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Human Resting CD4⁺ T Cells Are Constitutively Inhibited by TGFβ under Steady-State Conditions¹,²

Sabine Classen,³* Thomas Zander,³* Daniela Eggle,* Jens M. Chemnitz,* Benedikt Brors,† Ingrid Büchmann,* Alexey Popov,* Marc Beyer,* Roland Eils,† Svenja Debey,* and Joachim L. Schultze⁴*

Based on studies in knockout mice, several inhibitory factors such as TGFβ, IL-10, or CTLA-4 have been implicated as gatekeepers of adaptive immune responses. Lack of these inhibitory molecules leads to massive inflammatory responses mainly mediated by activated T cells. In humans, the integration of these inhibitory signals for keeping T cells at a resting state is less well understood. To elucidate this regulatory network, we assessed early genome-wide transcriptional changes during serum deprivation in human mature CD4⁺ T cells. The most striking observation was a “TGFβ loss signature” defined by down-regulation of many known TGFβ target genes. Moreover, numerous novel TGFβ target genes were identified that are under the suppressive control of TGFβ. Expression of these genes was up-regulated once TGFβ signaling was lost during serum deprivation and again suppressed upon TGFβ reconstitution. Constitutive TGFβ signaling was corroborated by demonstrating phosphorylated SMAD2/3 in resting human CD4⁺ T cells in situ, which were dephosphorylated during serum deprivation and rephosphorylated by minute amounts of TGFβ. Loss of TGFβ signaling was particularly important for T cell proliferation induced by low-level TCR and costimulatory signals. We suggest TGFβ to be the most prominent factor actively keeping human CD4⁺ T cells at a resting state. The Journal of Immunology, 2007, 178: 6931–6940.

Although much is known about activation, differentiation, and expansion of lymphocytes involved in Ag responses, the maintenance of lymphocyte homeostasis before and after an immune response is less well understood (1–5). As suggested by Grossman and colleagues (3), T cell homeostasis requires temporary T cell activation followed by either self-renewal or differentiation. This “balance of growth” of activated T cells was proposed to be regulated by feedback loops most likely on the level of APCs. The mechanisms involved are supposed to function in each differentiation compartment from the naive to the memory cell differentiation stage. To initiate differentiation or self-renewal, T cells must first undergo a transition between the resting and an activated state.

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CTL-A4, a different phenotype has been shown in IL-10-deficient mice with less acute inflammatory diseases mainly manifesting in the intestine (28). This bowel disease may be due to uncontrolled chronic inflammation induced by enteropathogens.

In mice there is clear evidence that TGFB is an important factor restraining the size of the T cell compartment (20, 22, 29–31). Mice defective in TGFB develop symptoms of a lymphoproliferative disease 2–3 wk after birth. Due to the pleiotropic effects of TGFB, studying the role of TGFB on T cell homeostasis in TGFB−/− mice is hampered by unrelated effects also responsible for the observed phenotype (22, 29). To circumvent such limitations, transgenic mice with a dominant-negative TGFB-RII specifically expressed in T cells were previously introduced by Gorelik and Flavell (20, 29). Mice expressing such dominant-negative TGFB-RII within the T cell compartment show disruption of homeostasis, a strong inflammatory response, and significant signs of autoimmunity (20, 22). These mice are characterized by a reduced number of naive T cells with a concomitant increase of memory T cells, of which a significant fraction demonstrates an activated phenotype. Furthermore, these cells show spontaneous differentiation in vitro in response to T cell activation. Taken together, numerous inhibitory mechanisms responsible for preventing T cells from being activated have been revealed in murine models. But even in the murine system their integration into homeostatic circuits is not fully understood.

In humans even less is known about the major signals involved in T cell homeostasis, which is mainly due to the inability of in vivo manipulation of single genes. It is tempting to speculate that the same factors involved in murine T cell homeostasis are also involved in human T cell homeostasis, especially because many of these factors have similar roles during induction of immunity. However, so far no experimental evidence exists supporting such a postulate.

Because T cell homeostasis seems to be mainly regulated by exogenous stimuli, we hypothesized that deprivation of resting human T cells of any exogenous signals should reverse intracellular signaling cascades actively keeping T cells at a resting state. We further postulated that such changes should certainly be recognizable on the genomic level. To this end, we interrogated genomewide transcriptional changes of human mature CD4+ T cells in response to deprivation of exogenous signals. Using this unbiased approach, we demonstrate TGFB to be the most prominent inhibitory factor detected that constitutively acts on mature human resting CD4+ T cells keeping them at a “resting phenotype.”

