

Soluble Ig-Like Transcript 3 Inhibits Tumor Allograft Rejection in Humanized SCID Mice and T Cell Responses in Cancer Patients¹

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Attempts to enhance patients' immune responses to malignancies have been largely unsuccessful. We now describe an immune-escape mechanism mediated by the inhibitory receptor Ig-like transcript 3 (ILT3) that may be responsible for such failures. Using a humanized SCID mouse model, we demonstrate that soluble and membrane ILT3 induce CD8⁺ T suppressor cells and prevent rejection of allogeneic tumor transplants. Furthermore, we found that patients with melanoma, and carcinomas of the colon, rectum, and pancreas produce the soluble ILT3 protein, which induces the differentiation of CD8⁺ T suppressor cells and impairs T cell responses in MLC. These responses are restored by anti-ILT3 mAb or by depletion of soluble ILT3 from the serum. Immunohistochemical staining of biopsies from the tumors and metastatic lymph nodes suggests that CD68⁺ tumor-associated macrophages represent the major source of soluble ILT3. Alternative splicing, resulting in the loss of the ILT3 transmembrane domain, may contribute to the release of ILT3 in the circulation. These data suggest that ILT3 depletion or blockade is crucial to the success of immunotherapy in cancer. In contrast, the inhibitory activity of soluble ILT3 on T cell alloreactivity *in vitro* and *in vivo* suggests the potential usefulness of rILT3 for immunosuppressive treatment of allograft recipients or patients with autoimmune diseases. *The Journal of Immunology*, 2007, 178: 7432–7441.

The development of new strategies to promote immune responses in malignancies and certain viral diseases or to suppress their activation in autoimmune diseases and transplantation is critical in overcoming the limited efficacy of conventional therapies. Research in this area has been fueled by the discovery that regulatory T (Treg)³ cells and tolerogenic APC modulate the immune response to self and non-self Ags. The concept has emerged that bidirectional interactions between APC and Ag-experienced T cells can initiate either a tolerogenic or an immunogenic pathway (reviewed in Refs. 1–3). Thus, there is increasing evidence that the immune response can be inhibited by various CD4⁺ and CD8⁺ Treg cells, which participate in innate and adaptive immunity (3, 4).

Naturally arising CD4⁺ and CD8⁺ Treg develop during the normal process of T cell maturation in the thymus, play an essential role in preventing autoimmune diseases, and are characterized by the constitutive expression of CD25 (the IL-2R α -chain) and forkhead-winged helix transcription factor FOXP3. After TCR triggering, natural Treg cells inhibit immune responses *in vivo* and *in vitro* in an Ag-nonspecific, APC-independent, and MHC-nonspecific manner. Natural Treg are anergic, do not produce cytokines, and suppress effector T cells by cell-to-cell contact (reviewed in Refs. 5–10).

Adaptive CD4⁺ and CD8⁺ Treg cells are Ag induced, develop in the periphery, and exert their function either by secreting inhibitory cytokines (such as IL-10 and TGF- β) or directly tolerizing the APC with which they interact (5, 10, 11). In humans, tolerogenic APC express high levels of inhibitory receptors such as Ig-like transcript (ILT)3 and ILT4, induce T cell anergy, and elicit the differentiation of Ag-specific CD4 and CD8 Treg cells (12, 13). For the sake of consistency with previous publications, we will refer to CD8⁺ Treg cells as T suppressor (Ts) cells (3, 11–13). The expression of ILT3 and ILT4 was shown to be confined to dendritic cells (DC), monocytes, and macrophages (14–16).

In previous studies, we demonstrated that chronic *in vitro* stimulation of human T cells with peptide-pulsed autologous APC or with allogeneic APC resulted in the generation of MHC class I-restricted CD8⁺ Ts that inhibit the activation and effector function of Th and CTLs with cognate specificity (3, 11–13). Alloantigen-specific CD8⁺ Ts induce the up-regulation of ILT3 and ILT4 on monocytes and DC, rendering them tolerogenic (12). Tolerogenic ILT3^{high}ILT4^{high} DC induce anergy in alloreactive CD4⁺CD45RO⁺CD25⁺ T cells, converting these cells into

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³ Abbreviations used in this paper: Treg, regulatory T; Ct, crossing threshold; DC, dendritic cell; hu-SCID, humanized SCID; ILT3, Ig-like transcript 3; mILT3, membrane-bound ILT3; sILT3, soluble ILT3; TAA, tumor-associated Ag; TAM, tumor-associated macrophage; Ts, T suppressor.

Treg cells that perpetuate the suppressor cell cascade by tolerizing other APC (13).

We recently demonstrated that the extracellular region of ILT3 is endowed with immunomodulatory properties. Both membrane-bound ILT3 (mILT3) and soluble ILT3 (sILT3) inhibited T cell proliferation in MLC, anergizing CD4⁺ Th cells, suppressing the differentiation of IFN- γ -producing CD8⁺ CTL, and inducing the differentiation of alloantigen-specific CD8⁺ Ts in primary 7-day MLC (17).

In view of recent studies unraveling the role of Treg/Ts and tolerogenic DC in tumor growth and metastasis, we explored the possibility that mILT3 and sILT3 participate in the induction of T cell anergy and differentiation of Treg cells in patients with cancer (10, 18–20). Understanding the role of ILT3 in the progression of malignant diseases and suppression of alloimmune responses may open the way to new therapeutic approaches.

Materials and Methods

Human subjects

We studied previously untreated patients with colorectal ($n = 44$) and pancreatic ($n = 17$) adenocarcinoma. A cohort of patients with advanced stage melanoma ($n = 46$), enrolled for treatment with high IL-2 doses, was also included. The patients ranged in age from 20 to 68 years. Serum samples from healthy age-matched blood donors ($n = 90$) were used as controls. Patients gave written informed consent. The study was approved by the Institutional Review Board of Columbia University, University of Rome "La Sapienza," and Campus Bio-Medico University. Tumor tissue used for molecular analysis was obtained from the Pathology Tissue Bank of the Herbert Irving Comprehensive Cancer Center of Columbia University under an Institutional Review Board-approved protocol. Buffy coats obtained from healthy blood donor volunteers were purchased from the New York Blood Center and used for injection into SCID mice.

Human tumor cell lines

The myelomonocytic KG1, melanoma SK-ME-28, and pancreatic carcinoma PANC-1 cell lines were obtained from American Type Culture Collection. KG1 tumor cells transfected with ILT3 (KG1.ILT3) or with the empty vector alone (KG1.MIG) were generated, as previously described (12, 17). The KG1 tumor cells display high expression of the CD34 markers, characteristic of stem cells. The melanoma RE280 and T567A cell lines were a gift from D. Rimoldi (Ludwig Institute of Cancer Research, Lausanne, Switzerland). Cell lines were maintained in RPMI 1640 medium (Mediatech) supplemented with 10% heat-inactivated FCS.

Animals

C.B-17 SCID female mice (Taconic Farms) were used at 5–8 wk of age. All protocols involving animals were approved by the Columbia University Animal Care Committee. The animals were housed individually in microisolator cages and were fed autoclaved food and water. Serum IgG levels were determined by sandwich ELISA using reagents from Alpha Diagnostics International. SCID animals were considered leaky at IgG levels $>1 \mu\text{g/ml}$ and excluded from experimental use.

Generation, transplantation, and treatment of humanized SCID (hu-SCID) mice

Human PBMC were isolated from fresh peripheral blood by Ficoll-Hypaque centrifugation. As indicated by flow cytometric analysis, these PBMC contained $<1\%$ CD34⁺ cells. SCID mice were reconstituted with 3×10^8 human PBMC by i.p. inoculation and are referred to as hu-SCID mice. Animals demonstrating signs of graft-vs-host disease or failing to reconstitute with human T cells were excluded from analysis by prior design. Circulating human T cells were evaluated by flow cytometry. Heparinized retro-orbital venous samples were obtained 2, 4, and 8 wk after reconstitution; the erythrocytes were lysed; and the phenotype of circulating human leukocytes was determined.

