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Alloantigen specific $CD8^+CD28^-$ $FOXP3^+$ T suppressor cells induce $ILT3^+$ $ILT4^+$ tolerogenic endothelial cells, inhibiting alloreactivity

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Abstract

Endothelial cells have been shown to activate T cell responses to alloantigens, triggering transplant rejection. However, they may also play a role in tolerance induction. Using RT–PCR we show here that alloantigen specific $CD8^+CD28^-$ T suppressor cells generated *in vitro* are FOXP3 positive and interact with human endothelial cells. This interaction results in the induction of inhibitory receptors and down-regulation of costimulatory and adhesion molecules, thus rendering endothelial cells tolerogenic. In turn, tolerized endothelial cells elicit the differentiation of $CD8^+CD28^-$ $FOXP3^+$ T suppressor cells. Taken together our data demonstrate a functional and phenotypic overlap between tolerogenic dendritic cells and endothelial cells. Furthermore, alloantigen specific $CD8^+CD28^-$ $FOXP3^+$ T cells, which trigger the upregulation of inhibitory receptors in endothelial cells, are present in the circulation of heart allograft recipients in quiescence as demonstrated by flow cytometry, RT–PCR and luciferase transcription assays. Their detection facilitates the identification of patients who may benefit from partial or complete cessation of immunosuppressive therapy, a goal of obvious importance given the morbidity and mortality associated with chronic immunosuppression. Modulation of endothelial cells in favor of promoting tolerance may be important for long-term survival of organ allografts.

Introduction

The induction of immunological tolerance remains a major challenge for prolonging allograft survival, treating autoimmune diseases and avoiding immune responses to therapeutic proteins.

Infiltrates of allospecific $CD4^+$ T cells, activated via the direct and indirect allorecognition pathways, as well as $CD8^+$ T cells are found in human heart allografts during episodes of acute rejection (1–3). However, such infiltrates do not always expand and/or cause injury to the myocardium (4). Frequently, T cell infiltrates regress spontaneously without rejection therapy, suggesting that some regulatory mechanisms are at play (2,4).

Immunoregulatory mechanisms such as those mediated by naturally occurring or antigen-induced regulatory T cells have been shown to inhibit the growth and functional maturation of alloreactive T effector cells (5–11). There is evidence that naturally occurring $CD4^+CD25^+$ regulatory T cells (natural T_R) prevent autoimmunity and mediate transplantation tolerance in rodents (5–8) and humans (9–11). These cells are characterized by the constitutive expression of CD25, the interleukin (IL) 2 receptor alpha, and forkhead transcription factor FOXP3 (12–14). Natural T_R inhibit conventional $CD4^+CD25^-$ T cell activation in response to antigens or mitogens in an antigen-nonspecific, MHC non-restricted and cytokine-independent manner via T cell–T cell interaction (5–14). However, their mechanism of action is still unclear.

A distinct population of antigen-primed T cells, characterized by their $CD8^+CD28^-$phenotype and lack of cytotoxic activity has also been shown to display regulatory functions in human transplant recipients and in a murine autoimmune disease model (15,16). As opposed to natural T_R cells, human $CD8^+CD28^-$ T suppressor (T_S) cells are antigen specific, MHC class I-restricted, and interact directly with antigen-presenting cells (APC) (17–20). T_S render APC tolerogenic, inducing the downregulation of costimulatory molecules and upregulation

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of the inhibitory receptors, immunoglobulin-like transcripts (ILT)3 and ILT4 (15). ILT3 and ILT4 display long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motifs (ITIM), which mediate inhibition of cell activation by recruiting tyrosine phoshatase SHP-1 (21,22). Both of these molecules were invariably co-expressed on dendritic cells (DC) rendered tolergenic by exposure to T_S or to certain cytokines such as IL-10 and interferon (IFN) alpha (23).

Studies of rejection-free recipients of heart transplants have demonstrated the presence of circulating $CD8^+CD28^-$ T cells which inhibit CD40L-induced upregulation of costimulatory molecules and enhance ILT3 and ILT4 expression on donor APC (15,24). Since T_S require direct contact with professional APC of donor origin in order to inhibit T cell allorecognition via the direct pathway (15,17–19), it is unclear how they maintain quiescence once donor APC have migrated out of the graft. However, it was recently demonstrated that in mice non-professional APC, such as graft endothelial cells (EC), directly activate cytotoxic $CD8^+$ T cells (Tc), triggering acute rejection in the absence of indirect allorecognition (25). We have explored the hypothesis that the interaction of T_S with graft EC results in suppression of the direct pathway of allorecognition and maintenance of quiescence in human heart allograft recipients.

We provide evidence that allospecific $CD8^+CD28^-$ human T_S express FOXP3 and induce upregulation of ILT3 and ILT4 in EC, converting them to a tolerogenic state. The interaction between allospecific $CD8^+CD28^-$ human T_S and EC is bi-directional since tolerogenic $ILT3^+ILT4^+$ EC induce the *in vitro* differentiation of $CD8^+CD28^-$ T_S in unprimed populations of human $CD8^+$ T cells. We found that allospecific $CD8^+CD28^-$ $FOXP3^+$ T cells which induce the upregulation of inhibitory receptors in EC carrying donor HLA class I antigens are present in the circulation of rejection-free patients.

Methods

Transplant patients

Citrate anticoagulated whole blood was obtained from recipients of cadaver donor heart transplants treated at New York Presbyterian Medical Center. The average number of HLA mismatches between donor/recipient pairs was 2.8 ± 0.2 for HLA-A and HLA-B and 1.8 ± 0.2 for HLA-DR. All patients were treated with standard immunosuppressive therapy (cyclosporine, steroids and azathioprine). The mean age of the patients was 53.1 ± 8.7, ranging from 28 to 64. The diagnosis of coronary artery vasculopathy (CAV) was made as previously described (2). All experiments were done in compliance with the relevant laws and Institutional Good Clinical Practice guidelines and were Institutional Review Board approved.

Endomyocardial biopsies

Endomyocardial biopsies were performed by standard transjugular approach weekly for the first month and then at progressively longer intervals to a baseline schedule of every 6 months. A minimum of four biopsy fragments were fixed in 4% buffered formalin, paraffin embedded, and multiple hematoxylin and eosin stained sections from three levels in the block were examined. An additional fragment was frozen for RT–PCR of ILT3 and ILT4 and/or immunohistochemical studies. Histologic grades were assigned according to the criteria of the International Society for Heart Transplantation (ISHLT) (4). Acute allograft rejection was defined as ISHLT grades 3A or higher.

Immunohistochemistry

For detection of ILT4 expression, endomyocardium sections from frozen biopsies were fixed for 10 min at room temperature in PBS with 4% paraformaldehyde. Sections were then washed with 0.1% saponin in PBS and incubated with rat anti-human ILT4 IgG for 1 h. Sections were next incubated with goat anti-rat IgG conjugated with biotin followed by streptavidin–horseradish peroxidase (HRP). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 prior to incubation of HRP. Tissue biotin was blocked by treatment with avidin and then biotin (Dako, Carpinteria, CA). The color was developed with DAB (diaminobenzidine; Dako). Samples stained only with goat anti-rat IgG were used as controls.

Molecular typing of HLA class I and class II antigens

HLA genotypes of transplant recipient/donor pairs and healthy blood or EC donors were determined by PCR with sequence specific primers (SSP) using commercially available kits (One Lambda, Los Angeles, CA).

Generation of alloreactive T cell lines (TCLs) and T cell clones (TCCs)

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were separated by Ficoll–Hypaque centrifugation. Responding PBMCs (1×10^6/ml) were stimulated in 24-well plates with irradiated (1600 rad) monocyte-derived DC (1×10^5/ml) obtained from allogeneic PBMCs. Cells were cultured for 7 days in complete medium [RPMI 1640 supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 2 mM 1-glutamine and 50 μg/ml of gentamycin (Gibco-BRL, Grand Island, NY)]. After 7 days, responding cells were collected, washed and rechallenged with the original stimulating cells. Three days later, recombinant human (rh) IL-2 (Boehringer Mannheim, Indianapolis, IN) was added (10 U/ml) and the cultures were expanded for an additional 4 days.

On day 14, $CD8^+$ T cells were separated using CD8 isolation kit II (Miltenyi Biotech, Auburn, CA). $CD8^+CD28^-$ T cells were obtained from the $CD8^+$ T cell suspension by depletion of $CD28^+$ cells using goat anti-mouse IgG beads (Dynal, Lake Success, NY) coupled with mAb to CD28 (Becton Dickinson). The purity of the $CD8^+CD28^-$ T cell suspension was >98%.