Materials and Methods

Peripheral blood samples

Following approval by the institutional review board, blood samples from healthy blood donors were collected after written informed consent had been obtained.

Isolation of CD4+ T cells, CD8+ T cells, B cells, and monocytes

CD4+ T cells were isolated from blood samples by using a RosetteSep CD4+ enrichment kit (StemCell Technologies); purity was >90% as determined by flow cytometry. For comparing SMAD2 and SMAD3 phosphorylation in CD4+ T cells, CD8+ T cells, B cells, and monocytes, cells were isolated by positive selection using CD4, CD8, CD19, or CD14 microbeads (Miltenyi Biotec).

Serum deprivation and T cell treatment

CD4+ T cells were cultured in serum-free medium (AIM V [Invitrogen Life Technologies] plus ExCell 640 1:1 (JRH Biosciences)) for 2, 8, 12, or 18 h (serum deprivation). TGFB (R&D Systems) was added after serum deprivation for 1, 2, or 8 h. In other experiments, 50% freshly isolated human serum, in combination with different concentrations of the ALK5 inhibitor SB431542 (Tocris Bioscience), was added for 2 h to CD4+ T cells after serum deprivation.

Immunofluorescence for SMAD2 and SMAD3

T cells and B cells were isolated from venipuncture blood samples by using RosetteSep T cell enrichment and B cell enrichment kits (StemCell Technologies). Cells were centrifuged on glass coverslips, fixed with 4% paraformaldehyde (Sigma-Aldrich), and permeabilized in 0.2% Triton X-100 (Invitrogen Life Technologies) before blocking for 0.5 h (1% fish skin gelatin [Sigma-Aldrich], 10% goat serum [DakoCytomation]) in PBS. Slides were incubated with anti-phospho-SMAD3 (Merck) followed by secondary Ab (Alexa Fluor 568 goat anti-rabbit IgG [H + L]; Invitrogen Life Technologies). Subsequently, cells were incubated with mAbs against CD4 (Novocastra), CD8 (DakoCytomation), or CD19 (Serotec) followed by secondary Ab (Alexa Fluor 488 goat anti-mouse IgG [H + L]; Invitrogen Life Technologies). Afterward, cells were incubated with 4',6'-diamidino-2-phenylindole dihydrochloride (Invitrogen Life Technologies) for nuclear staining. Photographs were taken with a Zeiss Axiosplan microscope (×63 magnification) and Zeiss AxioVision Rel 4.5 software.

For control stainings, freshly isolated CD4+ T cells were cultured for 24 h under serum-free conditions and either analyzed directly or treated with 30 ng/ml TGFB for 1 h.

Cell lysis and Western blot

Cells were lysed (5 ml of 1% Triton X-100 [Promega], 750 μl of 150 mM NaCl [Roth], 250 μl of 50 mM Tris-HCl [Invitrogen Life Technologies], 50 μl of Phosphatase Inhibitor Cocktail 1 [Sigma-Aldrich]), 50 μl of Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich), protease inhibitor (Roche, Complet Mini), 10 μl of 1 M PMSF), lysates resolved by SDS-PAGE, proteins were transferred to nitrocellulose, and blots were probed with appropriate primary and secondary Ab combinations. The following Abs were used: anti-phospho-SMAD3/SMAD1 Ab (Cell Signaling), anti-phospho-SMAD2 Ab (Cell Signaling), anti-SMAD2/3 (BD Biosciences), anti-β-actin Ab (Chemicon International), anti-mouse IgG-HRP (DakoCytomation), anti-rabbit IgG-HRP (DakoCytomation).

Microarray procedure

When using the Affymetrix platform RNA, isolation, quantification, target preparation, and hybridization were performed as described previously (32). Biotin-labeled cRNA preparation for the Illumina platform was performed using the Ambion Illumina RNA amplification kit (Ambion Europe). Biotin-labeled cRNA (1.5 μg) was hybridized to Sentrix whole 6×2 genome bead chips (Illumina) and scanned on the Illumina BeadStation 500x (33). Table I represents a summary of all microarray experiments performed within this study.

Data analysis and software

For data collection, assessment and statistical analysis we used Affymetrix Microarray Analysis Suite 5.0, Affymetrix Data Mining Tool 3.0, Illumina BeadStudio, and R language (Bioconductor project). In R language, the vsn method and the quantile method were used for data normalization of Affymetrix Illumina microarrays, respectively. Unpaired t tests were calculated as appropriate. For visualization and gene ontology assessment, we used GenMAPP and MAPTfinder (both from Gladstone Institutes, University of California, San Francisco) (34). All heat maps were visualized using MAYDAY (PAS-group, University Tübingen) (35). For further gene ontology analysis, the R platform was used.