Concomitant with the humanizing treatment, mice were injected s.c. on the right flank with 2×10^6 tumor cells. The tumor cell lines used for transplantation were as follows: KG1, KG1.MIG, KG1.ILT3, SK-ME-28, RE280, and T567A. Groups of mice transplanted with wild-type KG1 or other human tumor cell lines were treated by i.p. injection of ILT3-Fc (250 $\mu\text{g/day}$) for the first 10 days following tumor and PBMC injection. Control hu-SCID mice received daily doses (250 μg) of human IgG

(Sigma-Aldrich) or were left untreated. Each experimental group consisted of 10 hu-SCID mice. Tumor growth was recorded every 3 days. For flow cytometry, immunohistochemistry, molecular, and functional studies, additional SCID mice were humanized by the same method, transplanted, treated, and sacrificed after 8 wk.

Histology and immunohistochemistry

KG1 and KG1.ILT3 tumors growing in hu-SCID mice were harvested after 8 wk and processed for paraffin-embedded sections. Immunostaining was performed using mouse anti-human CD4 and CD8 mAb (DakoCytomation) or isotype-matched, nonbinding control Abs.

Frozen and fixed biopsies from the patients were examined for CD68 and ILT3 expression. Slides containing paraffin sections were dried, deparaffinized, rehydrated, and washed. Sections were stained with goat anti-human ILT3 polyclonal Ab (R&D Systems) or mouse anti-human CD68 mAb (DakoCytomation) washed in TBS/20% Tween 20 (DakoCytomation) and then incubated with secondary rabbit anti-goat IgG or anti-mouse IgG Ab (Vector Laboratories). Color was developed, using the Vectastain Elite ABC kit (Vector Laboratories) diaminobenzidine⁺ (DakoCytomation).

FACS analysis

Flow cytometry studies were performed on a FACSCalibur instrument using five-parameter acquisition (BD Biosciences). The following mAbs were used: anti-HLA ABC FITC, CD4 FITC, CD8 FITC, CD14 PE, CD19 PE, CD25 PE, CD34 PE, CD56 PE, IL-2 PE, IFN- γ PE, IL-10 PE, TGF- β PE, and CD3 CyChrome (all from BD Biosciences). All stainings were performed at 4°C. For cytokine studies, purified CD4 and CD8 T cells were activated for 6 h on CD3 Ab-coated plates (BD Biosciences) in the presence of 1 $\mu\text{g/ml}$ CD28 mAb (BD Biosciences), Brefeldin A (1 $\mu\text{g/ml}$) was added to the culture for the last 3 h of incubation at 37°C. Cells were first stained with surface markers, then for intracellular cytokine expression using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. For each marker, a corresponding isotype-matched Ab conjugated with the same fluorescent dye was used as a negative control. The percentage of human CD4 and CD8 T cells present in the peripheral blood, spleen, and draining axillary and inguinal lymph nodes was determined.

Ts cell assays

Human CD4⁺ and CD8⁺ T cells were sorted from lymph nodes and spleen of hu-SCID mice at 8 wk using the CD4 or CD8 isolation kits (StemCell Technologies). Sorted CD4⁺ or CD8⁺ T cells were added at increasing numbers (from $1-4 \times 10^4$) to a fixed number (10^4) of unprimed allogeneous CD3⁺CD25⁻ T cells and stimulated for 6 days in MLC with irradiated KG1 cells. The percentage of inhibition of proliferation at various suppressor to responder ratios was calculated according to the following formula: $(1 - \text{cpm}(\text{Th} + \text{T}_s \text{ vs KG1})/\text{cpm}(\text{Th} \text{ vs KG1})) \times 100$.

To determine whether ILT3-containing sera from cancer patients induce Ts, we first primed CD3⁺CD25⁻ T cells isolated from fresh peripheral blood of healthy volunteers with irradiated APC from allogeneic donors differing by two HLA-DR Ags from the responder. Cultures were performed in medium supplemented with serum depleted or not depleted of sILT3. Three sera from patients with carcinoma of the pancreas and two of colon were used in five independent experiments. CD8⁺ T cells, magnetically sorted from these cultures after 7 days, were added to MLC containing unprimed CD4⁺CD25⁻ T cells from the same responder and APC from the original stimulator. [³H]Thymidine was added to the cultures 18 h before harvesting, and incorporation was determined by scintillation spectrometry using a LKB 1250 Betaplate counter (PerkinElmer). Mean cpm of triplicate cultures, the SD from the mean, and percentage suppression were calculated.

Generation of mAb anti-ILT3 (clone ZL5.7)

Six- to 8-wk-old female BALB/c mice were given an initial i.p. immunization of 100 μg of ILT3-Fc protein in PBS mixed with CFA at a 1:1 ratio (v/v) (Sigma-Aldrich). The mice received two additional injections i.p. of ILT3-Fc in IFA (Sigma-Aldrich). A final booster immunization with ILT3-Fc in PBS was given 2 wk after last injection. Mice were sacrificed 3 days later. Spleen cells were isolated and fused with a mouse myeloma fusion partner, Sp2/0-Ag14 (American Type Culture Collection), using 50% (w/v) polyethylene glycol (Sigma-Aldrich). Supernatants were first screened by ELISA for reactivity for ILT3-Fc and human IgG. Supernatants from clones that react with ILT3-Fc, but not with human IgG, were further tested by flow cytometry for their ability to stain KG1.ILT3 and KG1 (untransfected) cells. Supernatants of clone ZL5.7 (IgG1) identified

ILT3-Fc protein in ELISA (but did not cross-react with h IgG) and efficiently stained KG1.ILT3 cells for flow cytometry (while not staining KG1 cells).

Sandwich ELISA detection of serum ILT3

Maxisorp 96-well plates (Nalge Nunc International) were coated overnight at 4°C with 1.0 µg/well anti-ILT3 mAb (clone ZL5.7), and free binding sites were blocked with 5% BSA solution for 1 h at room temperature. After washing with PBS-T (PBS plus 0.1% Tween 20), 100-µl serum samples were added to the wells in 1/3 and 1/9 dilutions. After 1 h of incubation at room temperature, the plate was washed and 100 µl of anti-ILT3 biotinylated polyclonal Ab (R&D Systems) was added at a concentration of 33 ng/ml to each well, followed by 1-h incubation at room temperature. After washing, 100 µl of 1 µg/ml HRP-conjugated streptavidin (BD Biosciences) was added to each well and incubated for 1 h at room temperature. After washing, the plate was developed using 100 µl of tetramethylbenzidine substrate (Sigma-Aldrich). Reactions were stopped using 100 µl/well 1 N H₂SO₄, and the plates were read at 450 nm. For calibration of each sandwich ELISA, standards in the range of 0–1500 ng/ml rILT3-Fc diluted in 10% FCS were run on each plate with the test samples.

Immunoprecipitation and Western blotting

Assays were conducted, as previously described (17). Briefly, KG1 and KG1.ILT3 cells (1×10^6) were homogenized and (1-ml) aliquots of serum samples previously tested by ELISA for the presence of sILT3 were thawed from storage, then vigorously centrifuged and precleared with 0.1 vol of agarose-protein G beads (Millipore). Immunoprecipitation of ILT3 was conducted using the same agarose-protein G beads as used for pre-clearing plus 5 µg/ml mAb anti-ILT3 (ZM3.8). Western blotting was conducted using a biotinylated anti-ILT3 detection Ab (R&D Systems) and the ECL detection system.

Serum inhibition studies

PBMC from healthy blood donors were separated from buffy coats by density gradient centrifugation. CD3⁺ T cells were obtained using the T cell isolation kits (Miltenyi Biotec), according to the manufacturer's instructions. CD25⁺ T cells were depleted from CD3⁺ responding cells by use of CD25 beads (Miltenyi Biotec). All cell cultures were performed in complete medium (RPMI 1640 supplemented with 2 mM L-glutamine and 50 mg/L gentamicin from Mediatech, at 37°C in a 5% CO₂, humidified incubator).

CD3⁺CD25⁻ T cells from healthy responders (5×10^4 /well) were stimulated in 6-day MLC with irradiated (5000 rad) KG1 cells (2.5×10^4 /well). T cell alloreactivity in normal serum was established by adding to 12 replicate wells pooled serum from healthy blood donors to a final concentration of 20%. Sera from individual patients with colorectal or pancreatic cancer, in which sILT3 was detected, were added to triplicate reactions at a final concentration of 20%. Serum from the same patient was tested in parallel triplicate cultures, to which 5 µg/ml anti-ILT3 mAb or mouse IgG was added. For some experiments, the same serum was tested in parallel before and after depletion of sILT3.