$CD8^+CD28^-$ T cells from TCL were cloned by limiting dilution at 0.5 T cells per well in 96-well U-bottom plates (Costar, Corning Inc., Corning, NY). Irradiated (3000 rads) PBMC (1×10^5) and lymphoblastoid JY cells (2×10^4) (from ATCC, Manassas, VA) were added to each well. Cultures were grown in RPMI 1640 medium containing 5% pooled human serum (Sigma Chemical, St Louis, MO), and rh IL-2 50 U/ml (Boehringer Mannheim) and 1 μg/ml phytohemagglutinin-L (PHA-L; Sigma). Medium without PHA-L was replenished at 5-day intervals. Proliferating T cell clones were expanded and tested for suppressor activity.

Generation of monocyte-derived DC

Monocytes were obtained from PBMCs using a Monocyte Negative Selection Kit (Dynal, Lake Success, NY). Immature DC were generated by culturing monocytes in 6-well plates at a concentration of 2×10^6 cells per well for 7 days with granulocyte macrophage colony stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) and interleukin (IL)-4 (R&D Systems), as previously described (15).

Endothelial cell culture

Human EC (HUVEC and HAEC) from Cambrex (Walkersvillle, MD) were cultured in EC growth medium (EGM BulletKit, Cambrex, Walkersville, MD) containing 2% bovine calf serum, 10 μg/ml of recombinant human epithelial growth factor (rh EGF), 1.0 mg/ml hydrococortisone, 3 mg/ml bovine brain extract (BBE), 100 U/mL penicillin/streptomycin, and 50 μg/mL gentamicin. For functional assays the EC were used between the second and fourth passages. To modulate ECs, recombinant human interferon (IFN)-γ (100 ng/ml) or tumor necrosis factor (TNF)-α (50 ng/ml) or a mixture of IL-10 (10 ng/ml) and IFN-α (1000 U/ml) (R&D systems, Minneapolis, MN) were added to the cultures 48 h prior to use.

When used for T cell priming, cytokine treated or untreated EC were plated at 10^4 cells/well in 96-well flat bottom culture plates (Costar). Purified $CD8^+$ T cells in complete medium were added at 10^5 cells/well. On day 3, rhIL-2 (Boehringer Mannheim) was added to the cultures. On day 7, T cells were collected and transferred to 96-well plates containing fresh cytokine or untreated EC. T cells were stimulated as above for an additional 7 days, then harvested and tested.

EC transfection and reporter gene luciferase activity assay

The 766 bp of the 5′-flanking region of the ILT4 gene was cloned into the *Sac*I and *Bgl*II sites, upstream of the pGL3 basic luciferase reporter gene (pGL3/766). For ILT3, 1034 bp of the 5′ flanking region was cloned into pGL3 basic reporter gene (pGL3/1034). Transient transfection of human umbilical cord vein EC (HUVEC) was performed by electroporation according to the manufacturer's recommendation (HUVEC Nucleofector, Program U1, Amaxa, Germany). HUVEC were co-transfected with 2 μg of pGL3/766 or pGL3/1034 and 1 μg of pGL3 TK-renilin luciferase reporter DNA. Purified $CD8^+CD28^-$ T_S cells were added 24 h later and incubation was allowed to proceed for an additional 15 h. After removal of T cells, HUVEC were harvested and cell lysates subjected to luciferase assay using a Promega dual-luciferase reporter assay kit (Promega, Madison, WI) and TD 20/20 Luminometer (Turner Biosystems, Sunnyvale, CA) according to the manufacturer's instructions. Experimental luciferase reporter gene activity was normalized with renilin luciferase activity.

Semi-quantitative RT–PCR

Total RNA was extracted from endomyocarditis biopsies and T cells (obtained from TCL, TCC or PBMC) using the RNAqueous–4PCR kit (Ambion, Inc., Austin, TX) following the manufacturer's recommendation. First-strand cDNA was synthesized with a cDNA synthesis kit (Roche Diagnostic, Indianapolis, IN). The following primers were used in PCR reactions. ILT3: 5′ primer ACGTATGCCAAGGTGAAACACT; 3′ primer CATTGTGAATTGAGAGGTCTGC (expected size 493 bp). ILT4: 5′ primer GCATCTTGGTGGCCGTCGTCCTAC; 3′ primer CCCAAAGTTCCCAGCATCTCCTCA (expected size 551 bp). CD8: 5′ primer AGGTGCTTGAGTCTCCAACGG; 3′ primer TGCCTCATCCCTGTATCTGCTAGT (expected size 504 bp). CD40: 5′ primer TTTCTGATACCATCTGCGAGCC; 3′ primer TCTCCTGCACTGAGATGCGAC (expected size 420 bp). CD83: 5′ primer ATGTCGCGCGGCCTCCAG; 3′ primer TCATACCAGTTCTGTCTTGTGAGGAG (expected size 950 bp). CD54: 5′ primer AAAACACTAGGCCACGCATCT; 3′ primer GGCCTTTGTGTTTTGATGCTAC (expected size 253 bp). CD58: 5′ primer AGATGAGCTCTTTTAACTCAAGCGAAA, 3′ primer GGGTGGGAAAAAAGCATGTGTA (expected size 230 bp). CD62e: 5′ primer AAGGTACACACACCTGGTTGC; 3′ primer TTCTCCAGAGGACATACACTG (expected size 562 bp). CD106: 5′ primer GAAGAAAAAAGCGGAGACAGGAG; 3′ primer GGAGGATGCAAAATAGAGCACGAG (expected size 217 bp). FOXP3: 5′ primer TTGGACAAGGACCCGATGCCCAACCCC; 3′ primer CCCTGGCAGGCAAGACAGTGGAAACCTC (expected size 1350 or 1450 bp). 5′ primer TGTCAGTCCACTTCACCAAGCC; 3′ primer CCTTCTCATCCAGAAGATGGTCC (expected size 724 or 619). 5′ primer TCCCAGAGTTCCTCCACAAC; 3′ primer GCAAGACAGTGGAAACCTCAC (expected size 465 bp). Perforin: 5′ primer TACAGCTTCAGCACTGACACGG; 3′ primer GAGCTTCACATAGGCATCCGT (expected size 825 bp). IL10: 5′ primer; TCATTCTATGTGCTGGAGATGG; 3′ primer; GCTCACCATGACCCCTACC (expected size 146 bp). TGFβ: 5′ primer; AAGATAACCACTCTGGCGAGTCG; 3′ primer, CAGAGCTCCGAGAAGCGGTAC (expected size 180 bp). GAPDH: 5′ primer CGGAGTCAACGGATTTGGTCGTAT; 3′ primer AGCCTTCTCCSTGGTGGTGAAGAC (expected size 362 bp). PCR reactions were done at 30 cycles in a 20 μl volume and PCR products were analyzed in an agarose gel stained with ethidium bromide. Samples were normalized with the use of GAPDH expression.

Flow cytometry analysis

Flow cytometry studies were done with a FACScan (Becton Dickinson). CaliBRITE beads from Becton Dickinson were run under the FACSComp program to calibrate the instrument. Human $CD8^+$ T cell subsets were defined by staining with phycoerythrin (PE)-conjugated mAbs to CD28 and CyChrome (CYC) conjugated mAbs to CD8. For EC staining the following mAb were used: FITC–CD31, PE–CD40, PE–CD54, PE–CD62E, PE–CD83, PE–CD106, CYC–CD58, CYC–HLA ABC and CYC–HLA DR (all from Becton Dickinson) and ILT3–PC5 (Coulter, Miami, FL).

For ILT4 staining EC were incubated with 25 μl of rat anti-human ILT4 mAb (clone 42D1, a generous gift from Dr Marco Colonna), then washed and stained with R-PE conjugated goat anti-rat IgG (Caltag, Burlingame, CA). Rat IgG was added as a blocking agent prior to staining with FITC–CD31 (Becton Dickinson). For each cell surface or intracellular marker, a corresponding isotype-matched control antibody conjugated with the same fluorescent dye was used. Six parameter analyses (forward scatter, side scatter and four fluorescence channels) were used for list mode data analysis.

Proliferation assay

EC monolayers (at 1×10^4 cells per well) were seeded in 96-well flat bottom plates (Costar) and rh IFN-γ (R&D Systems) was added to each well at 400 ng/ml. After 48 h, supernatant was removed and plates were washed twice with complete medium. Mitomycin C (Sigma) was added to each well at 10 μg/ml. After 2 h of incubation, plates were washed four times. Primed $CD4^+$ T cells, obtained from each TCL using CD4 isolation kit II (Miltenyi Biotech, Auburn, CA) were added to each well at 1×10^5 cells/well. In parallel control cultures $CD4^+$ T cells (1×10^5) were added to mitomycin C treated allogeneic monocyte-derived DC (1×10^4) in 96-well U bottom plates (Costar).