To better understand changes in transcriptional regulation, an algorithm was developed that integrates gene ontology information provided by the international gene ontology (GO) consortium, a quantitative distance analysis between different experimental groups (here time points), a calculation of the statistical power of the method based on a permutation approach and a visualization of the data. The algorithm will be described in more detail elsewhere (D. Eggel, T. Zander, and J. L. Schultz, manuscript in preparation).

Briefly, in a first step, so-called “gene spaces” are determined which are based on GO classifications. A gene space is composed of genes within a specific GO identification (ID). GO IDs defining a gene space are restricted to one of the specified categories: biological process, molecular function, or cellular component. Only those GO IDs which are represented within the least five probe sets on the array in use are used for further analysis. In the second step, pairwise centroid distances using the Euclidean distance are calculated between the different experimental groups. The third step is a

5 Abbreviations used in this paper: GO, gene ontology; ID, identification; aAPC, artificial APC; FC, fold change; PIGF, phosphatidylinositol glycan class F.
significance analysis of the calculated distances. In this study, group assignments of the samples are randomly permuted followed by recalibration of distances between centroids. This is done 1000 times. Corresponding p values are determined as the fraction of iterations where the centroid distance obtained from the permuted groups is greater than the centroid distance in the original data. In the next step, a network of contributing genes is being constructed and visualized using Cytoscape. Therefore, gene spaces meeting a specified significance criterion are determined and genes included in the respective gene space (contributing genes) are extracted. The network of contributing genes is constructed by drawing edges between genes belonging to the same GO IDs and is subsequently visualized in Cytoscape.

All microarray data can be accessed using the National Center for Biotechnology Information (GEO accession number).

**Real-time-PCR**

Five hundred nanograms of RNA were reverse transcribed using the Transcripter First Strand cDNA Synthesis kit (Roche Diagnostics). RT-PCR was performed with a LightCycler TaqMan master kit and a Universal Probe Library Assay on a LightCycler 1.3 instrument (Roche Diagnostics). Analysis was performed with LightCycler3 and RelQuant software (Roche Diagnostics) using a calibrator normalized relative quantification based on the \( \beta_{2}-\)microglobulin expression. Primers used are listed in Table II.

**Functional T cell assays**

CD4\(^+\) T cells were cultured in serum-free medium and TGF\(\beta\) was supplemented as described (1 ng/ml). The cells were stimulated 16 h after serum deprivation with artificial APCs (aAPC) comprised of magnetic beads (Dynal Biotech) coated with the following Abs: 5% anti-CD3 (OKT3; Janssen-Cilag), 14% anti-CD28 (9.3, a gift from Drs. C. June and J. Riley, Abramson Cancer Research Center, University of Pennsylvania, Philadelphia, PA), and 81% anti-MHC class I (W6/32) as previously described (36). Before stimulation with aAPCs, CD4\(^+\) T cells were labeled with CFSE (Invitrogen Life Technologies). After four days of culture, T cell proliferation was assessed by flow cytometry.

**Results**

**Significant transcriptional changes in human CD4\(^+\) T cells after short-term serum deprivation**

To assess factors keeping T cells at a resting state, we exposed purified human CD4\(^+\) T cells to an environment depleted of blood-derived soluble factors present in serum. Early genome-wide transcriptional changes were assessed using Affymetrix microarrays (Fig. 1A). Filtering based on fold changes (FC) and significance (variable probe sets, FC >1.5 or FC < -1.5 and \( p < 0.05 \)) revealed a high number of genes (878 genes, 443 up- and 435 down-regulated) with altered transcription after 2 h of serum deprivation in highly purified CD4\(^+\) T cells. Changes of transcription even further increased at a later time point (910 genes at 8 h; 593 up- and 317 down-regulated) (Fig. 1A). When hierarchical clustering using all variable probe sets was performed, time of serum withdrawal was the major factor separating the sample groups (Fig. 1B).

