THE JOURNAL OF IMMUNOLOGY

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J Immunol 2010;185;5714-5722; Prepublished online 8 October 2010;
doi:10.4049/jimmunol.1001732
http://www.jimmunol.org/content/185/10/5714

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

BCL6 Is Required for Differentiation of Ig-Like Transcript 3-Fc–Induced CD8$^+$ T Suppressor Cells

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Ig-like transcript 3 (ILT3) is an inhibitory receptor expressed by tolerogenic dendritic cells. When human CD8$^+$ T cells are allostimulated in the presence of recombinant ILT3-Fc protein, they differentiate into antigenic specific T suppressor (Ts) cells that inhibit CD4 and CD8 T cell effector function both in vitro and in vivo. ILT3-Fc–induced CD8$^+$ Ts cells express high amounts of BCL6 that are crucial to their function. Knockdown of BCL6 from unprimed human T cells prevents their differentiation into Ts cells, whereas ex vivo overexpression of BCL6 converts CD8$^+$ T cells into Ts cells. NOD/SCID mice transplanted with human pancreatic islets and humanized by injection of human PBMCs tolerate the graft and develop BCL6^{high} CD8$^+$ Ts cells when treated with ILT3-Fc before or after the onset of rejection. This indicates that ILT3-Fc acts through BCL6 and is a potent immunosuppressive agent for reversing the onset of allo- or possibly autoimmune attacks against pancreatic islets. ***The Journal of Immunology*****, 2010, 185: 5714–5722.**

Pancreatic islet transplantation is a potential cure for type 1 diabetes (T1D), an autoimmune disease of increasing frequency in the population. However, traditional immunosuppressive drugs do not secure insulin independence after transplantation because they have toxic effects on the islets and/or fail to prevent rejection or recurrence of autoimmune damage to the graft (1–3).

The immune response against self (autologous) and nonself (allogeneic) pancreatic islets is mediated by Ag-specific T cells. The possibility of preventing the onset and progression of the immune attack against autologous or allogeneic islets by adoptive transfer of T regulatory (Treg) cells has been the subject of numerous studies. However, adoptive transfer of ex vivo expanded autologous Treg cells from patients with autoimmune diseases may be inefficient because of their reduced numbers and/or functional defects associated with the disease per se (4, 5).

In recent years, it has become apparent that T cells have a great degree of functional plasticity that is dictated by the microenvironment in which they become activated, as well as by the functional state of the APCs with which they interact. The flexibility of activated effector cells may permit their conversion into T suppressor (Ts) cells by manipulating the signals that they receive (6, 7). Agents inducing such changes may facilitate the development of immunological tolerance.

In previous studies, we demonstrated that allostimulation of T cells in the presence of membrane or soluble Ig-like transcript 3 (ILT3) induces the differentiation of CD8$^+$ Ts both in vitro and in vivo (8–10). ILT3 (also known as LILRB4, LIR5, or CD85k) is an inhibitory receptor that is structurally and functionally related to killer cell inhibitory receptors. The cytoplasmic region of ILT3 contains ITIMs that inhibit cell activation by recruiting the tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 (11, 12). The extracellular Ig-like domain of ILT3 binds to T cells, shaping their functional differentiation into Ts/Treg cells (8–10, 13–15).

Upregulation of ILT3 on the membrane of APCs renders them tolerogenic to T cells with cognate specificity, eliciting the differentiation of Ag-specific Ts/Treg cells (13–15). Similarly, tolerogenic APCs are generated by exposure to IL-10, IFN-α, IFN-β, or vitamin D3 receptor agonists that induce the upregulation of ILT3 (13–16). Although ILT3 expression by dendritic cells (DCs) is required for induction of Treg cells, DC pretreatment with vitamin D3 receptor agonist leads to induction of Treg cells irrespective of the presence of neutralizing anti-ILT3 mAb, indicating that ILT3 expression is dispensable for the capacity of 1,25 (OH_2D_3)-treated DCs to induce CD4$^+$ FOXP3$^+$ T cells (17). As a corollary of its tolerogenicity, silencing of ILT3 expression in APCs increases synthesis of proinflammatory cytokines and chemokines, enhancing their capacity to activate IFN-γ– and IL-17–secreting T effector cells (18). The extracellular domain of ILT3 also displays intrinsic immunosuppressive activity. Soluble ILT3, engineered as an ILT3-Fc fusion protein, was shown to induce tolerance to allogeneic human pancreatic islet transplants in humanized NOD/SCID mice (hu-NOD/SCID) (10).

Because autoimmune diseases like T1D are usually diagnosed after their onset, we sought to determine whether the destruction of islets by effector T cells can be blocked by treatment with ILT3-Fc. We used hu-NOD/SCID mice transplanted with allogeneic islets

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Received for publication May 25, 2010. Accepted for publication September 3, 2010.

This work was supported by Juvenile Diabetes Research Foundation Grant 1-2009-550.