T cell reactivity in pooled normal serum was normalized to 100%. Reactivity of the same responder's T cells in cultures containing sera from individual cancer patients was expressed as percentage of reactivity seen in normal serum. Reactivity in patient serum supplemented with anti-ILT3 mAb or mouse IgG control was calculated in the same manner.

Quantitative real-time PCR

Total RNA was isolated from human cells using the RNAqueous Kit (Ambion), and reverse transcribed using the First Strand cDNA Synthesis Kit (Roche Diagnostics). Quantitative real-time PCR was performed using TaqMan gene expression assays (Applied Biosystems). The gene expression assays used in these studies are identified by the HUGO gene name and the manufacturer's part number as follows: GAPDH (4326317E), GZMB (Hs00188051_m1), PRF1 (Hs00169473_m1), IL-2 (Hs00174114_m1), IFNG (Hs00174143_m1), and IL-10 (Hs00174086_m1). The forward and reverse primers are contained in different exons of the targeted transcript to prevent amplification of genomic DNA.

The data were acquired using the 7300 RT-PCR instrument and the packaged 7300 SDS 1.3.1 software (Applied Biosystems). The manufacturer's protocols and recommended amplification conditions used were as follows: one cycle at 50°C (2 min) and 95°C (10 min), followed by 50 cycles at 95°C (15 s) and 60°C (1 min). Each assay plate included no template negative controls and a control cDNA. The relative amount of gene expression was calculated according to the following formula: $2^{-\Delta Ct}$,

in which $\Delta Ct = (Ct(\text{gene}) - Ct(\text{GAPDH}))$ and Ct is the crossing threshold value returned by the PCR instrument for every gene amplification.

Isolation and cloning of alternatively spliced ILT3 transcripts

Total RNA was isolated from archived tumor tissue obtained from 13 patients with colon adenocarcinoma. For amplification of ILT3-specific cDNA, 1–3 µl of individual RNA preparations was added to 25 µl of One-Step RT-PCR (SuperScript One-Step RT-PCR; Invitrogen Life Technologies) reactions together with ILT3 (exons 3, 4, 7, and 8)-specific primers. The following primers were synthesized by a commercial vendor (Invitrogen Life Technologies): exon 3 forward, 5'-CCCCTCCCAACCC ACCCTC; exon 4 forward, 5'-CTGCCGGTCTCTTGTGACC; exon 7 reverse, 5'-AGAGGAGGAGGGAGAGAAGCAGGATG; and exon 8 reverse, 5'-TGGAGGACGTTGGAAATCAGC. The thermal profiles typically consisted of 1 cycle of 50°C (30 min) and 94°C (2 min), followed by 40 cycles of 94°C (20 s), 60°C (30 s), and 72°C (30 s), and one cycle of 72°C (7 min). PCR amplification products were fractionated by 1.3% agarose gel electrophoresis. DNA with variant sizes were purified, cloned into a TA cloning vector using TOPO cloning kit (Invitrogen Life Technologies), and completely sequenced from both strands.

Construction and expression of an alternatively spliced variant, δ ILT3p45

cDNA for the alternatively spliced ILT3 variant (δ ILT3p45) was obtained from colon carcinoma biopsies by RT-PCR using an exon 4 primer 5'-CTGCCGAGTCTCTTGTGACC and an exon 11 primer 5'-ATCGAATT CGGATTAGTGGATGGCCAGAG. A DNA fragment (750 bp) was excised from the gel and subcloned into the pCR2.1-TOPO vector (Invitrogen Life Technologies). The 390-bp *EcoRI* fragment, which comprises exons 4–11 of alternatively spliced ILT3, was excised from the recombinant vector to replace the 596-bp *EcoRI* fragment of the normally spliced ILT3 cDNA. The full length of δ ILT3p45 cDNA (1.1 kb) was subsequently subcloned into *BglII* and *XhoI* sites of a retroviral GFP vector, MIG (12). The 2.5-kb *BglII/SalI* DNA fragment of δ ILT3p45-MIG, which contains δ ILT3p45-IRES-GFP, was then subcloned into *BamHI* and *XhoI* sites of a lentiviral vector FUGW under ubiquitin promoter control (21). ILT3 sequences in all cloning or expression vectors were confirmed by DNA sequencing from both ends. The splice variant was expressed in CHO-S and 293T cells, as previously described (17). The supernatant and cell lysates were collected and tested by ELISA and Western blot analysis, respectively, using anti-ILT3 Abs, as described above.

Statistics

ELISA data were analyzed using the nonparametric Mann-Whitney *U* test to compare sILT3 in sera from healthy individuals and patients with cancer. The statistical difference between T cell reactivity in serum from healthy individuals and patients with cancer in the presence or absence of anti-ILT3 mAb or mouse IgG was analyzed using paired two-tailed Student's *t* test. Calculations were performed using BMDP release 7 software (BMDP Statistical Software).

Results

ILT3 induces tolerance to allogeneic tumors

To explore the immunomodulatory effect of sILT3 and mILT3, we transplanted hu-SCID mice with the myelomonocytic cell line KG1 (wild-type KG1), KG1.MIG (transfected with empty vector), or KG1.ILT3 (transfected with full-length ILT3) (12). In nonhumanized SCID mice, all tumors grew at roughly the same rate, reaching a cross-sectional area of 268 ± 50 mm² by day 60. Groups consisting of 10 hu-SCID mice each received the following: 1) wild-type KG1; 2) KG1.MIG; 3) KG1.ILT3; 4) wild-type KG1 plus human IgG throughout the first 10 days posttransplantation; or 5) wild-type KG1 plus rILT3-Fc throughout the first 10 days posttransplantation. The s.c. transplants of wild-type KG1 or KG1.MIG were rejected by hu-SCID hosts, generating no tumor (groups 1 and 2). In contrast, when KG1-ILT3 cells were transplanted s.c. in hu-SCID mice (group 3), they generated tumors that grew to a large size (>50-mm² cross-sectional area) within 2 mo. Furthermore, hu-SCID mice transplanted with wild-type KG1 cells and treated with rILT3-Fc protein (group 5) developed tumors that grew more aggressively, reaching a cross-sectional area of 240 mm² within 60 days. Control hu-SCID mice transplanted with