**Quantitative analysis reveals the TGF\(\beta\) signaling pathway as a major target after short-term serum deprivation**

Next, we were interested in determining which biological systems mainly contribute to these changes of gene expression. Therefore, we applied a systems biology approach based on GO. In short, a set of gene spaces was defined as the group of genes given by one GO ID in the category biological process (www.geneontology.org). Only those GO IDs were used that are represented with at least five probe sets on the HGU133A Affymetrix array (Fig. 1C). Of the 18,455 currently known GO IDs, 9,805 comprise biological processes. 2,616 are present on the HGU133A array, but only 1,336 of them include at least 5 probe sets (Fig. 1C). Within these 1,336 predefined gene spaces, Euclidean distances were calculated between the different sample groups (time points \( t = 0, 2, \) and 8 h). Significance of the obtained distances was validated by permutation analysis. As demonstrated in Fig. 1D, 384 GO IDs were identified to be on a significance level below 0.1%, 180 GO IDs between 0.1 and 1%, and 230 GO IDs between 1 and 5% after 8 h of serum deprivation.

When analyzing the most significant GO IDs, it became apparent that genes belonging to the biological terms cell cycle, cell growth, and transcription regulation were major contributors to differences in gene expression after serum deprivation (Fig. 1D).

**Table II. RT-PCR primer**

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Universal Probe Library</th>
</tr>
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<tbody>
<tr>
<td>CXCR4</td>
<td>TGGTCTCCGTGAGAGAAGATGCT</td>
<td>TCCGAGTTAAGAGATGAGA</td>
</tr>
<tr>
<td>KLF10</td>
<td>TTAAGATGTTCCCATGAGAGG</td>
<td>TCACTGAAAGGCAAAACCCT</td>
</tr>
<tr>
<td>SLAMF1</td>
<td>ACCGGAGAACCCCTATCAG</td>
<td>CTGCCCCATGTTTTGTTTTCCT</td>
</tr>
<tr>
<td>B2M</td>
<td>TTTCTGGCCTGAGGCTAT</td>
<td>TCAGGAAATTTTGACTTTCCATTC</td>
</tr>
</tbody>
</table>
<1%). To corroborate the GO-based approach, a set of genes containing previously described TGFβ1 target genes (37, 38) was subjected to GO analysis. We postulated that these TGFβ target genes should again reveal significant changes in gene expression associated with serum deprivation. Indeed, this set of genes was even more significantly changed in human primary CD4+ T cells (error rate <0.1%) (Fig. 1D).

To further evaluate the specificity of our results, GO IDs containing genes associated with immune regulation were studied. Strikingly, none of these GO IDs reached a level of significance exceeding 1% (three GO IDs with an error rate between 1 and 5%, and eight GO IDs with an error rate >5%). Taken together, the genome-wide screen for transcriptional changes and a quantitative bioinformatics approach revealed that changes in TGFβ-related genes are major contributors to the overall transcriptional changes observed after serum deprivation in human CD4+ T cells.

Serum-deprived human CD4+ T cells present a TGFβ loss signature

To visualize the impact of serum deprivation on TGFβ-related genes, we adapted the TGFβ signaling map provided by GenMAPP and added target genes significantly changed in gene expression (FC >1.5 or FC <−1.5, p <0.05 at t = 8 h) after serum deprivation (Fig. 2A). After 2 h, inhibitors of TGFβ signaling (SMURF1 and 2, SMAD7, TGFβ, and SKI) were significantly up-regulated while at the same time many genes known to be induced by TGFβ including JUNB, JUNB, GADD45B, ZFP36L2, ID2, BHLB2, and KLF11 were down-regulated. This was further pronounced after 8 h: 13 known target genes of TGFβ were significantly reduced in expression, while three genes usually repressed by TGFβ (ID3, MYB, and phosphatidylinositol glycan class F (PIGF)) were induced (Fig. 2A). To further support these findings, we performed an additional experiment extending the time of serum deprivation to 12 and 18 h (Fig. 2B, plotted are only genes differentially expressed after 8 h). For those TGFβ target genes, we observed a similar expression pattern at the later time points, further supporting that the transcriptional control of these genes by TGFβ is lost after serum deprivation.

Identification of novel TGFβ target genes in resting CD4+ T cells

To investigate which genes are main targets of TGFβ in resting CD4+ T cells, human CD4+ T cells were first serum-deprived for 18 h followed by a single pulse of TGFβ1 (10 ng/ml). TGFβ target genes were defined as genes showing transcriptional changes after serum deprivation (FC >1.5 or FC <−1.5, p <0.05 at t = 8 h after serum deprivation) and counterregulation after addition of TGFβ1 (FC >1.25 or FC <−1.25, p <0.05 at t = 8 h following addition of TGFβ1). As demonstrated in Fig. 3A, most of the known TGFβ target genes identified as significantly regulated during serum deprivation (see Fig. 2B) were indeed counterregulated after addition of TGFβ1, with only a few exceptions.