$CD8^+CD28^-$ T cells from allospecific TCL were first tested for FOXP3 expression. $FOXP3^+$ T cells were added (at 1×10^5) to cultures containing primed $CD4^+$ T cells and either EC or DC. Monoclonal antibodies to ILT3 (clone ZM3.8, a generous gift from Dr Marco Colonna) or mixtures of mAb to ILT4 and mAb to HLA class I (W6/32) (ATCC, Manassas, VA) or rh IL-2 (10 U/ml) were added to the cultures where indicated. Triplicate cultures were set up for each condition. After 2 days of incubation, cultures were pulsed with tritiated thymidine and harvested 18 h later. [^{3}H]thymidine incorporation was measured by scintillation spectrometry.

Statistical analysis

Fisher exact test for two-tail analysis was used for determining the significance of differences between two groups.

Results

FOXP3 expression in T_S cells

FOXP3 encodes a member of the forkhead/winged helix family, which acts as a transcriptional repressor (26) and has been shown to be important for the function of T_R cells. In both rodents and humans FOXP3 is selectively expressed in $CD4^+CD25^+$ natural T_R, representing the most reliable marker for their identification (12–14).

To determine whether FOXP3 is also expressed in $CD8^+CD28^-$ T_S we generated allospecific TCL. $CD8^+CD28^-$ T cells from these TCL were separated, tested for suppressor activity and then cloned by limiting dilution. FOXP3 expression was assessed by semi-quantitative RT–PCR. $CD8^+CD28^-$ T_S cells from TCL and TCC expressed FOXP3. No expression was detected in primed $CD8^+CD28^+$ T cells from these TCL. $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells from fresh peripheral blood did not express FOXP3. Unfractionated $CD4^+$ T cells and sorted $CD4^+CD25^+$ T cells from the same samples of blood were $FOXP3^+$ while the $CD4^+CD25^-$ fraction was $FOXP3^-$ (Fig. 1A).

To examine the structure of the FOXP3 transcript expressed by $CD8^+CD28^-$ T_S we used primers complementary to the 5′ and 3′ untranslated regions of the FOXP3 transcript to amplify the coding region of this gene. Sequence analysis of seven isolated clones and comparison with the GenBank database indicated the presence of a novel, alternatively spliced form of the previously described FOXP3 gene. Alignment of amino acid sequences of the two FOXP3 isoforms showed that the newly identified isoform lacks a 35 amino acid region corresponding to position 71 to 105 of the previously described FOXP3 protein product (Fig. 1B).

To establish whether this novel FOXP3 isoform, which we refer to as FOXP3α, is common to $CD8^+CD28^-$ T_S and $CD4^+CD25^+$ natural T_R cells, we generated a primer pair which allows size discrimination of FOXP3α from FOXP3 (Fig. 1C). $CD4^+CD25^+$ T cells, but not $CD4^+CD25^-$ and $CD8^+$ T cells from fresh peripheral blood of 20 donors expressed both FOXP3 and FOXP3α. $CD8^+CD28^-$ T cells from allospecific TCL generated from these donors were FOXP3 and FOXP3α positive (Fig. 1D). However, when TCC derived from one of these TCL were tested, exclusive expression of canonic FOXP3 was seen in 20 clones, while FOXP3α was expressed in 30 clones (Fig. 1D). Further characterization of TCC by RT–PCR using specific primers showed that they were PRF1 negative (Fig. 1E) as well as IL-10 and TGF-β negative (data not shown). The functional relevance of the differential expression of canonic FOXP3 and FOXP3α is currently being investigated.

EC activation is inhibited by allospecific T_S cells

Serially passaged HUVEC and aortic EC (HAEC) do not display HLA–DR, CD80, CD86 (27,28) or detectable amounts of ILT3 or ILT4. They stimulate peripheral blood $CD8^+$ T cells *in vitro* inducing the generation of alloreactive T_C which express PRF1 and IFN-γ intracellularly (29,30). IFN-γ treated EC express HLA class II as well as high levels of HLA class I and CD58 and activate both alloreactive $CD4^+$ and $CD8^+$ T cells from allogeneic blood donors (31–33).

To study the effect of allospecific $CD8^+CD28^-$ $FOXP3^+$ T_S on serially passaged human EC we selected HUVEC, which shared at least one HLA-A and HLA-B allele with the DC used for T_S priming. $CD8^+CD28^-$ T cells from eight different TCLs were tested by RT–PCR for expression of FOXP3 and PRF1 prior to use. HUVEC were co-incubated for 18 h with T_S at a 1:10 ratio and then analyzed by flow cytometry for expression of cell surface markers. The expression of co-stimulatory molecules CD40 and CD58 was downregulated while the inhibitory receptor ILT4 was induced. CD83 was not expressed on untreated HUVEC neither before nor after incubation with $CD8^+CD28^-$ $FOXP3^+$ T_S (Fig. 2A and B).

We next studied the effect of T_S on activated HUVEC. For this, HUVEC were treated for 48 h with IFN-γ or TNF-α which upregulate the expression of costimulatory and adhesion molecules involved in EC–T cell interaction. Treatment of EC with IFN-γ induced the upregulation of HLA class I, HLA class II, CD40, CD54 and CD58, consistent with previous studies (29–33). Analysis of CD83, a marker expressed by mature allostimulatory DC (34), showed that IFN-γ activated EC also express CD83 (Fig. 3A). When IFN-γ pretreated EC were incubated for 18 h with T_S primed to at least one of their HLA class I antigens they showed decreased expression of CD40, CD54, CD83 and HLA–DR and high expression of ILT4 (Fig. 3B). Treatment of EC with TNF-α induced the upregulation of CD40, CD62E, CD83 and CD106 (Fig. 3C). Upon incubation with T_S, EC showed lower expression of these cell surface molecules, yet exhibited high levels of ILT4 (Fig. 3D). This phenotype was confirmed by flow cytometry and RT–PCR using eight different