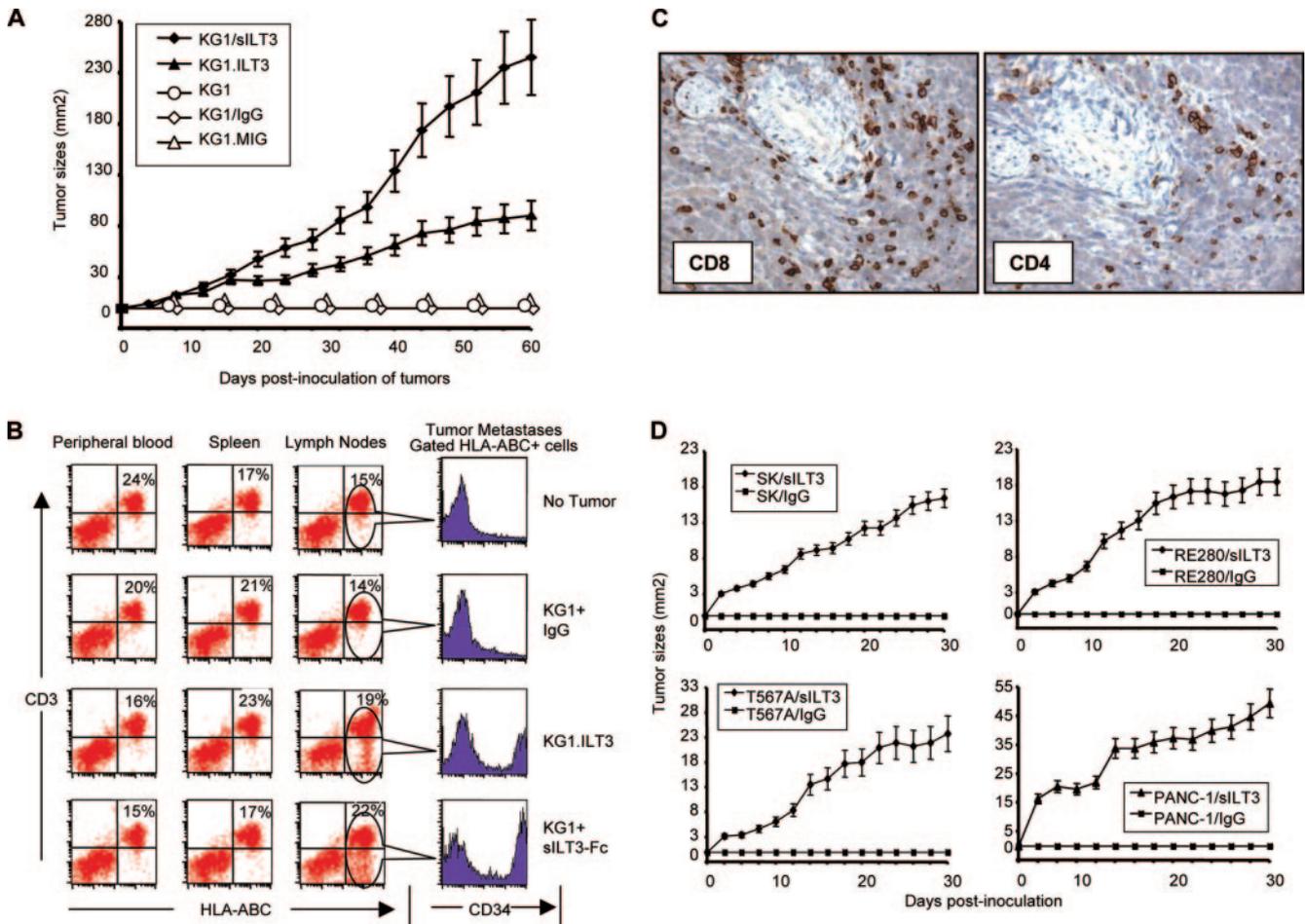


FIGURE 1. Effect of ILT3 on tumor allografts. sILT3 and mILT3 induce tolerance to allogeneic tumors in hu-SCID mice. *A*, Tumor growth was assessed in 5 groups, each consisting of 10 hu-SCID mice transplanted with KG1 and treated with rILT3-Fc (◆), human IgG (◇), not treated (○), and transplanted with empty vector-transfected KG1.MIG (△) or ILT3-transfected KG1 (▲). *B*, Engraftment of human T cells in blood, lymph nodes, and spleen of hu-SCID mice. HLA class I⁺ CD34⁺ tumor metastasis in regional lymph nodes was seen in mice with KG1 and KG1.ILT3 tumors. *C*, Immunohistochemical staining of CD4⁺ and CD8⁺ human T cells infiltrating KG1 tumor growing in hu-SCID mice treated with rILT3-Fc. *D*, Growth of melanoma and pancreatic carcinoma cell lines in hu-SCID mice treated or not treated with rILT3-Fc.

wild-type KG1 and treated with human IgG (group 4) developed no tumors (Fig. 1*A*). The data suggested that rILT3-Fc as well as mILT3 inhibited the rejection of allogeneic KG1 tumor transplant.

To exclude the possibility that the fate of the transplanted tumor cells was determined by the number of human PBMC that reached the periphery, rather than by their exposure to ILT3, we monitored leukocyte engraftment in the peripheral blood of all 10 mice from each experimental group. We found no engraftment of CD14⁺ monocytes, CD19⁺ B cells, or CD56⁺ NK cells in hu-SCID mice. The percentage of human CD3⁺ T cells varied from mouse to mouse (5–25%) within each group. There were no obvious differences between hu-SCID mice that rejected the tumor grafts (groups 1, 2, and 4, showing an average of 10.3 ± 5.4 , 13 ± 3.5 , and $12 \pm 4.0\%$, respectively); mice that tolerated the tumor (groups 3 and 5, showing 12.7 ± 5.3 and $10.7 \pm 4.5\%$, respectively); and mice that were humanized, but not transplanted ($10.4 \pm 5.1\%$). Lymph nodes draining the growing KG1 or KG1.ILT3 tumors (which highly express the CD34 marker) contained, in addition to human T cells, a population of CD34⁺HLA class I⁺ cells that are metastatic KG1 tumor cells. The spleens of these tumor-bearing mice, however, showed no metastatic KG1 cells (Fig. 1*B*).

Immunohistochemical examination of KG1 tumors growing in hu-SCID hosts showed focal infiltrates of CD8⁺ and diffuse, but

sparse CD4⁺ T cell infiltrates. There was no tumor cell necrosis or changes suggestive of rejection in the proximity of these infiltrates (Fig. 1*C*), suggesting that T cells infiltrating the tumor were rendered anergic or converted into Treg/Ts upon exposure to ILT3.

The tolerogenic effect of ILT3-Fc on hu-SCID mice transplanted with human tumors was further proven in experiments for which we used melanoma (SK-ME-28, RE280, and T567A) and pancreatic carcinoma PANC-1 cell lines. Untreated hu-SCID mice rejected the tumors, whereas ILT3-Fc-treated mice showed rapid tumor growth, demonstrating unambiguously that sILT3 inhibits the capacity of human T cells to reject allogeneic tumor transplants (Fig. 1*D*).

sILT3 and mILT3 induce CD8⁺ Ts

In previous studies, we have shown that CD8⁺ T cells, allostimulated in the presence of mILT3 or sILT3, not only lose cytotoxic activity, but also acquire suppressor function (17). To determine whether this phenomenon also occurs in vivo, we tested the suppressor activity of human T cells from hu-SCID mice sacrificed 8 wk following tumor transplantation. For these assays, CD4⁺ and CD8⁺ T cells isolated from draining lymph nodes or from the spleen of tumor-grafted or nongrafted hu-SCID mice were added to MLC containing unprimed autologous T cells and irradiated KG1-stimulating cells. The response of 10^4 unprimed T cells to

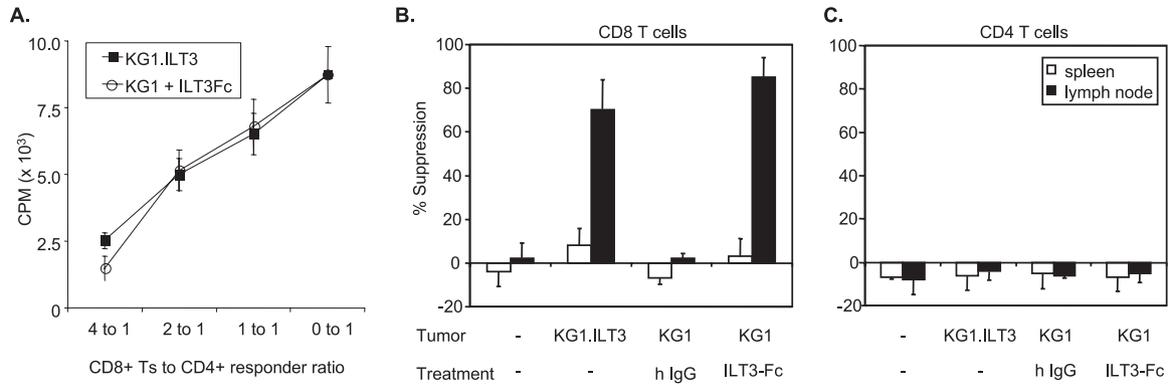


FIGURE 2. Suppressive capacity of human T cells from transplanted hu-SCID mice. *A*, CD8 T cells from the lymph nodes draining KG1 (○) or KG1.ILT3 tumors (■) suppress the response of unprimed CD4 T cells to KG1, in a dose-dependent manner. CD8 (*B*) and CD4 (*C*) T cells from lymph nodes and spleen of hu-SCID mice with growing KG1 or KG1.ILT3 tumors and from untreated mice that have rejected KG1 tumors were tested for their capacity to inhibit the MLC response of unprimed CD4 T cells to KG1 at a 4:1 suppressor to effector cell ratio.