In addition to known TGFβ target genes we identified 42 novel genes that so far have not been recognized as TGFβ target genes in other cellular systems (Fig. 3B). Although most of the known TGFβ target genes were down-regulated during serum deprivation and restored following TGFβ1 pulse (Fig. 3A), the majority of the new target genes were found to be suppressed by TGFβ1 (Fig. 3B). This might be explained by previous strategies that mainly identified TGFβ target genes solely by exposing cells to increased concentrations of TGFβ1 (37). Applying a GO approach, four major groups of genes were identified: genes encoding for membrane-associated proteins (transport and signaling), proteins with nuclear localization (transcriptional regulation), proteins involved in cell cycle regulation, and genes of unknown function. Six genes...
(RDH11, SLC35A1, VDP, PIGF, B3GALT2, and GNPAT) associated with intracellular membranes, especially of the Golgi apparatus and the endoplasmic reticulum, are repressed by TGFβ. Except for PIGF, a key enzyme involved in GPI anchor biosynthesis, the function of the other genes in T cells is still elusive. Expression of three extracellular membrane proteins, CXCR4, FLT3LG, and SLC7A5, is significantly reduced upon serum deprivation, while ICAM-2, an adhesion molecule, and SLAMF1 (CD150) a costimulatory molecule, are suppressed by TGFβ. SLAMF1 has been shown to be constitutively expressed in T cells and increased expression of SLAMF1 is clearly associated with T cell activation (39). Our data further suggest that the level of constitutive SLAMF1 expression is under the control of TGFβ with increased expression in the absence of TGFβ.

The second group of genes codes for proteins with nuclear localization. In addition to regulators of transcription known to be TGFβ target genes (KLF10, JUN, and MYB), expression of seven novel genes involved in transcriptional regulation (HDAC2, SF1, ZFP36, RNPC1, RACGAP1, YWHAE, and IFI16) was shown to be constitutively expressed in T cells and increased expression of SLAMF1 is clearly associated with T cell activation (39). Our data further suggest that the level of constitutive SLAMF1 expression is under the control of TGFβ with increased expression in the absence of TGFβ.

To verify these findings by a second technique, we performed real-time PCR for exemplary target genes (Fig. 3). CD4+ T cells were cultured under the same culture conditions. Similar to the microarray data, mRNA for CXCR4 and KLF10 was significantly down-regulated following serum deprivation and stayed low over the whole culture period (up to 26 h). In contrast, after a single pulse of TGFβ1 (10 ng/ml) at 18 h, the expression levels of both genes were counterregulated, almost restoring the levels of CXCR4 expression to baseline and significantly exceeding baseline levels for KLF10. Exemplary for a gene suppressed by TGFβ1 RT-PCR results obtained for SLAMF1 are shown. mRNA for SLAMF1 was significantly up-regulated following serum deprivation and was highly transcribed over the whole culture period (up to 26 h). Addition of TGFβ1 (10 ng/ml) after 18 h of serum deprivation reduced the expression to baseline levels.

Ablation of TGFβ signaling is associated with reduced SMAD phosphorylation

The genome-wide transcription analysis suggested that constitutive TGFβ signaling controls resting T cells on the transcriptional level. Phosphorylation of receptor-regulated SMAD2 and SMAD3
is an early event following binding of TGFβ to its cognate receptor complex. Loss of transcriptional control by TGFβ should therefore be accompanied by loss of SMAD phosphorylation. To study SMAD3 phosphorylation on the single cell level, immunofluorescence analysis was performed on human lymphocytes from venipuncture blood immediately after isolation. As depicted in Fig. 4, all freshly isolated CD4+ T cells were cultured under serum-free conditions for up to 18 h before 10 ng/ml TGFβ1 was added, and the transcriptional changes were assessed 1, 2, or 8 h after addition of TGFβ1. In control cultures, no TGFβ1 was added. A, Heat map displaying FCs of known TGFβ target genes. Genes were selected based on differential expression (FC > 1.5 or FC < −1.5, p < 0.05) at 8 h after serum deprivation compared with the 0-h time point. FCs for the 8, 12, and 18 h after serum deprivation were computed in comparison to the 0-h time point. FCs for the 1, 2, and 8 h after addition of TGFβ1 were computed in comparison to the 18-h time point of serum deprivation. FCs are color coded showing genes being up- and down-regulated in red and blue, respectively. B, Identification of novel TGFβ target genes. Genes were selected based on differential expression after serum deprivation (0- vs 8-h time point; FC > 1.5 or FC < −1.5, p < 0.05) and counterregulation following addition of TGFβ1 (FC > 1.25 or FC < −1.25, p < 0.05). FCs were computed and are color coded in the same way as in A. Genes marked with an asterisk are known TGFβ target genes. C, Regulation of CXCR4, KLF10, and SLAMF1 mRNA assessed by RT-PCR. The relative expression levels compared with B2-microglobulin are plotted. Data represent mean ± SD of three independent experiments. □, T cells before culture; ■, T cells under serum-free conditions; ▪, T cells after addition of TGFβ1 (10 ng/ml). Statistically significant differences (paired t test, p < 0.05) are marked with an asterisk.
Ablation of TGFβ signaling is associated with reduced SMAD phosphorylation