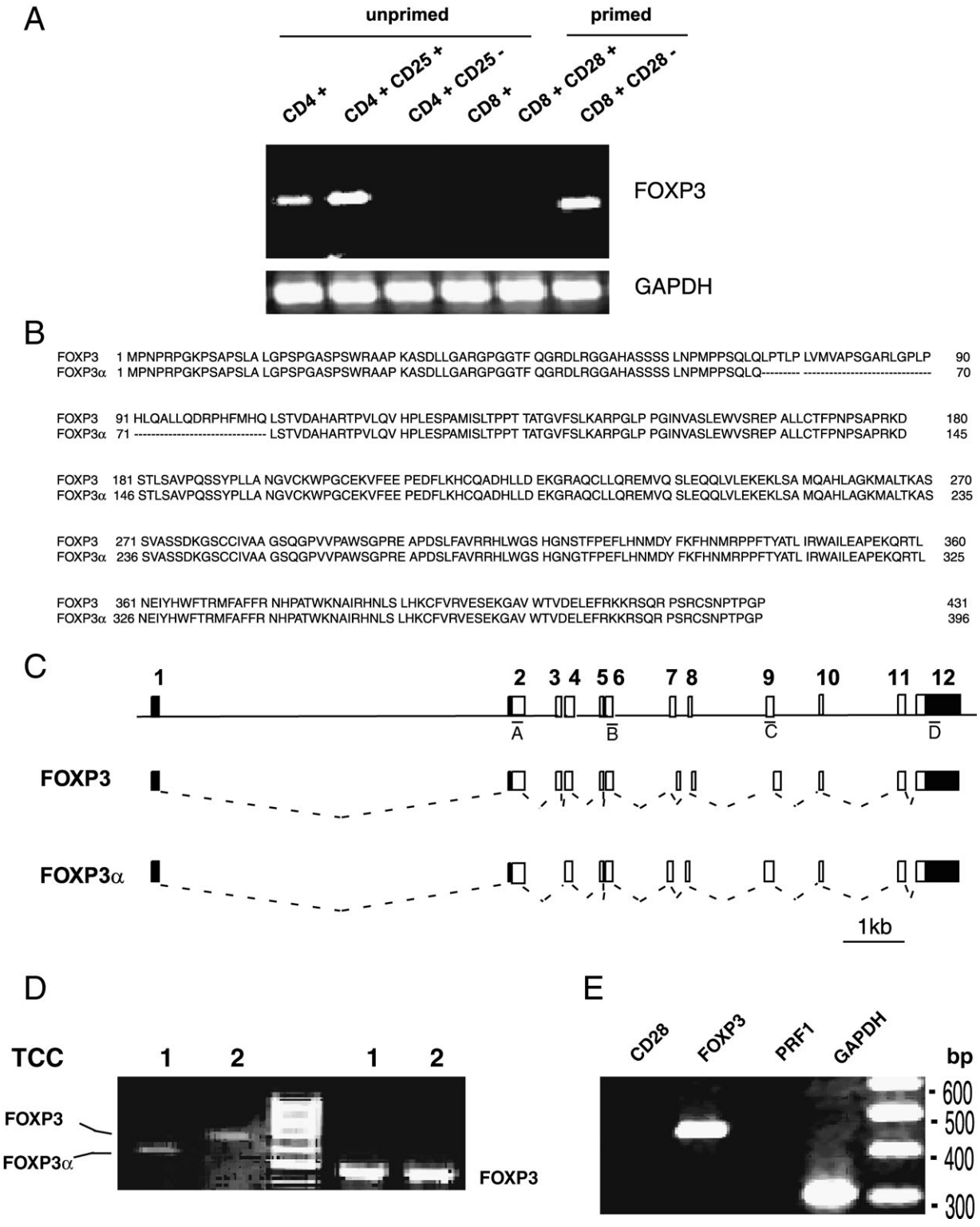

Fig. 1. A novel FOXP3 isoform is expressed in the $CD8^+CD28^-$ T cells. (A) Semi-quantitive RT–PCR analysis of FOXP3 expression in T cells. Lane 1 unfractionated $CD4^+$; lanes 2 and 3 fractionated $CD4^+CD25^+$ and $CD4^+CD25^-$; lane 4 unfractionated $CD8^+$; and lanes 5 and 6 primed $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells from allospecific TCL. (B) Sequence alignment of FOXP3 and FOXP3α protein products showing the absence of a 35 amino acid region in the N-terminus of FOXP3α. (C) Genomic and transcript structures of the FOXP3 gene. A, B, C and D indicate location of primers used for RT–PCR analysis in (D). (D) RT–PCR analysis of FOXP3 and FOXP3α in primed $CD8^+CD28^-$ TCCs showing PCR products obtained in two independent clones (1 and 2) with primers from region A and B (left two lanes) and from C and D (right two lanes). (E) RT–PCR analysis of $CD8^+CD28^-$ T cell clones.

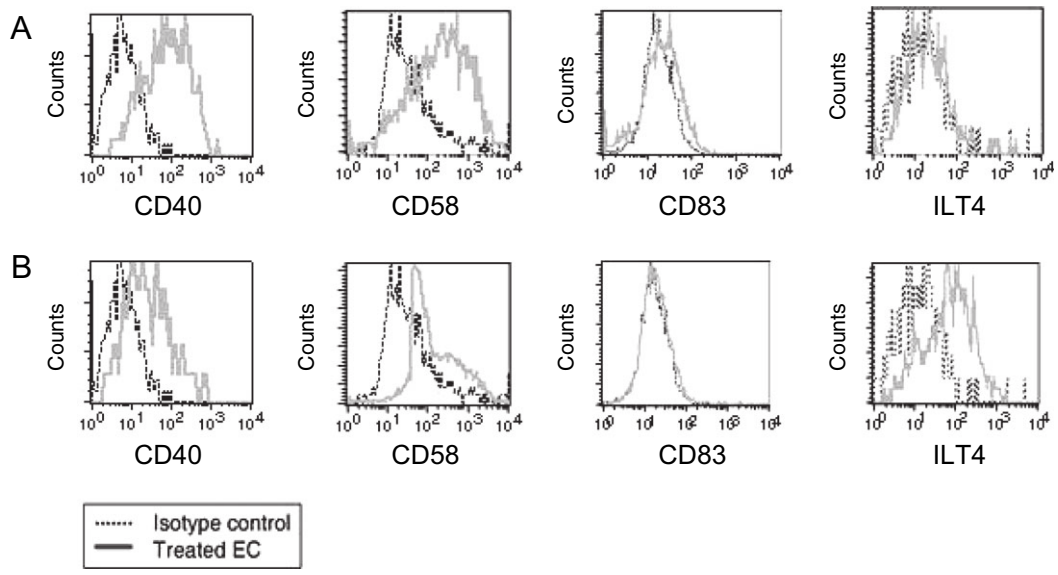

Fig. 2. Expression of activating and inhibitory molecules on non-activated EC. (A) EC were incubated without, or (B) with allospecific $CD8^+CD28^-$ $FOXP3^+$ $PRF1^-$ T_S cells prior to staining.

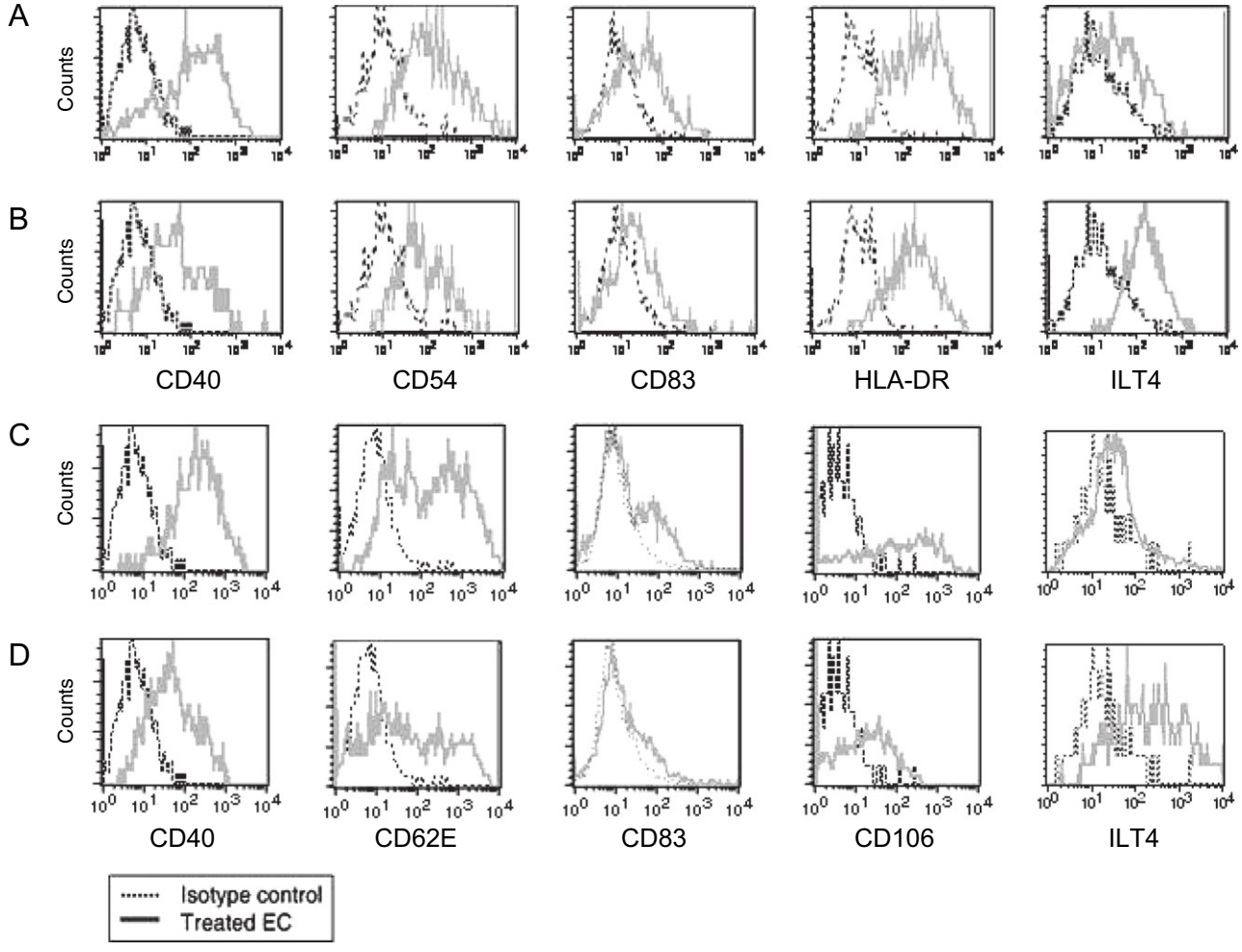

Fig. 3. Expression of activating and inhibitory molecules on activated EC. (A) IFN-γ activated EC before, or (B) after incubation with T_S, (C) TNF-α activated EC before, or (D) after incubation with T_S.

T_S–EC combinations. The induction of ILT4 was accompanied by induction of ILT3. Upon exposure of EC to T_S ILT3 was detected by cell surface staining and RT–PCR in both non-activated and IFN-γ activated EC (Fig. 4). $CD8^+CD28^-$ or $CD8^+CD28^+$ T cells from fresh peripheral blood had no effect on the expression of cell surface molecules on EC (data not shown). Similar results were also obtained when HAEC instead of HUVEC were exposed to T_S (data not shown). Hence, the phenotypic overlap between DC and EC includes the expression of activation/maturation markers, such as CD83, and of inhibitory receptors such as ILT3 and ILT4.