KG1-stimulating cells ranged from 8,000 to 10,000 cpm for different healthy responders.

Human CD8⁺ T cells from lymph nodes of hu-SCID mice treated with ILT3-Fc and transplanted with wild-type KG1, as well as CD8⁺ T cells from hu-SCID mice transplanted with KG1.ILT3, inhibited T cell proliferative responses to KG1 by >20, 40, and 80% at a 1:1, 2:1, and 4:1 ratio of Ts to T effector cells, respectively. The inhibitory activity of CD8⁺ T cells from the lymph

nodes of tumor-bearing mice was dose dependent, reaching 80% at a 4:1 ratio (Fig. 2*A*). CD8⁺ T cells from the same animals' spleen had no suppressor activity, suggesting that CD8⁺ Ts migrated from the tumor only to the draining lymph nodes and/or that they differentiated into Ts only in tumor cell-infiltrated lymph nodes. CD4⁺ T cells from the lymph nodes or spleen of these tolerant mice were not able to inhibit the MLC response to KG1. CD4 and CD8 T cells from the lymph nodes and spleen of hu-SCID mice

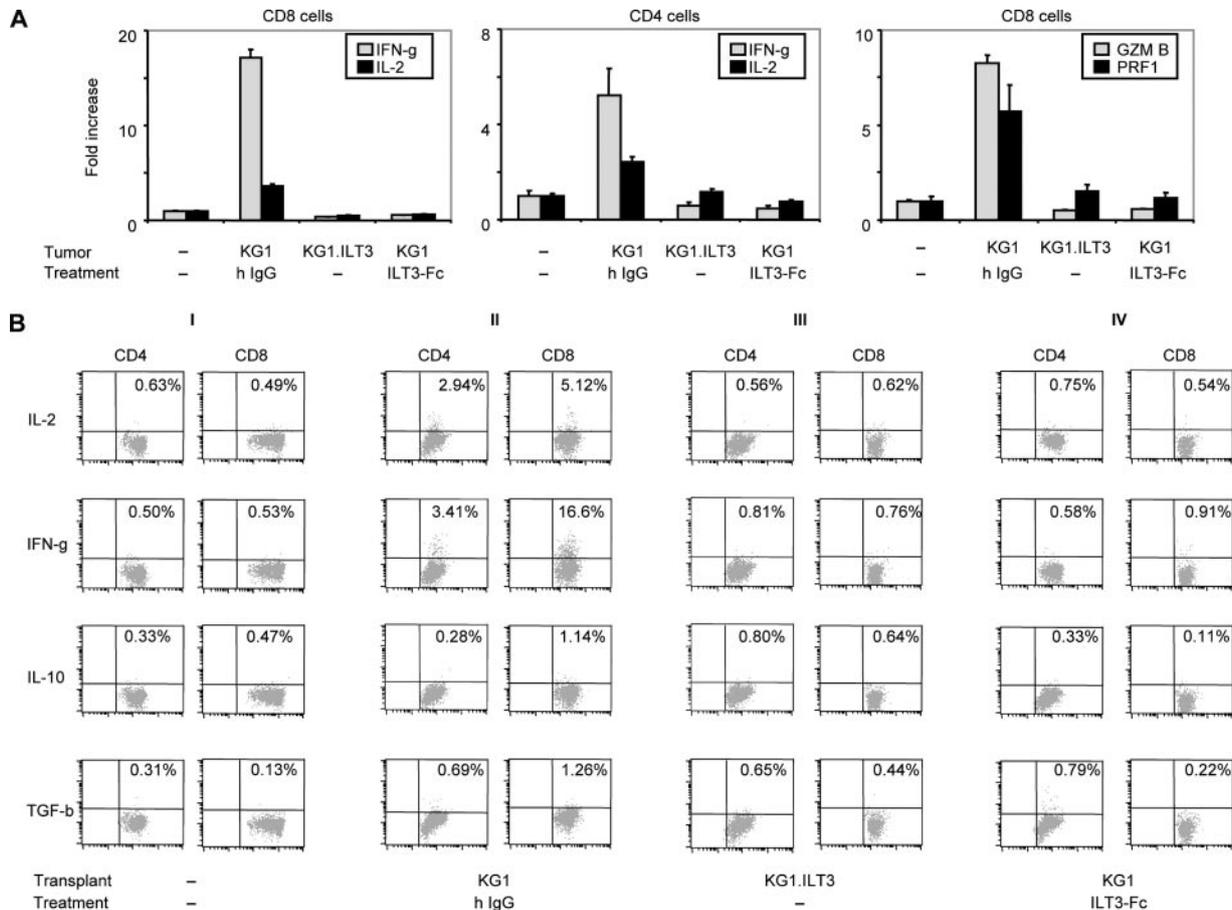


FIGURE 3. Cytokine profile of CD4 and CD8 T cells from hu-SCID mice. *A*, IL-2 and IFN- γ were detected by quantitative real-time PCR in lymph nodes draining the transplantation site of rejected, but not of tolerated KG1 tumors. Granzyme B and perforin were expressed at high levels in CD8⁺ T cells from hu-SCID mice that rejected the tumor. *B*, IL-2 and IFN- γ were detected by flow cytometry only in CD4⁺ and CD8⁺ T cells from lymph nodes of mice rejecting the tumor (group II).