Next, we determined whether serum deprivation would be accompanied by loss of phosphorylation of SMAD molecules. Indeed, SMAD2 and SMAD3 phosphorylation were significantly reduced after 2 h and basically undetectable after 8 h (Fig. 6, A and B) while the amount of total SMAD protein remained constant during serum deprivation, ruling out that loss of total SMAD protein is responsible for the decrease of SMAD phosphorylation. Therefore, signaling events distal of the TGFβ receptor become inactive in human resting CD4+ T cells shortly after removal of TGFβ. If removal of TGFβ leads to loss of SMAD phosphorylation, reconstitution with TGFβ should restore SMAD phosphorylation in serum-deprived resting CD4+ T cells. Therefore, CD4+ T cells were serum deprived for 18 h to reduce SMAD phosphorylation to undetectable levels and afterward stimulated with increasing concentrations of TGFβ1. As shown in Fig. 6C, as little as 0.001 ng/ml TGFβ1 induced significant phosphorylation of SMAD3 and at 0.01 ng/ml TGFβ1 SMAD2 phosphorylation was clearly detectable 2 h after TGFβ1 addition. As we have shown constitutive phosphorylation of SMAD molecules in resting CD4+ T cells from peripheral blood, we postulated that freshly isolated human serum, containing active TGFβ, should also restore SMAD phosphorylation following serum deprivation. Again, T cells were cultured under serum-free conditions.

FIGURE 5. SMAD2 and SMAD3 are phosphorylated in primary human CD4+ T cells. A, In four individuals, p-SMAD2 and p-SMAD3 were analyzed in highly purified CD4+, CD8+, CD14+, and CD19+ cells by Western blotting using Abs specifically detecting phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3). The relative amount of p-SMAD2 and p-SMAD3 in comparison to β-actin was measured by densitometry. Shown here are mean ± SD of the data after normalization to CD4+ cells. B, p-SMAD2, p-SMAD3, and total SMAD2/3 were analyzed in highly purified CD4+ T cells derived from peripheral blood of five healthy individuals by Western blotting. – , The negative control (T cells after serum deprivation); +, the positive control (T cells stimulated with 10 ng/ml TGFβ1). C, The relative amount of p-SMAD2 and p-SMAD3 in comparison to β-actin (see B) was measured by densitometry.

FIGURE 4. SMAD3 phosphorylation in human lymphocytes. Immunofluorescence analysis of venipuncture blood samples; cells were stained for either CD4, CD8, or CD19 (green, Alexa 488) and p-SMAD3 (red, Alexa 568). Nuclear staining with 4',6'-diamidino-2-phenylindole (blue). The overlay is depicted in the top row. A, CD4+ T cells analyzed after 24 h of serum deprivation (− TGFβ1) and after an additional incubation period of 1 h with 30 ng/ml TGFβ1 (+ TGFβ1). B, Freshly isolated cells were either stained with Abs against CD4, CD8, or CD19 as well as p-SMAD3 mAb. One representative experiment of four is shown.
for up to 24 h (Fig. 6D) with subsequent addition of serum to the culture for 2 h. At this point, SMAD phosphorylation was significantly increased and exceeded baseline levels. To demonstrate that this effect was due to signaling via the TGFβ RIIL, we used the TGFβ receptor kinase inhibitor SB431542. As shown in Fig. 6D, serum-induced SMAD phosphorylation was decreased by this inhibitor in a concentration-dependent manner, further supporting that SMAD phosphorylation in human resting T cells is controlled by TGFβ. The observed signaling events downstream of TGFβ are independent of other signals such as TCR stimulation or costimulation, because the effects were observed in the absence of further T cell stimulation.