Taken together, these results indicate that T_S modulate DC or EC in favor of promoting tolerance by inducing an alternative activation state (35,36).

In previous studies we and others have shown that treatment of immature, monocyte-derived DC with IFN-α and IL-10 or vitamin D3 results in the induction of a tolerogenic phenotype characterized by the high expression of ILT3 and ILT4 (23) and decreased expression of costimulatory molecules (23–38). Under the same treatment conditions with IL-10 plus IFN-α, serially passaged HUVEC also expressed high cell surface levels of ILT4 (Fig. 5A) and ILT3 (Fig. 5B) as well as decreased levels of CD40 and CD58 (data not shown). Induction of ILT3 and ILT4 occurred together in IFN-α and IL-10 treated EC as previously shown to be the case for DC (23). We, therefore used the mixtures of IFN-α and IL-10 as proxies (for $CD8^+CD28^-$ T_S) to induce the expression of ILT3 and ILT4 on EC and determine whether $ILT3^+ILT4^+$ EC elicit the generation of $CD8^+CD28^-$ $FOXP3^+$ T_S cells. In four independent experiments, allostimulation of $CD8^+$ T cells with $ILT3^+$ $ILT4^+$ EC (treated with the cytokine mixture and then extensively washed) resulted in an increase in the size of the population of $CD8^+CD28^-$ T cells from 30 ± 6% (Fig. 5C) before priming to 70 ± 5% after two rounds of stimulation (Fig. 5D). RT–PCR studies demonstrated that these cells expressed FOXP3 (Fig. 5F) but not PRF (data not shown). In contrast, $CD8^+$ T cells stimulated repeatedly with $ILT3^-$ $ILT4^-$ EC showed no increase in the frequency of $CD8^+CD28^-$ T cells. Instead 80 ± 5% of the cells were $CD8^+CD28^+$ (Fig. 5E). T cells from these cultures were $FOXP3^-$ (Fig. 5F) yet $PRF1^+$ by RT–PCR (data not shown). Hence, $ILT3^+ILT4^+$ EC induce the differentiation of $CD8^+CD28^-FOXP3^+$ T_S cells.

To better understand the mechanisms by which T_S can regulate ILT4 transcription in EC, we transfected EC with pGL3 luciferase reporter gene containing 766 bp of the 5′-flanking region of the ILT4 gene. In parallel EC were transfected with pGL3 containing 1034 bp of the 5′-flanking region of the ILT3 gene. EC from two donors (one carrying HLA-A 0101, B 0801 the other HLA-A 0301, B 0702) were used. Luciferase activity was measured 18 h after incubation of transfected EC with medium only, unprimed $CD8^+$ T cells, or allospecific $CD8^+CD28^-FOXP3^+T_S$. The increase of luciferase activity was

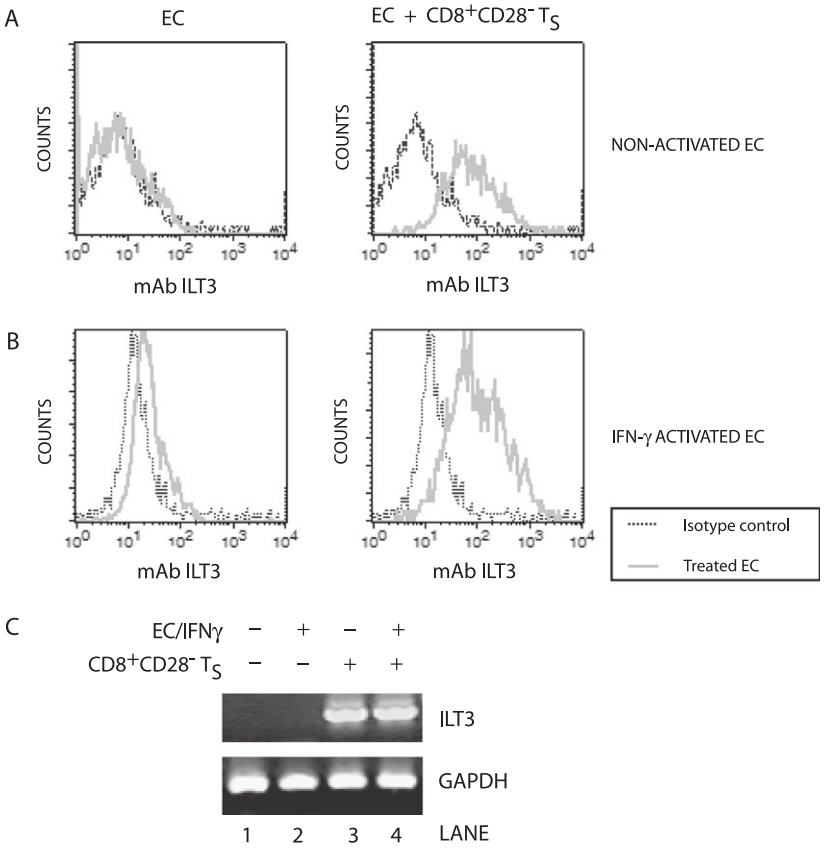

Fig. 4. Expression of ILT3 on non-activated and IFN-γ activated EC. Flow analysis of ILT3 on (A) non-activated or (B) activated EC in the presence or absence of T_S. (C) RT–PCR of ILT3 in EC treated (lane 2 and 4) or not treated (lane 1 and 3) with IFN-γ and with (lane 3 and 4) or without T_S (lane 1 and 2).

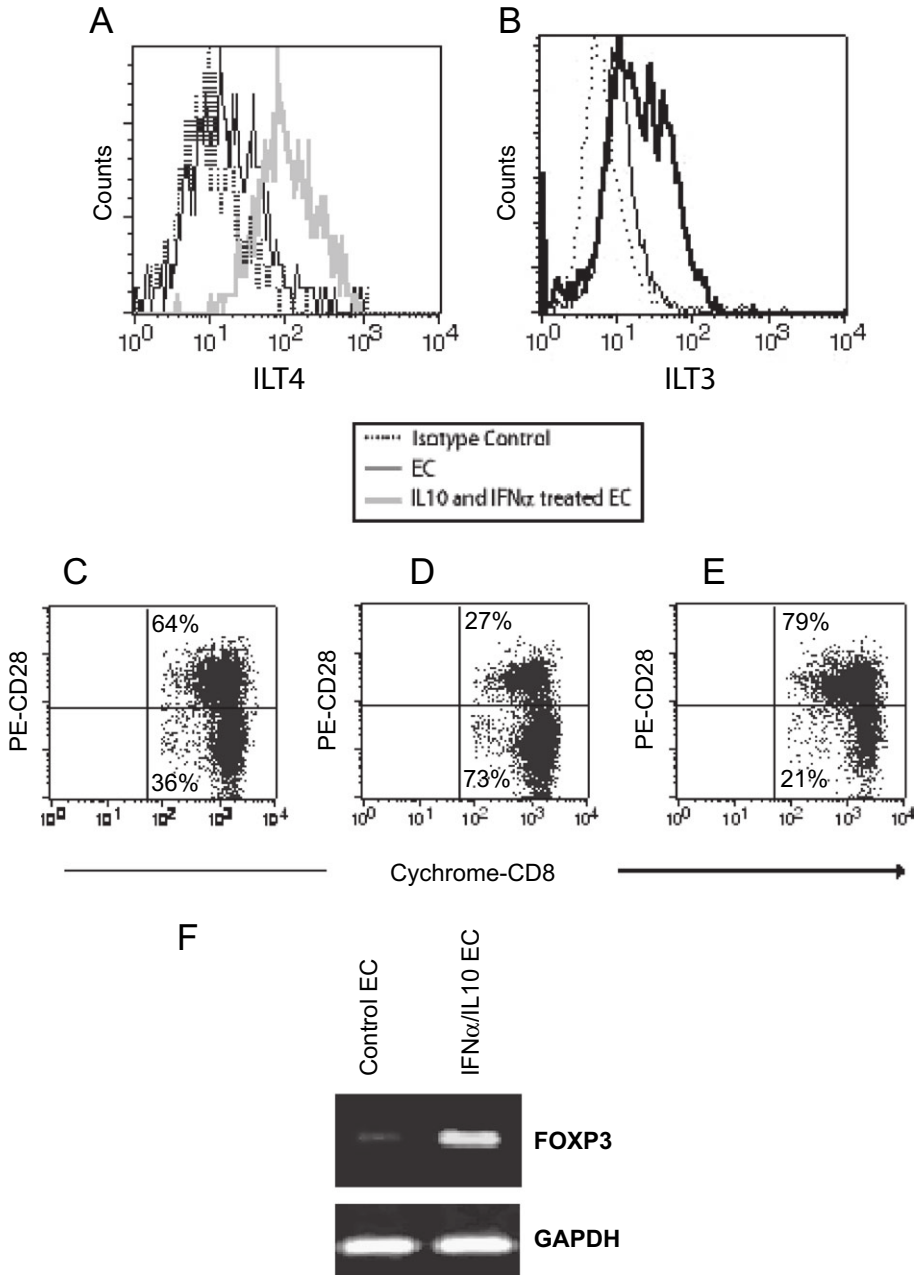

Fig. 5. Induction of $CD8^+CD28^-$ $FOXP3^+$ T cells by $ILT4^+$ $ILT3^+$ EC. (A) ILT4 and (B) ILT3 expression in EC before and after treatment with IFN-α and IL-10. (C) Expression of CD28 on unprimed $CD8^+$ T cells, (D) $CD8^+$ T cells primed with IL-10 and IFN-α treated EC, and (E) $CD8^+$ T cells primed with untreated EC. (F) FOXP3 expression in $CD8^+$ T cells primed with IL-10 and IFN-α treated EC or with untreated control EC.