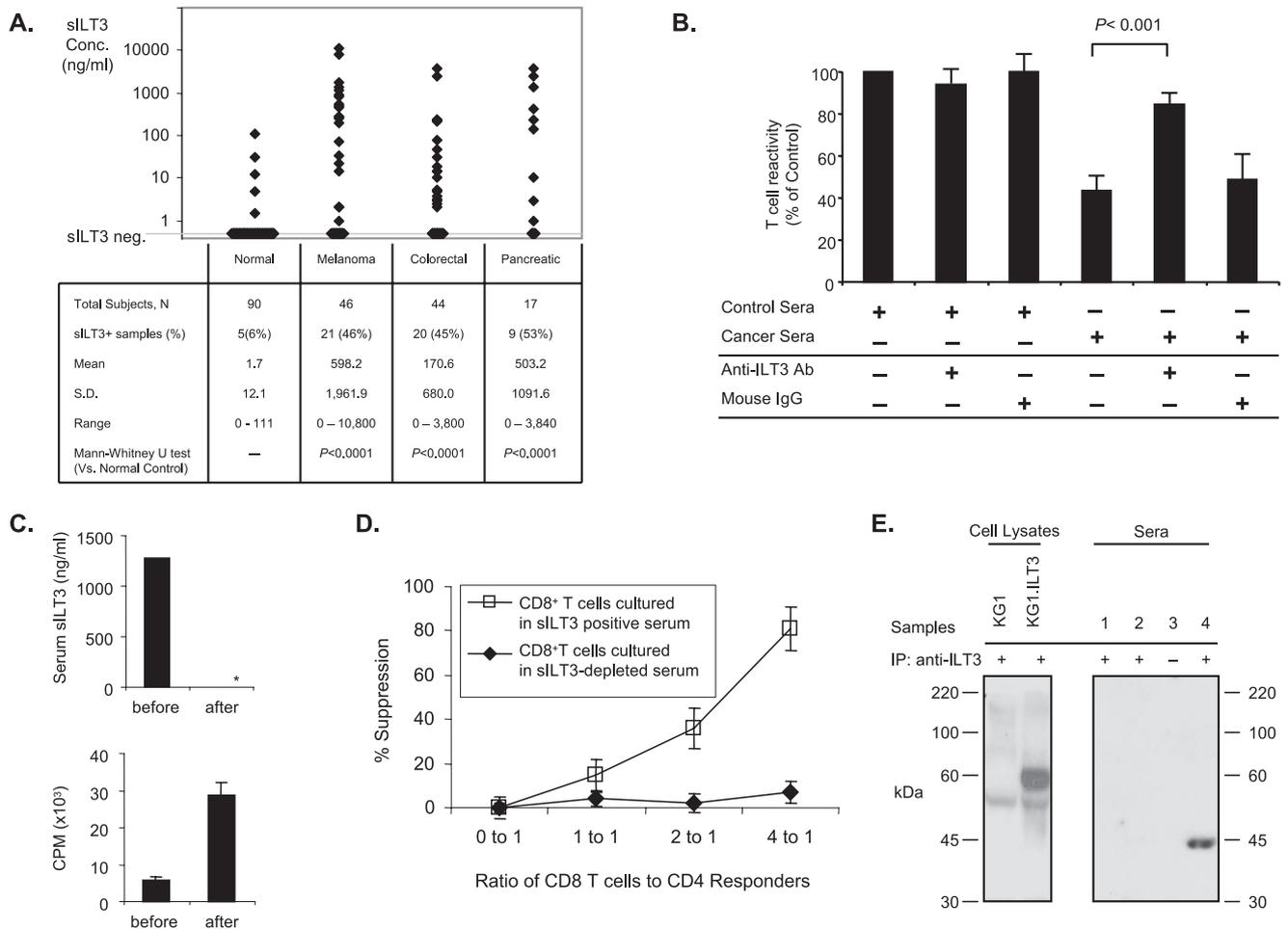


FIGURE 4. Detection of ILT3 in human sera. *A*, Sera from cancer patients and healthy blood donors were tested for sILT3 by ELISA. *B*, Healthy donor T cells were tested for MLC reactivity to irradiated KG1-stimulating cells in cultures containing pooled (ILT3-negative) sera from healthy controls or ILT3-positive sera from individual cancer patients ($n = 29$) in the presence or absence of anti-ILT3 mAb or mouse IgG. *C*, The MLC-inhibitory activity of a cancer patient’s serum was tested before and after depletion of sILT3. *D*, CD8⁺ T cells allostimulated in cultures containing ILT3-positive sera inhibited the alloreactivity of autologous, unprimed CD3⁺ T cells in a dose-dependent manner. *E*, Immunoprecipitation and Western blot analysis of an ILT3-positive serum prior to (lane 4) and after depletion of ILT3 (lane 3) and of two ILT3-negative control sera (lanes 1 and 2).

without tumor transplants or from IgG-treated controls that rejected the KG1 tumor transplant showed no suppressor activity (Fig. 2, *B* and *C*).

To further characterize human T cells from transplant draining lymph nodes, their cytokine profile was determined by flow cytometry and quantitative real-time PCR. CD8⁺ T cells were also tested by quantitative real-time PCR for granzyme B and perforin expression. The results obtained with both methods showed induction of IFN- γ and IL-2 in CD4⁺ and CD8⁺ T cells from mice rejecting their tumor transplants, but not in mice with growing tumors (Fig. 3). No IL-10- or TGF- β -producing T cells were detected in lymph nodes of any group of mice (Fig. 3*B*). CD8⁺ T cells from mice rejecting the tumor showed much higher levels of granzyme B and perforin compared with tolerant, tumor-bearing mice, exposed to ILT3 (Fig. 3*A*). These data demonstrate that both mILT3 and sILT3 energize CD4⁺ T cells, suppress the generation of CTL, and induce the differentiation of CD8⁺ Ts in tumor-invaded lymph nodes.

Sera from patients with cancer contain sILT3

The finding that sILT3 induces tolerance to allogeneic human tumors in hu-SCID mice and that it promotes the differentiation of CD8⁺ Ts cells led us to hypothesize that, if present in the circu-

lation of patients with cancer, sILT3 may also abolish T cell responses against tumor Ags.

Sandwich ELISA studies detected sILT3 in only 6% of healthy blood donors (5 of 90), whereas >40% of patients with melanoma, colorectal, or pancreatic carcinoma had ILT3 in their sera. There was also a significantly higher amount of sILT3 in patients (mean values of 170, 503, and 598 ng/ml in colorectal carcinoma, pancreatic carcinoma, and melanoma, respectively) compared with controls (mean 1.7 ng/ml) (Fig. 4*A*).

To assess the effect of serum ILT3, we tested the MLC-inhibitory activity of sera from 20 patients with colorectal and 9 with pancreatic carcinoma. For these assays, T cells from healthy individuals were stimulated with irradiated KG1 cells in cultures to which pooled sera from healthy controls or individual sera from cancer patients were added to a final concentration of 20%. Each patient’s serum was tested in triplicate cultures to which 5 μ g/ml anti-ILT3 mAb or mouse IgG (used as control) was added. Sera from cancer patients showed strong MLC-inhibitory activity that was abolished, however, by anti-ILT3 mAb, but not by control mouse IgG (Fig. 4*B*). To further substantiate the finding that serum ILT3 inhibits T cell reactivity in MLC, we selected sera containing >1000 ng/ml sILT3 and tested their inhibitory activity before and after depletion of serum ILT3 on mAb ILT3-coupled Sepharose

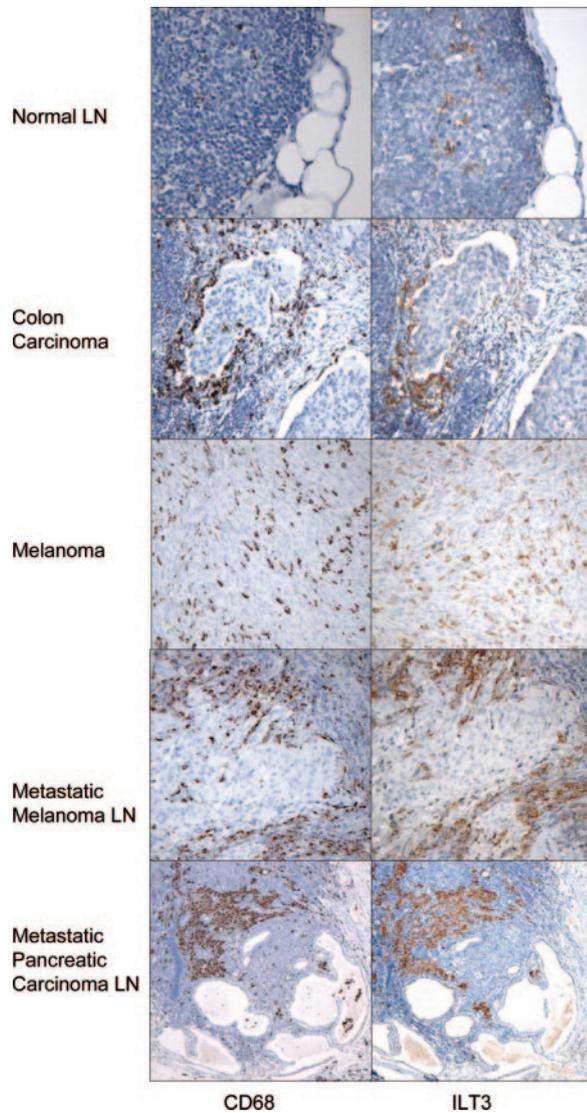


FIGURE 5. Immunohistochemical staining of macrophages with anti-CD68 and anti-ILT3 Abs in patients' tumors and lymph nodes.

columns. Complete depletion of sILT3 was demonstrated by ELISA and Western blot analysis. A representative experiment is illustrated in Fig. 4C, which shows that a sILT3-containing serum was inhibitory before, but not after, depletion of sILT3. This indicates that sILT3 is responsible, at least in part, for the capacity of sera from cancer patients to inhibit T cell reactivity in vitro.

sILT3 induces the differentiation of CD8⁺ T_s cells in humans

We next explored the possibility that similar to rILT3-Fc, serum ILT3 induces the in vitro differentiation of CD8⁺ T_s (17). For this, we stimulated CD3⁺CD25⁻ T cells from healthy responders with allogeneic APC in cultures supplemented with sera from cancer patients showing >1000 ng/ml sILT3. In parallel cultures, the same sera were used after depletion of sILT3. CD8⁺ T cells, magnetically sorted from these cultures after 7 days, were added to MLC containing unprimed CD4⁺CD25⁻ T cells from the same responder and APC from the original simulator. As illustrated in Fig. 4D, CD8⁺ T cells primed in the presence of serum containing sILT3 induced dose-dependent inhibition of T cell proliferation, whereas CD8⁺ T cells primed in ILT3-depleted serum had no T_s activity. ELISA and Western blot analysis confirmed the presence of sILT3 in serum before depletion and the removal of sILT3 after passage over Sepharose beads coated with anti-ILT3 mAbs (Fig. 4E). The molecular mass of serum ILT3 was ~45 kDa. Taken together, these data demonstrate that ILT3 contained in cancer patients' sera inhibits normal T cell reactivity to allogeneic APC and induces the differentiation of T_s.