**FIGURE 7.** CD4⁺ T cell proliferation is increased after TGFβ withdrawal. Freshly isolated CD4⁺ T cells were cultured in serum-free medium for 16 h and then stimulated with the indicated ratios of CD3/CD28/MHC-I aAPC in the presence or absence of 1 ng/ml TGFβ1. A, CD4⁺ T cell proliferation was assessed by CFSE labeling. The percentage of cells dividing at least once is indicated inside the respective histogram plot. B, Displayed is the percentage of cells dividing at least for the indicated numbers of cycles (e.g., 1⁺, all dividing cells; 2⁺, cells that divided two or more times; 5⁺, cells that divided five or more times) depending on the presence (●) or absence (○) of TGFβ1 for the indicated ratios of aAPC:T cells.

**Removal of constitutive TGFβ signaling leads to increased T cell proliferation**

The transcriptional analysis revealed significant changes of gene expression associated with cell cycle regulation (Fig. 1D). Does this have a functional consequence for CD4⁺ T cells? Ablating TGFβ signaling in resting CD4⁺ T cells should lead to a more pronounced activation and proliferation because entry of the cells into the cell cycle should be enhanced in the absence of TGFβ-dependent regulators.

To address the removal of TGFβ before activation of resting human CD4⁺ T cells, the cells were deprived from serum for 16 h and stimulated with increasing concentrations of aAPC (ratios aAPC:T cell from 1:10 to 2:1) comprised of magnetic beads coated with suboptimal concentrations of anti-CD3 and anti-CD28 mAbs (36) in the presence or absence of TGFβ1 (1 ng/ml). When only low concentrations of aAPC were present (1:10 ratio), approximately one-half of the T cells underwent cell division (46.5%) in the absence of TGFβ1, while addition of TGFβ1 decreased the percentage of proliferating cells to 25% (Fig. 7A). Even more important, whereas a significant number of cells reached four or five cell divisions in response to low Ag (1:10 ratio) in the absence of TGFβ1, virtually no cells had divided five times and only 4% divided four times in presence of TGFβ1 (Fig. 7B).

Albeit CD4⁺ T cell proliferation was always lower in the presence of TGFβ1, this difference was less pronounced at higher concentrations of aAPC, suggesting that TGFβ1 is particularly able to inhibit T cell proliferation at low levels of Ag and/or costimulation. When increasing the amount of aAPC to very high concentrations, aAPC were not able to activate TGFβ1-treated CD4⁺ T cells to the same extent as T cells in the absence of TGFβ1. Taken together, the lack of TGFβ1 signaling, as a consequence of serum deprivation, leads to an enhanced capacity of human resting T cells to respond to low Ag concentrations.

**Discussion**

From elegant studies using knockout mice, it became apparent that T cell homeostasis is tidily regulated by extrinsic factors. Although positive signals via the TCR derived from MHC-peptide complexes on APCs (6–8) and IL-7 signaling (9–13) have been well established (1–3), negative regulators are less well integrated in
current models of T cell homeostasis (3). In mice lacking inhibitory molecules such as TGFβ, CTLA-4, or IL-10, profound pathophysiology with severe lymphoproliferative disease has been demonstrated (20–26). A role of TGFβ during T cell differentiation and proliferation has been described in dominant-negative TGFβ-ReI mice (20). In human T cells, the role of inhibitory factors for the balance between the resting and the active state of human T cells is far less understood. To address this important issue, we applied an unbiased genomics approach. We hypothesized that potentially negatively regulating factors constitutively acting on T cells in vivo can be removed by depriving highly enriched CD4⁺ T cells from their natural environment and that this would be accompanied by specific transcriptional changes. In this study, we demonstrate that a strictly quantitative assessment of genome-wide gene expression changes combined with a search for GO-defined biological processes reveals the TGFβ pathway to be the major exogenous inhibitory signaling pathway constitutively repressing human CD4⁺ T cells in vivo. Several of the TGFβ target genes induced upon TGFβ stimulation were shown to be under the permanent control of TGFβ in resting T cells. Moreover, this approach led to the identification of numerous novel TGFβ target genes, which have not yet been recognized as such in other cell types.

Constitutive TGFβ signaling in resting CD4⁺ T cells was additionally demonstrated by constitutively phosphorylated SMAD2 and SMAD3, two early events after receptor ligation by TGFβ. This phosphorylation was completely lost during serum deprivation and quickly restored after addition of either exogenous TGFβ or freshly isolated human serum. By further isolating CD4⁺ T cell subsets (naive, memory, conventional, and regulatory cells), we demonstrated that both SMAD2 and SMAD3 are constitutively phosphorylated in all CD4⁺ T cells. In contrast, in CD8⁺ T cells, only SMAD2 phosphorylation occurred, whereas in B cells, only phosphorylated SMAD3 was detectable. No phosphorylation of SMADs was apparent in CD14⁺ monocytes. Either TGFβ-induced cell signaling is not dependent on phosphorylation of both SMAD2 and SMAD3 in cells other than CD4⁺ T cells, or the effect of TGFβ can be mediated by phosphorylation of only one SMAD molecule. It might also be possible that other pathways might be more important in CD8⁺ T cells, B cells, or monocytes to exert the TGFβ inhibitory effect within these cells. Alternatively, these cells are not as dependent as CD4⁺ T cells on TGFβ signaling to be kept in a resting state.

