCs in different states of maturity has
results in the differentiation of T cells with suppressive function (37–39). Addition of exogenous IL-10 and TGF-β was demonstrated to induce murine CD4+CD25+ cells stimulated in an MLR to decrease constitutively producing primarily IL-10 and/or TGF-

Other systems used culture conditions based on purified T cells and cytokines to derive cells with apparent regulatory function. TGF-β has been demonstrated to induce expansion of regulatory populations resembling thymic-derived CD4+CD25+ cells or Th3 cells when either CD4+CD45RA− cells or CD4+CD25− human PBL, respectively, were stimulated with Ag in the presence of the cytokine (29, 41).

Other cytokines have also been used to differentiate cells in vitro with some regulatory properties. The first regulatory T cell populations to be derived from peripheral T cells in vitro were the TR1 cells. TR1 cells were derived initially from mouse or human CD4+ T cells stimulated through their Ag receptors in the presence of exogenous IL-10 (27). Interestingly, Bacchetta et al. (42) demonstrated, that like the TGF-β-derived cells described in this study, TR1 cells required IL-15 for efficient expansion. These cells were demonstrated in vitro and in vivo to possess clear regulatory T cell functions, but examination of their transcription factor usage indicated that GATA3 was the primary T cell differentiation-related transcription factor in these cells. Our finding that human PBL stimulated in the presence of exogenous IL-10 results in relative enhancement of GATA3 message usage is consistent with the potential to derive human TR1 cells.

Stimulation of murine CD4+CD62Lhigh cells with Ag in the presence of APCs, vitamin D₃, and dexamethasone resulted in T cells similar to TR1 cells, producing high levels of IL-10 (43). In the human system, TR1 cells have been differentiated in vitro from CD4+ cord blood cells or naïve, CD4+CD45RA− peripheral blood T cells by stimulation through the TCR in the presence of exogenous IL-10 and IFN-α (44). Addition of TGF-β to these cells, with or without IL-10, did not result in the outgrowth of cells with typical TR1 cytokine profiles, and TGF-β appeared to generally inhibit IL-10, IL-4, IFN-γ, and IL-2 production. Only low levels of IFN-γ were produced by TGF-β-treated cultures. These results and the conclusion that TGF-β treatment did not enhance differentiation of TR1 cells are consistent with our findings.

Phenotypically, we have demonstrated that human CD4+CD45RA− cells activated in vitro in the presence of TGF-β express cell surface molecules consistent with those expressed by both in vitro-derived and thymus-derived regulatory T cells. Human and mouse CD4+CD25+ thymic-derived regulatory T cells are consistently reported to express CD4, CD25, CD69, CD103, CD122, CTLA-4, HLA-DR, and GITR (9, 22, 25, 45–49). Because these molecules are all activation Ags on lymphocytes, during the in vitro differentiation of Th1, Th2, and TGF-β-derived cells, all these molecules appeared on all cell types at some point during activation. However, as shown, when the differentiated cells returned to a resting state, TGF-β-derived cells retained expression of these cell surface proteins of this phenotype, similar to the phenotype of natural thymus-derived CD4+CD25+ cells. Regulatory T cells from both mice and humans are described as generally producing primarily IL-10 and/or TGF-β when stimulated, with variable reports of low levels of IL-4, IL-5, and IFN-γ. Specifically, TR1 cells appear to produce primarily IL-10 (27, 42, 50), whereas Th3 cells and CD4+CD25− cells produce more TGF-β (51, 52). Like TR1 cells, the TGF-β-derived cells described in this study require exogenous IL-15 (42) for efficient proliferation. However, based on cytokine production, the cells described in this study are more similar to Th3 or CD4+CD25+ cells than to TR1 cells. Most in vitro assays of the suppressive abilities of regulatory T cells have demonstrated that addition of regulatory cells to responding cells in ratios ranging from 1:10 to 1:1 produces suppression of mitosis by the responders. The TGF-β-derived cells reactions and inhibiting graft-vs-host disease in vivo (40). Other systems used culture conditions based on purified T cells and cytokines to derive cells with apparent regulatory function.
appear to function similarly. Therefore, in all respects these cells appear to represent regulatory T cells.

The mechanisms by which regulatory cells suppress T cell proliferation varies by the type of regulatory cell studied. Thymic-derived CD4+CD25+ cells appear to require cell-cell contact to suppress proliferation (53, 54), whereas Th1 cells appear to use IL-10 (28, 55, 56). Inhibition of cell-cell contact reduces suppression by the TGF-β-derived cells described in this report by ~50%, whereas addition of anti-TGF-β or anti-IL-10 has no effect. These results appear to be more consistent with the mechanism of action described for Th3 cells than that for Th1 cells. They also suggest that some other soluble factor produced by the cells may account for some of the suppressive activity. Because the cells used in these experiments are lines and not clones, it is not possible to determine whether these results are produced by a single type of regulatory cells using both cell-cell contact and secretion of an unidentified suppressive factor or whether differentiation of cells in the presence of TGF-β results in the development of more than one type of regulatory cell.

The ability of TGF-β to alter the cytokine production and transcription factor usage of differentiated T cells has been described. TGF-β is known to inhibit both IFN-γ production by inhibiting expression of TBX21 (57) and the production of Th2 cytokines and GATA3 by Th2 cells (58, 59). TGF-β is produced by a variety of cell types in the body and is often produced in association with healing. The observations in this paper suggest that naive lymphocytes, drawn to a site of previous injury, might be activated during the healing process in the presence of sufficient quantities of TGF-β to result in the differentiation of a population of peripherally derived regulatory T cells. These cells could then play an important physiological role in containing the natural, protective, immune responses and promoting normal healing, augmenting a more homeostatic role possibly belonging to the thymus-derived regulatory T cells.

PKCθ has been shown to play an important role in the proliferation of peripheral T cells. Peripheral T cells, but not thymocytes, from PKCθ knockout mice proliferate poorly in vitro when stimulated with CD3, CD3 δCD28, TPA plus ionomycin, and anti-CD3 in MLR; in vivo responses to keyhole limpet hemocyanin were also impaired (60). Various data strongly suggest that effector T cells (Th1 and Th2) use the caspase-recruitment-domain-containing 11 (CARD11)/PKCθ pathway to activate NF-κB to turn on the production of inflammatory cytokines (61, 62). The expression of NF-κB and components of this transcription factor has been shown to increase during allograft rejection. Treatment of animals with CD154 Ab resulting in permanent graft acceptance and tolerance inhibited the expression of NF-κB and related proteins (63). Other inhibitors of the NF-κB pathway, such as deoxyspergualin (64), have been used successfully in vivo to induce long-term allograft survival and tolerance (65). Recently, data showing that PKCθ knockout mice readily developed tolerant or anergic T cell responses have more closely linked the inhibition or absence of PKCθ, resulting in decreased activation of NF-κB, to the development of tolerance (32). Our data suggest that human Th1 and Th2 cells may also more heavily use the PKCθ activation pathway for proliferation than do TGF-β-derived regulatory cells. Taken together, these data suggest that regulatory T cells may not be as dependent on the PKCθ pathway for activation of NF-κB to expand and respond to stimuli. Additional examinations of the biochemical pathways used by effector and regulatory T cells may permit the identification of targets for new therapeutic agents that uniquely target effector T cells and spare regulatory T cells, enhancing the development of tolerance.

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References