Expression of ILT3 in tumors

Immunohistochemical studies of specimens from patients with melanoma demonstrated no ILT3 staining of tumor cells. However, strong mILT3 and cytoplasmic ILT3 staining was seen in cells surrounding and infiltrating the tumor. These cells were most likely of myeloid/histiocytic lineage because they coexpressed CD68, a macrophage marker.

Studies of archival tumor biopsies from patients with adenocarcinoma of the pancreas also revealed strong mILT3 staining of CD68⁺ macrophages. Examination of adjacent lymph nodes with no tumor cell infiltrates showed only few, weakly staining histiocytes. In sharp contrast, lymph nodes containing metastatic carcinoma demonstrated massive peritumoral infiltration by CD68⁺ histiocytes presenting intense membrane staining for ILT3. Similar observations emerged from the examination of colorectal carcinoma (Fig. 5).

Alternatively spliced ILT3 mRNAs

To test the possibility that sILT3 may be transcribed by alternatively spliced mRNA, we designed ILT3-specific primers that can amplify mRNA from either exon 3 (first Ig-like domain) or exon 4 (second Ig-like domain) to exon 7 (transmembrane domain) or exon 8 (cytoplasmic domain) for RT-PCR analysis. We used exon 4- and exon 8-specific primers to investigate possible alternative splice variants of this region of the ILT3 gene. Along with the normally spliced ILT3 PCR product (515 bp), we detected additional ILT3 bands (296 bp) in 11 of 13 tumor biopsies from colon

FIGURE 6. Identification of alternatively spliced ILT3 in tumor biopsies. Schematic representation of the structure of the ILT3 gene and of the mRNA deletion mutant. RT-PCR analysis of ILT3 mRNA in normal PBMC and tumor tissue from patients with colon carcinoma. All spliced variants were cloned and fully sequenced. Arrows indicate the position of primers; *second lane* is the negative control with no RNA added.

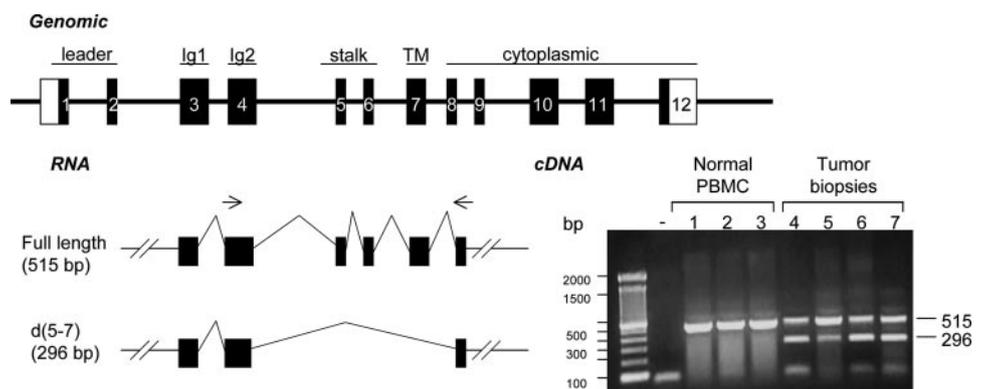
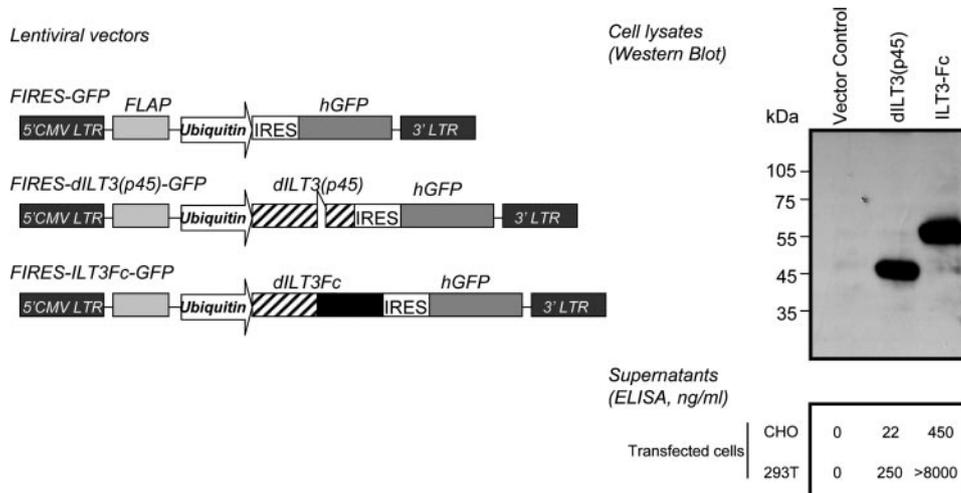


FIGURE 7. Alternatively spliced mRNA encodes sILT3 protein. Lentiviral vectors were constructed and used to transfect cloned, alternatively spliced ILT3 cDNA into CHO-S and 293T cells. Empty vector and ILT3-Fc constructs were used as controls. Western blot and ELISA analysis show that deletion of exons 5–7 from ILT3 messages results in a 45-kDa soluble form of the ILT3 protein.



adenocarcinoma patients (Fig. 6). Sequencing of the 296-bp amplified product showed the absence of exons 5–7, whereas the other exons of ILT3 were normally spliced, indicating that alternative splicing of ILT3 messages has occurred in these tumors. We proceeded to clone this ILT3 alternative splice variant in CHO-S and 293T cells and showed that these transfectants expressed sILT3. The sILT3 protein was detected both in the cell lysates and culture supernatants of the transfected cells, and Western blot assays showed it to be of the same size (~45 kDa) as that identified in cancer patients' sera (Fig. 7). This experiment proves unambiguously that the splicing variants encode sILT3, although it does not allow us to discriminate between the possibility that it is produced by tumor cells with ectopic expression of cytoplasmic ILT3 or by macrophages with high expression of mILT3.

Discussion

There is an emerging recognition that tumor growth elicits specific immune responses mediated by CD8⁺ and CD4⁺ T cells that may delay progression and be potentially harnessed to eradicate malignant disease (reviewed in Ref. 10). T cell-based immunotherapy has recently attracted much attention largely due to its success in experimental animal models and to the identification of tumor-associated Ags (TAA) in certain malignancies (10, 20). Because activated DC are highly efficient in stimulating immune responses, numerous clinical studies have used DC loaded in various ways with TAA (DC vaccines) to induce CD8 and CD4 T cell responses against complexes formed by TAA-derived peptides with MHC class I and class II molecules (reviewed in Ref. 22). However, most immunotherapy trials have met with limited success, failing to demonstrate significant clinical responses (22, 23). It is believed that combination immunotherapy such as adoptive transfer of *in vitro* primed T cells and subsequent vaccination may foster enhanced memory T cell responses (24, 25).

Potential mechanisms responsible for tumor escape from immunosurveillance include dysfunction of Th and CTL, expansion of CD4⁺CD25⁺ Treg cells, loss or down-regulation of HLA class I Ags or tumor-specific differentiation Ags, defective signaling through death receptor ligands such as FAS ligand and TRAIL, lack of appropriate costimulation by immature or tolerogenic DC, and production of immunosuppressive cytokines by tumor cells or infiltrating APC (26–29).