Because transcriptional changes of genes associated with cell cycle regulation indicated the potential for enhanced cell cycle entry, we analyzed whether decreased TGFβ signaling would also have functional consequences for subsequent T cell activation. Indeed, the loss of TGFβ signaling under serum deprivation significantly increased the capacity of resting T cells to proliferate in response to low-level TCR stimulation. TGFβ seems to be the major negative regulator of T cell homeostasis in humans not only inhibiting T cell differentiation and proliferation, but also keeping T cells at the resting state.

The effect of TGFβ signaling has been clearly demonstrated to be exclusively cell and context dependent (41). Although many aspects of TGFβ signaling in epithelial cells and fibroblasts have been discovered, far less is known in T cells, particularly human T cells. TGFβ, especially at high concentrations, has been clearly shown to be a major immunoinhibitory factor (42–46). Especially in cancer patients, elevated levels of TGFβ have been associated with reduced T cell proliferation and function (47–50). It needs to be stressed that former work has almost exclusively focused on elevated levels of TGFβ and its effect on human immune cells. However, there is little evidence so far that TGFβ plays a physiological role under steady-state conditions in human CD4⁺ T cells.

The chosen approach has also led to the identification of several novel TGFβ target genes, especially genes that were suppressed in human CD4⁺ T cells in vivo. Many of these genes are currently of unknown function, especially in T cells. However, some biological processes or associations with cellular components seemed to be overrepresented among the new target genes, e.g., nuclear localization, membrane association, or cell cycle regulation, suggesting that TGFβ exerts specific and rather focused effects on human resting CD4⁺ T cells. The serum deprivation experiments also revealed novel aspects about the regulation of genes such as CXCR4, previously shown to be up-regulated in the presence of elevated levels of TGFβ (51). The significant decrease of CXCR4 mRNA in resting T cells after serum deprivation actually suggests, that CXCR4 expression is not only a result of elevated TGFβ levels but rather a function of constitutive TGFβ signaling under physiological conditions. This model of CXCR4 regulation would certainly fit the known function of CXCR4 for maintenance of recirculation of resting T cells in vivo (52).

The immediate loss of TGFβ signaling and transcriptional regulation of its downstream targets following withdrawal of TGFβ by serum deprivation might redefine our understanding of “resting T cells,” at least in humans. Resting T cells not only seem to be controlled by APCs as suggested by Grossman et al. (3) but also seem to be constitutively inhibited by TGFβ. Active inhibition of T cell activation therefore follows rules established in other biological systems that need to quickly respond to stimuli. In view of energy consumption, active repression of cell activation followed by proliferation and differentiation seems to be a rather ineffective approach. However, considering the enormous growth rates of viruses and other infectious agents, a prompt and sufficient activation and proliferation of specific immune cells by releasing an active blockade is most likely leading to an evolutionary advantage. We have embarked on this concept by studying the importance of TGFβ in the setting of T cell activation where the relative abundance of inhibitory signals, including TGFβ vs proinflammatory signals, decides whether T cells are activated or inhibited by modulating central molecular switches (T. Zander and J. L. Schultz, unpublished results). The functional outcome of removal of even low physiological concentrations of TGFβ further supports the hypothesis that T cells are constantly repressed by TGFβ.

The significant loss of SMAD2 and SMAD3 phosphorylation as an early response upon serum deprivation strongly suggests that the changes observed on the transcriptional level are mediated, at least in part, by the classical signaling cascade via TGFβ receptor and SMAD signaling. It will be an interesting question to determine whether other non-SMAD-mediated signaling pathways such as the MAPK pathway or the RAS pathway, which have been shown in other cell types (41, 53), are also involved in keeping the “resting phenotype” of human T cells.

Taken together, we provide evidence for a major role of TGFβ keeping human CD4⁺ T cells at a resting phenotype. We conclude that the activation of resting human T cells, particularly after a low level of Ag encounter, is actively and constitutively blocked by TGFβ. This TGFβ-mediated T cell inhibition, particularly at low levels of Ag, might also be an important mechanism in the prevention of autoimmune diseases.

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Disclosures

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