up to 12-fold and 17-fold when ILT4 and ILT3 transfected EC, respectively, were coincubated with $CD8^+CD28^-$ T_S primed to shared HLA class I alloantigens. The T_S effect was alloantigen specific as it did not occur when transfected EC were incubated with $CD8^+CD28^-$ T_S from TCL primed to APC expressing irrelevant HLA class I antigens (Fig. 6). Similarly, unprimed $CD8^+$ T cells, used as controls, did not trigger luciferase activity (Fig. 6). These experiments demonstrate that T_S induce ILT3 and ILT4 expression in EC at the transcriptional level and that this effect is allorestricted by HLA class I antigens.

$ILT3^+$ $ILT4^+$ EC are tolerogenic

We explored the possibility that $CD8^+CD28^-$ $FOXP3^+$ PRF^- T_S from TCL primed with allogeneic DC inhibit the capacity of activated $HLA–DR^+$ EC to stimulate the proliferation of primed $CD4^+$ T cells from the same TCL (A anti-B). Primed $CD4^+$ T cells were allostimulated in 3 day proliferation assays, with IFN-γ treated EC in the presence or absence of $CD8^+CD28^-$ $FOXP3^+$ PRF^- T_S. The stimulating EC shared HLA class I and class II (HUVEC B) or only class II alleles (HUVEC C) with the DC used for TCL priming. T_S from five different TCL strongly inhibited the proliferation of primed $CD4^+$ T cells in response

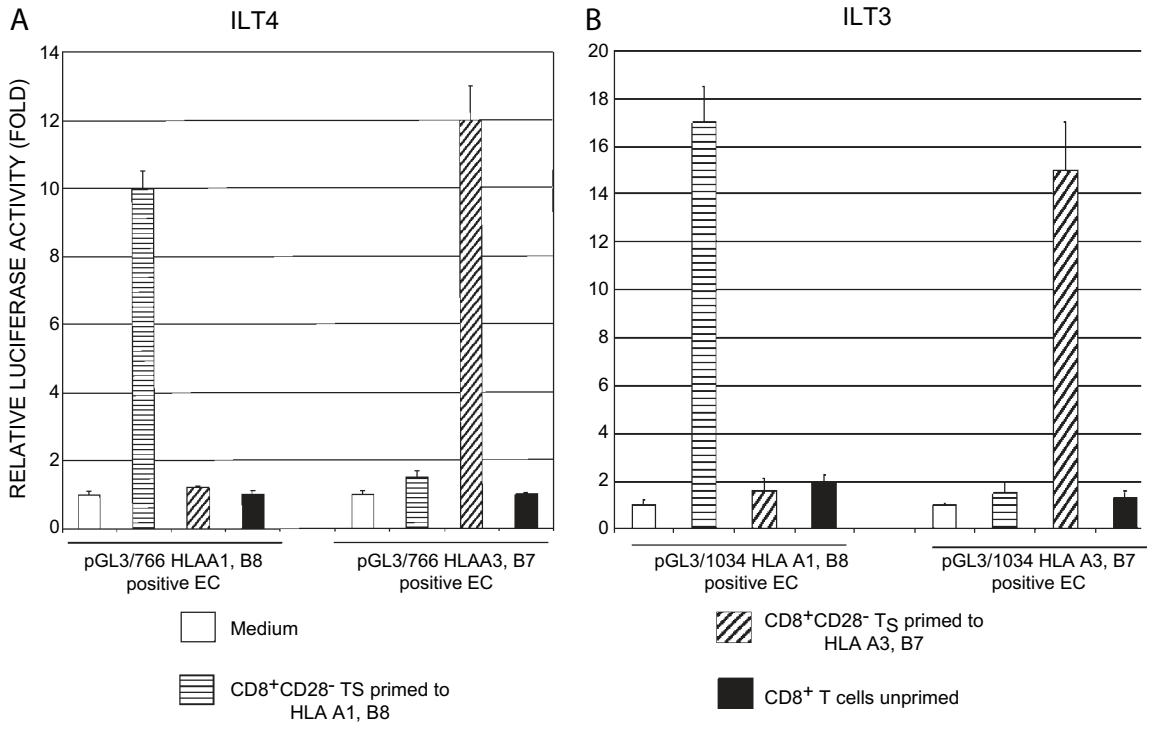

Fig. 6. Induction of ILT4 and ILT3 promoter activity in EC by allospecific $CD8^+CD28^-$ $FOXP3^+$ $PRF1^-$ T_S cells. EC from two HLA different donors were transfected with (A) 766 bp of the ILT4 promoter (pGL3/766) construct or (B) with 1034 bp of the ILT3 promoter (pGL3/1034) construct and co-cultured with T_S primed to their HLA class I or different HLA class I antigens. Control cultures were incubated without T cells or with unprimed $CD8^+$ T cells. ILT4 and ILT3 luciferase activity in EC cultured with T cells was compared to baseline values in cultures without T cells. Error bars represent SE of triplicate wells. Results are representative of six independent experiments.

to IFN-γ treated EC. The inhibitory effect of $CD8^+$ T_S on T_H proliferation to EC was MHC class I allorestricted (Fig. 7A).

To determine whether EC exposed to T_S become tolerogenic we pre-incubated INF-γ treated EC (HUVEC B) with allospecific T_S from TCL A anti-B for 18 h. Next, T_S were removed by repeated washing of EC. Complete removal of T_S was monitored by flow cytometry using mAb to CD8 and confirmed by RT–PCR (see Supplementary fig. 1, available at *International Immunology* Online). These conditioned HLA DR^+ EC, devoid of T_S, were then used in 3-day proliferation assays for stimulating primed $CD4^+$ T cells from the same TCL (A and B). T_S-treated $ILT3^+$ $ILT4^+$ EC induced little proliferation of allospecific $CD4^+$ T_H cells from the same TCL, whereas T_S-untreated EC induced strong proliferation. Addition of rhIL-2 to allospecific $CD4^+$ T_H cells restored their responsiveness to Ts-treated EC, indicating that $CD4^+$ T_H cells were rendered anergic by $ILT3^+$ $ILT4^+$ EC (Fig. 7B).

To determine whether anti-ILT3 mAb abrogate the T_S effect, mAb to ILT3 was added to cultures containing primed $CD4^+$ T_H, $CD8^+CD28^-$ T_S and IFN-γ-treated EC sharing HLA class I and HLA class II antigens with the APC used for priming. Anti-ILT3 mAb had no effect on T_H proliferation in cultures without T_S, yet restored at least in part T_H reactivity in the presence of T_S (Fig. 7C). This effect was optimal at a concentration of 1 μg/well (Supplementary fig. 2).

Similarly, to establish whether ILT4 contributes to the reduced stimulatory capacity of EC exposed to T_S we added mAb to ILT4 and HLA class I to cultures containing primed $CD4^+$ T_H cells, $CD8^+CD28^-$ T_S and IFN-γ-treated EC. The inhibitory effect of T_S on $CD4^+$ T_H proliferation in response to EC was reversed partially by the mixture of mAbs to ILT4 and HLA class I but not by either of the mAbs when used alone. This mixture of mAb had no effect on $CD4^+$ T cell proliferation in cultures without T_S (Fig. 7C). These data indicate that the effect of T_S on T_H proliferation in response to EC is mediated at least in part by ILT4. The combinations of mAbs to ILT3, ILT4 and HLA class I showed a synergistic effect, increasing T_H proliferation in the presence of T_S above the level seen when either mAb to ILT3 or mixtures of mAb to ILT4 and HLA class I were used alone (Fig. 7C and Supplementary fig. 2).

In vivo expression of ILT4 on donor EC

To understand the *in vivo* relevance of ILT3 and ILT4 expression on EC we studied serial endomyocardial biopsies, obtained within the first 12 months post-transplantation, from 30 heart allograft recipients. Nine of these 30 patients had at least one episode of acute rejection (histologic grade 3A or higher).