In this study, we describe the inhibitory effect of serum and mILT3, which may represent an additional mechanism that contributes to impaired T cell responses in patients with cancer. The experiments, which we describe, are an outgrowth of our previous

investigations in which we demonstrated that mILT3 and sILT3 (ILT3-Fc) induce T cell anergy and promote the differentiation of Ag-specific CD8⁺ T cells *in vitro*. Because no ILT3 ortholog has been found in rodents, we elected to use a hu-SCID mouse allotransplantation model. As a first attempt to find out whether ILT3 acts as an immunomodulatory molecule *in vivo*, we transplanted allogeneic tumors in hu-SCID mice. We found that hu-SCID mice rejected a wide variety of such tumor transplants, including myelomonocytic leukemic cells (KG1), pancreas carcinomas, and melanomas. Tumor allograft rejection was inhibited, however, by mILT3 and sILT3. Tumors grew faster and reached larger sizes in KG1-transplanted animals treated with sILT3 than in KG1.ILT3-grafted hu-SCID mice, probably because of the systemic, early, and continuous access of sILT3 to human T cells. Because engraftment of CD3⁺ T cells, but not of NK cells and macrophages was detected, it is apparent that tumor rejection was caused by CD3⁺ T cells. This conclusion is supported by the finding that both CD4 and CD8 T cells from lymph nodes draining the site of tumor rejection produced IL-2 and IFN- γ . In addition, CD8⁺ T cells from these lymph nodes expressed high levels of granzyme B and perforin. Although CD8⁺ T cells acquired suppressor function in tumor-bearing hu-SCID mice, CD4⁺ T cells from the same animals had no regulatory activity.

This finding is consistent with our previous *in vitro* studies that showed that CD4⁺ T cells allostimulated in the presence of sILT3 or mILT3 became anergic and thus unable to provide the help required for functional differentiation of IFN- γ -producing CD8⁺ CTL (17). Instead, alloactivated CD8⁺, but not CD4⁺ T cells from these cultures, differentiated into Ts, which acted in an allorestricted manner on priming APC, inducing them to up-regulate the inhibitory ILT3 receptor (17).

The finding that sILT3 inhibits the rejection of allogeneic human tumors led us to hypothesize that, if present in sera from patients with cancer, ILT3 may have a similar effect on T cell responses to autologous tumors even if these express immunogenic TAA. This hypothesis is supported by several lines of evidence. First, sera from cancer patients were shown to inhibit the *in vitro* reactivity of autologous or allogeneic T cells in MLC and PHA stimulation assays (30, 31). Second, elevated serum levels of IL-10 (a documented ILT3 inducer) (13) were also shown to correlate with poor clinical outcome (32). Finally, soluble forms of membrane-bound molecules, such as NKG2D ligands, which can potentially impair NKG2D-mediated immune function by blocking NKG2D receptors on NK and T cells, were also found in cancer sera, contributing to their inhibitory activity (33–35).

Our present study shows that sILT3, present in sera from a relatively high percentage of patients with various malignancies, strongly inhibits T cell responses in MLC. This inhibitory effect was partially abrogated by anti-ILT3 mAb or by depletion of ILT3 from the serum, substantiating the hypothesis that ILT3 is indeed the suppressive factor. Furthermore, T cell allostimulation in cultures containing sILT3⁺ sera from cancer patients resulted in the differentiation of allospecific CD8⁺ T cells. Taken together our findings that: 1) mILT3 and sILT3 inhibit tumor allograft rejection in hu-SCID mice; 2) sera from cancer patients contain large amounts (up to 10,000 ng/ml) of ILT3, inhibit T cell reactivity in MLC, and induce the differentiation of Ts in such cultures, suggest that ILT3 may inhibit patients' T cell reactivity to TAA expressed by autologous tumors and presented by professional APC.

There is evidence that successful immunotherapy and effective vaccination are hampered by the immunosuppressive activity of regulatory CD4⁺CD25⁺ T cells, which contribute to tumor evasion from immune surveillance (3, 18, 36). Stage-related increases in the frequencies of CD4⁺CD25⁺ Treg cells were found in numerous malignancies supporting this hypothesis (reviewed in Refs. 10 and 20).

CD8⁺ T cells that act in an Ag-specific manner, inhibiting the T cell-priming capacity of APC or which display their regulatory activity by producing IL-10 (similar to CD4⁺CD25⁺ Tr1 cells), have been described both in humans and rodents (3, 11, 12, 37–42). Their contribution to tumor escape from immunosurveillance, however, has received less attention, although the high frequency of noncytolytic TAA-specific CD8⁺ T cells in patients with metastatic melanoma may reflect the presence of Ts rather than of CTL effector cells (43, 44).

Our present results suggest that mILT3 and sILT3 could induce T cell anergy and promote the differentiation of CD8⁺ Ts within the tumor microenvironment or in sentinel lymph nodes. We found intensive mILT3 staining of tumor-associated CD68⁺ macrophages in colorectal and pancreatic carcinomas as well as in melanoma. The frequency of ILT3-expressing macrophages in tumor-infiltrated lymph nodes was much higher than that seen in noninvaded lymph nodes. There is evidence that cytokines present in the tumor microenvironment have the potential to induce the differentiation of recruited macrophages into tumor-associated macrophages (TAMs) that have an M2 phenotype and poor Ag-presenting capacity, and produce growth factors and extracellular matrix enzymes, facilitating tumor proliferation and invasion of surrounding tissue (45, 46). Recently, a population of TAMs, characterized by B7-H4 expression and the capacity to suppress TAA-specific T cell immunity, was identified in human ovarian carcinoma (47). In such carcinomas, plasmacytoid DC that express ILT3 constitutively (3, 40) induce the differentiation of CD8⁺ Treg cells (48). It is, therefore, possible that ILT3-high TAMs suppress T cell reactivity to the TAA that they process and present.

Ectopic expression of ILT3 by tumor cells, as recently found in chronic lymphocytic leukemia, may render tumor cells tolerogenic and provide an alternative source of both mILT3 and sILT3 (49). We cannot discriminate, however, between the possibility that Ts induce up-regulation and release of ILT3 from TAM or, alternatively, that tumor- or TAM-secreted ILT3 binds to the ILT3 ligand on activated CD4 T cells, rendering them anergic and, thus, unable to sustain the differentiation of CD8⁺ CTL (17).

Different mechanisms may account for the presence of sILT3 in patients' circulation. Our data are consistent with the possibility that sILT3 found in patients' sera is secreted by TAM expressing alternatively spliced variants of ILT3 that lack the transmembrane domain, as also shown to be the case for other immunoregulatory receptors (50). An alternative, not mutually exclusive mechanism

is that sILT3 production is associated with posttranslational proteolytic cleavage (33, 51) as in the case of NKG2D ligand. The possibility that ILT3 is released from APC is also suggested by our observation that a relatively high proportion of tumor-free patients with chronic infections such as hepatitis C and/or HIV displays serum ILT3 in conjunction with elevated levels of IL-10 (52, 53) (N. Suci-Foca, unpublished observations).

Importantly, in addition to IL-10, certain immunoregulatory agents such as IFN- α and calcitriol, the most active natural metabolite of vitamin D, which enhances ILT3 expression in DC, rendering them tolerogenic (13, 54), have been shown to display antineoplastic activity. The antineoplastic activity of Calcitriol occurs via different mechanisms, including inhibition of proliferation, induction of apoptosis and cell differentiation, reduction of invasiveness, and inhibition of angiogenesis (55, 56). Similarly, IFN- α has been used with some success in treatment of renal cell carcinoma (57). This cytokine, however, is produced primarily by plasmacytoid DC that express ILT3 constitutively and induce the differentiation of IL-10-secreting CD8⁺ T cells (38, 58). Thus, certain agents may display antineoplastic activity, despite their immunosuppressive potential. This implies that the progression of malignancies depends on the delicate balance between the patients' immunological competence and the aggressiveness of the tumor. The design of clinical protocols using potentially immunosuppressive agents should be carefully assessed to ensure their safety and efficacy for cancer therapy (55).

Cancer immunotherapy attempts to harness the exquisite power and specificity of the immune system for the treatment of malignancy. Attempts to manipulate the immune response rely on enhancement of T cell immunity by stimulation with immunogenic vaccines, adoptive therapy using tumor-specific CTL, and modification of host environment to improve the homeostatic expansion of infused T cells or to eliminate Treg cell subsets. However, all of these approaches may fail in the inhibitory milieu created by sILT3, which may paralyze T cell responses. Plasmapheresis with immunoabsorption of serum ILT3 may be necessary as a preliminary step before active or passive (adoptive) immunotherapy is initiated in patients with cancer. Identifying and blocking the ligand of ILT3 on activated T cells (17) may offer new strategies to enhance T cell immunity in cancer. In contrast, ILT3 may prove to be a valuable tool for suppressing T cell responses in transplantation and autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

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