ILT4 was not expressed on EC from biopsies obtained 1 and 2 weeks after transplantation. However, ILT4 was expressed on EC from at least two consecutive biopsies obtained from 16 out of the 21 rejection-free patients (Fig. 8A). Of the nine patients with acute rejection episodes one showed $ILT4^+$ EC on a single biopsy. Hence, the absence of acute rejection episodes during the first year post-transplantation is associated

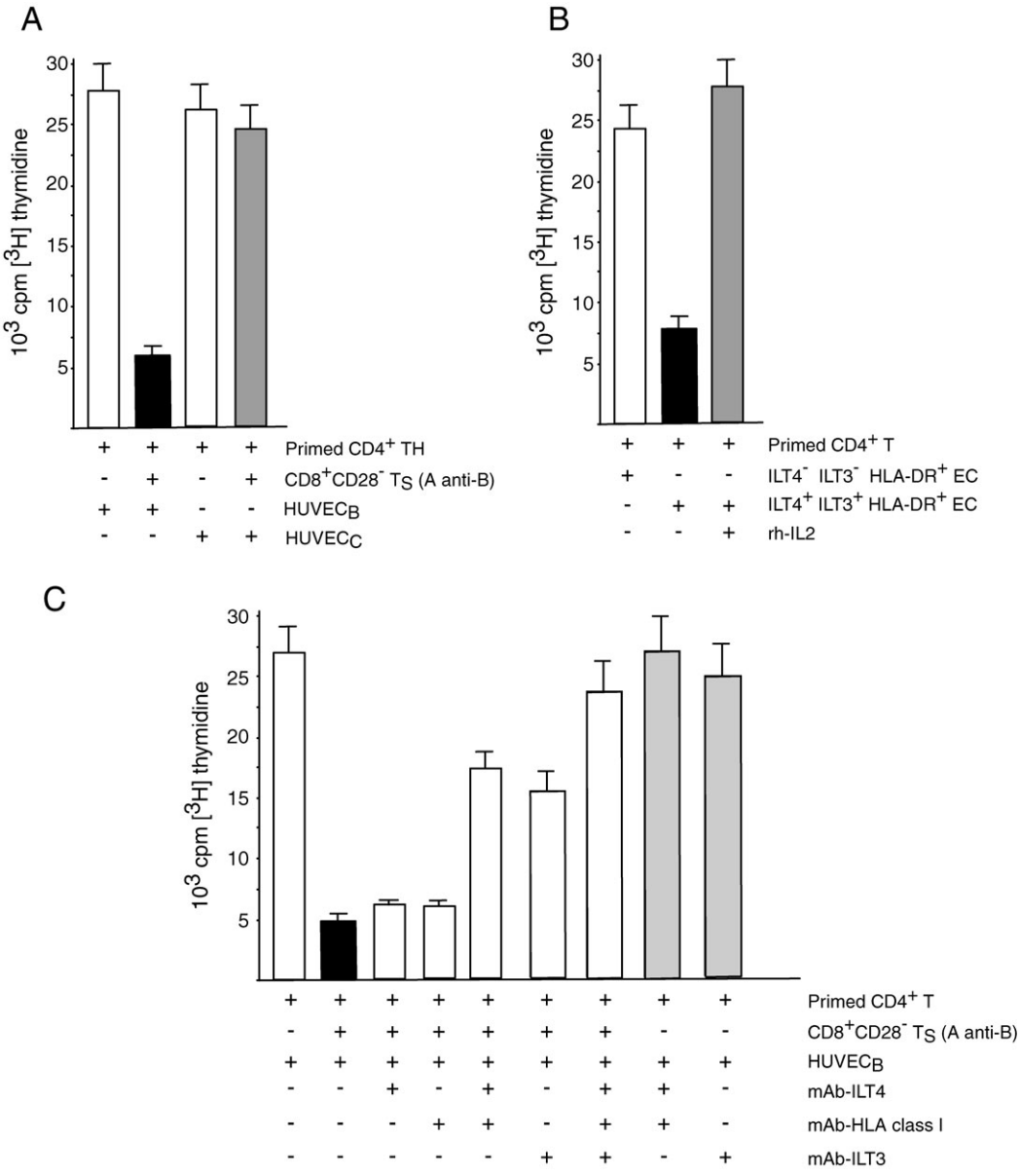

Fig. 7. ILT4$^+$ ILT3$^+$ EC are tolerogenic. (A) The inhibitory effect of T_S on T_H proliferation to EC is HLA class I allorestricted. (B) ILT4$^+$ ILT3$^+$ HLA–DR$^+$ EC induce T_H anergy. (C) The T_S inhibitory effect is partially abrogated by a mixture of mAb to ILT4 and HLA class I and/or by mAb to ILT3.

with ILT4 expression on graft EC ($P < 0.0009$, Fisher exact test).

We next investigated the possibility that CD8$^+$CD28$^-$ T cells which express FOXP3 and trigger ILT4 and ILT3 transcription in EC are present in the patients' circulation. A panel of 12 different HUVEC lines, representing a total of 24 distinct HLA-A and 24 HLA-B alleles, was transfected with pGL3 constructs containing 766 bp of the ILT4 promoter or 1034 bp of the ILT3 promoter upstream of the luciferase reporter gene. These transfected EC were used as targets for induction of ILT4 and ILT3 transcription. Twenty recipients, five with and 15 without a history of acute rejection, were selected for these studies based on the availability of EC matched to their donor for at least one HLA-A and B antigen. CD8$^+$CD28$^-$ T cells from the peripheral blood of these heart transplant recipients were tested for their capacity to trigger ILT4 and ILT3 transcription 10–12 months after transplantation. The frequency of CD8$^+$CD28$^-$ T cells in the peripheral blood of these patients ranged from 28 to 35% of the total CD8$^+$ T cell population and did not differ significantly from that seen in healthy controls.

Out of 15 rejection-free patients, 12 had CD8$^+$CD28$^-$ FOXP3$^+$ T cells which triggered the transcription of ILT3 and ILT4 in EC sharing HLA class I antigens with the donor but not in control EC mismatched from the donor. None of the five patients with a history of acute rejection had CD8$^+$CD28$^-$ T cells that induced ILT3 or ILT4 transcription in EC, as illustrated in Fig. 8(B–D).

To determine whether direct allorecognition of EC by T_S still plays a role late after transplantation when chronic rejection may occur, we next studied the presence of T_S in blood samples obtained 3 years following heart transplantation from

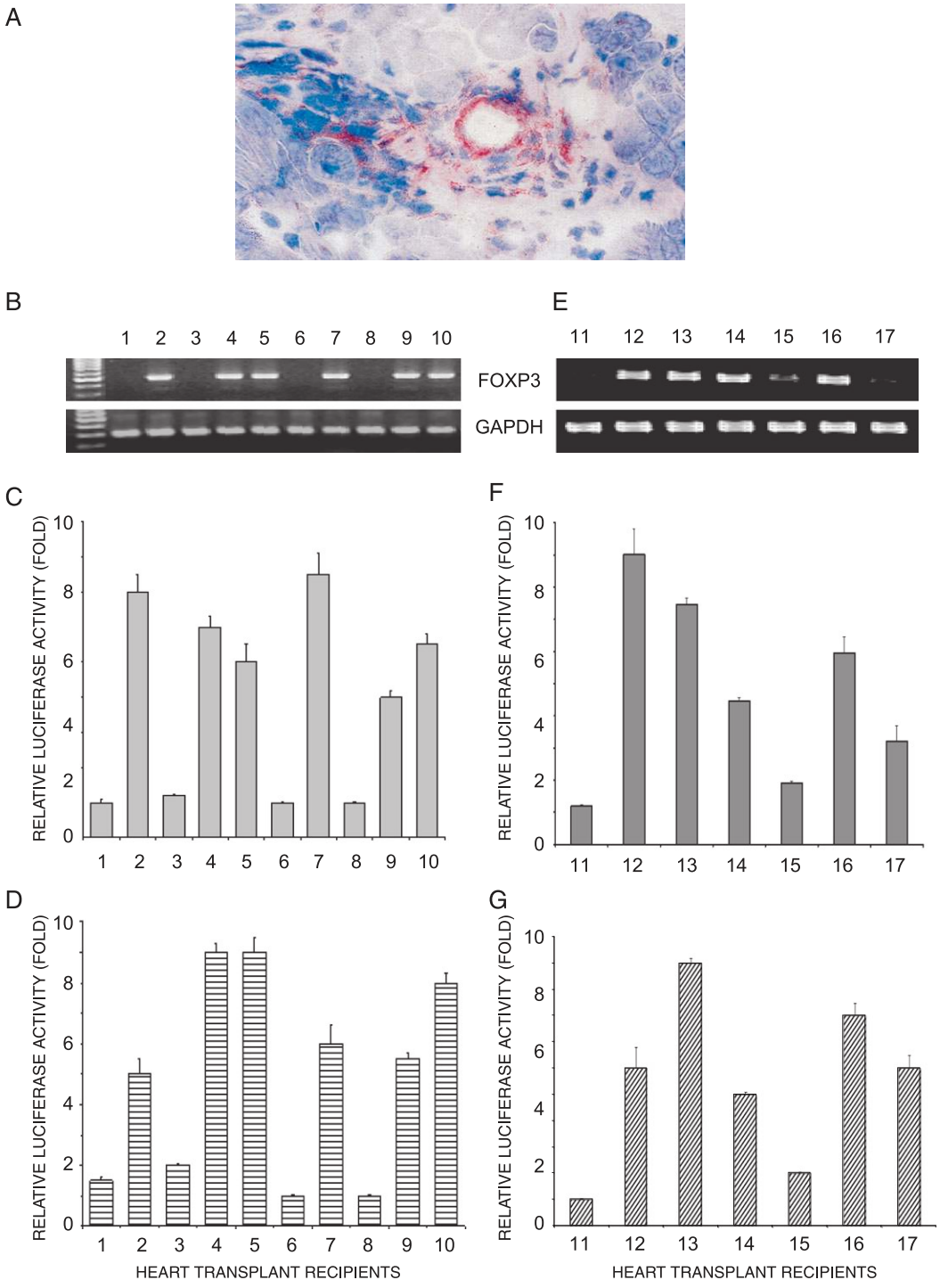

Fig. 8. ILT4 and ILT3 in heart allograft recipients. (A) ILT4 expression on EC of endomyocardial biopsy from a patient without rejection. (B) RT–PCR of FOXP3 in $CD8^+CD28^-$ T cells obtained after 10 months from recipients with (1, 3, 6, 8) and without (2, 4, 5, 7, 9, 10) rejection. (C) Induction of ILT4 and (D) ILT3 transcription in EC (expressing donor HLA class I antigens) by recipient $CD8^+CD28^-$ $FOXP3^+$ T cells in (B). (E) RT–PCR of FOXP3 in $CD8^+CD28^-$ T cells obtained after 3 years from recipients with (11 and 15) and without (12, 13, 14, 16 and 17) rejection. (F) Induction of ILT4 and (G) ILT3 transcription in EC expressing donor HLA class I antigens by recipient $CD8^+CD28^-$ $FOXP3^+$ T cells in (E).

a cohort of 14 different patients. This group included four recipients with coronary artery vasculopathy (CAV) indicative of chronic rejection. Seven of the 10 rejection-free patients had $CD8^+CD28^-$ $FOXP3^+$ T cells which triggered the transcription of ILT3 and ILT4 in donor-matched EC as illustrated in Fig. 8 (E–G). None of the four patients with chronic rejection had circulating $CD8^+CD28^-$ T cells that triggered either ILT3 or ILT4 in EC ($P < 0.01$).

These data suggest that the presence in the circulation of donor specific $CD8^+CD28^-$ $FOXP3^+$ T cells, which trigger ILT3 and ILT4 expression in EC, is associated with quiescence.

Discussion

Although tolerance to allogeneic grafts has been induced in rodents and adoptively transferred using $CD4^+CD25^+$ T_R cells, the underlying mechanism is not yet fully understood. It has been emphasized that the lack of antigen specificity displayed by natural T_R cannot be reconciled with the exquisite allospecificity of $CD4^+$ T cells, which transfer transplantation tolerance in some well defined experimental models (39).

However, alloantigen specificity is an attribute of *in vitro* or *in vivo* generated $CD8^+CD28^-$ T_S which inhibit the direct T cell recognition pathway by tolerizing the APC and blocking their allostimulatory capacity (15,17–20,23,24). Upregulation of inhibitory receptors ILT3 and ILT4 and down-regulation of costimulatory molecules is a critical feature of tolerogenic DC (15,17–20,23,24,37,38).

Our finding that T_S tolerize allogeneic APC, which in turn convert effector T cells into suppressor/regulatory T cells, fulfills the requirements of infectious tolerance and linked suppression described by Waldmann and collegues (39–41).

Our model predicts that the serial transfer of tolerance to allogeneic heart transplants in Waldmann and collegues (41) was mediated by T_R from the primary host which tolerized APC of the graft in secondary hosts. These tolerized APC triggered a new wave of T_S and T_R perpetuating tolerance through many generations of naive unmanipulated transplant recipients. Furthermore, since recognition of a single alloantigen by T_S or T_R is sufficient for tolerizing an APC, linked tolerance to any other antigen presented by the same APC is predicted to occur, according to our model (39–41). This model does not preclude the possibility that antigen receptors on effector T cells are the targets of T_R cells (42).

The effect of T_S on EC is reminiscent of the changes which they induce in allogeneic DC. In the present study we have demonstrated for the first time that the interaction of allospecific $CD8^+CD28^-$ $FOXP3^+$ T_S with activated EC induces the downregulation of costimulatory and adhesion molecules (such as CD40, CD54, CD58, CD62E, CD83, CD106) which mediate T cell interaction with EC and have been implicated in acute allograft rejection (33,43). Such changes are most likely caused by inhibition of NF-κB activation and reduced capacity of EC to transcribe these NF-κB-dependent cell surface molecules (44–50) when exposed to T_S (15, 20). However, T_S induce a distinct differentiation pathway in non-activated EC, and reprogram activated EC converting them into tolerogenic cells. Tolerogenic EC express the inhibitory receptor ILT3 and ILT4 which act as negative regulators of T cell responses to allogeneic APC (15,21,22). The ligand of ILT3 is not known yet. However, blocking of ILT3 with anti-ILT3 mAb (ZM3.8) abrogates the T_S effect.

The inhibitory receptor ILT4 has been shown to interact with a broad range of HLA class I molecules including HLA-A, B and G (21,22). Our finding that mixtures of mAbs to ILT4 and its ligand HLA class I abrogates the T_S effect, demonstrates that ILT4 is involved in suppression.

Compelling evidence that donor EC play an important role in triggering T_S, which inhibit the direct recognition pathway, is provided by three important findings. First, $CD8^+CD28^-$ $FOXP3^+$ T cells that trigger the upregulation of ILT4 in EC are present in the circulation of rejection-free heart allograft recipients. Second, ILT4 is expressed by EC from endomyocardial biopsies obtained from these quiescent patients. Third, the bi-directional interaction between T_S and EC perpetuates long-term quiescence as demonstrated by the persistence of T_S 3 years following transplantation in patients without chronic rejection.

Our results do not detract from our previous studies indicating the importance of the indirect allorecognition pathway in acute and chronic rejection of heart allografts (1–3). However, we demonstrated that both in the graft and in the periphery the frequency of allopeptide specific T cells was 10 000-fold lower than that of T cells involved in the direct allorecognition pathway (1–3,51). The demonstration that EC can elicit effectors or suppressors of the alloimmune response depending on their functional state supports the concept that direct allorecognition is a continuum throughout the lifespan of the transplant and is driven by donor EC.

These findings pertain both to acute and chronic rejection. Donor EC can elicit and become targets of alloreactive T_H cells as well as anti-HLA antibodies which induce EC activation, proliferation and obstruction of transplant vasculature (52). Since chronic rejection occurs within 10 years in ~40% of solid organ allografts, it is likely that regulatory mechanisms protect the graft in the remaining 60% of the recipients. Study of the capacity of $CD8^+CD28^-$ $FOXP3^+$ T cells from recipients' circulation to induce the upregulation of inhibitory receptors in EC, in an alloantigen specific manner, may permit the identification of patients who will benefit from partial or complete withdrawal of immunosuppression. This is an important aim in view of the morbidity and mortality associated with the long-term use of immunosuppressive drugs. Furthermore, the development of pharmaceutic agents that can act on DC and/or EC by upregulating inhibitory receptors, such as ILT3 and ILT4, may permit modulation of the immune response in patients with autoimmune diseases or transplants. The recent finding that *in vitro*-generated tolerogenic APC induce $CD8^+$ T regulatory cells which can suppress ongoing experimental autoimmune encephalomyelitis (53) supports the rationale for developing such new therapeutic strategies.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Abbreviations

DC	dendritic cells
EC	endothelial cells

ILT	immunoglobulin-like transcript
HAEC	human aortic endothelial cells
HUVEC	human umbilical vein endothelial cells
PBMC	peripheral blood mononuclear cells
PRF	perforin
Tc	cytotoxic T cells
TCC	T cell clones
TCL	T cell lines
T_R	T regulatory cells
T_S	T suppressor cells

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