The sequences presented in this article have been submitted to the Gene Expression Omnibus Web site under accession number GSE23922.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: DC, dendritic cell; hu-NOD/SCID, humanized NOD/SCID; ILT3, Ig-like transcript 3; MS, mass spectrometry; shRNA, short hairpin RNA; T1D, type 1 diabetes; Rej, Rejection; Tol, Tolerance; Treg, T regulatory; Ts, T suppressor; UNTR, untreated.

as a model, allowing T cell allosensitization and onset of rejection to occur before we started treatment with ILT3-Fc. This model is based on the hypothesis that activated $CD8^+$ T cells have a degree of functional flexibility that may permit the conversion of CTL precursors into Ts cells.

To better understand the mechanism of action of ILT3-Fc, we explored the molecular changes that it induces in $CD8^+$ T cells that acquire suppressor function. We now report that ILT3-Fc restores quiescence in 70% of mice treated after the onset of rejection, and that tolerance is mediated by ILT3-Fc–induced $CD8^+$ Ts cells that express high amounts of the transcriptional repressor BCL6.

Materials and Methods

Cell isolation

PBMCs were obtained from fresh buffy coats (New York Blood Center, Long Island City, NY) by gradient centrifugation. $CD3^+$ T cells were obtained by selective depletion of CD14, CD19, CD56, CD16, CD36, CD123, and glycophorin A-positive cells using CD3 isolation kits (Miltenyi Biotec, Auburn, CA). $CD25^+$ T cells were depleted from $CD3^+$ T cell suspensions. $CD4^+$ or $CD8^+$ T cells were magnetically sorted from $CD3^+$ T cells to a purity of 93–98% using CD4 or CD8 isolation kits (Miltenyi Biotec) for negative selection (8, 10).

ILT3-Fc protein

ILT3-Fc protein expressed and purified as previously described was analyzed by gel elecrophoresis and mass spectrometry (MS). MALDI and liquid chromatography-MS/MS analysis of tryptic digests showed no contaminants (8, 10).

Gene chip transcriptional profiling of Ts cells

Ts cells were generated from different donors by priming $CD3^+CD25^-$ T cells for 7 d with CD2-depleted allogeneic APCs in the presence or absence of ILT3-Fc (8). On day 7, cultures were restimulated for 4 h with the priming APCs. Magnetically sorted $CD8^+$ T cells from the ILT3-treated and nontreated counterparts were tested for Ts activity. Five different ILT3-Fc–treated T cell lines that inhibited the MLC response of autologous T cells by >80% at a 1:1 responder:suppressor ratio were selected for microarray studies. The untreated counterpart, which contained no Ts, was included as a control.

Total RNA was prepared from cells using the Absolutely RNA extraction kit (Stratagene, La Jolla, CA). cDNA obtained from 1 μg total RNA was used directly for synthesis and labeling of cRNA using Quick-Amp labeling kit and Cyanine CTP 2 color dyes (Agilent Technologies, Palo Alto, CA). The amount of cRNA synthesized was determined by a spectrophotometer (Model Ultraviolet-1700; Shimadzu Scientific Instruments, Columbia, MD). The quality of RNA was controlled using a Bioanalyzer (Agilent Technologies). Equal amounts (0.8 μg) of two different color cRNAs (CY3, ILT3 Fc treated; CY5, control) were spiked with the same color-labeled control RNA (adenoviral E1A) and hybridized to an array of 44,000 unique genes that were spotted on a glass slide (Agilent Technologies). After 16 h, slides were scanned with a computer-assisted scanner and analyzed.

Microarray images were analyzed with Feature Extraction Software (version 10.5; Agilent Technology) (19). Normalization was performed with limmaGUI, which is part of the Bioconductor package and runs under the *R* statistical computing environment. Raw intensities were normalized using the within-slides global lowess protocol without background correction, which is optimal for Agilent 2 color arrays. Differential expression was analyzed using Linear Models for MicroArrays. A cutoff of the Bayesian log-odds of differential expression, $B > 0$, corresponding to a p value of 0.001 and a Benjamini–Hochberg false discovery rate of 0.1 was used to consider genes for further cutoff and validation. The gene profile results were deposited online at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE23922. Both semiquantitative and real time RT-PCR were used to validate the microarray results for selected genes.

Real-time PCR

Total RNA was isolated with RNAqueous-4PCR kit (Stratagene). cDNA was synthesized using the first-strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Basel, Switzerland). Real-time PCR was performed using proprietary TaqMan gene expression probes (Applied Biosystems, Foster City, CA). Data were collected and analyzed with 7300 SDS 1.3.1 software (Applied Biosystems). The gene expression relative to the housekeeping gene GAPDH was calculated using the formula 2^{-ddCt}, where $ddCt = (Ct[\text{gene}] - Ct[\text{GAPDH}])$ and Ct is the crossing threshold value returned by the PCR instrument for every gene amplification.

Flow cytometry

Flow cytometry studies were performed on a FACSCalibur instrument using five-parameter acquisition (BD Biosciences, San Jose, CA). The following mAbs were used: anti–HLA-ABC, CD3, CD4, CD8, CD14, CD19, CD56, CD45, CD40, CD80, CD86 (all from BD Biosciences), and ILT3 (Beckman Coulter, Fullerton, CA). All stainings were performed at 4°C. For each marker, a corresponding isotype-matched Ab conjugated with the same fluorescent dye was used as a negative control.

For T cell proliferation assays, responding $CD4^+$ T cells were labeled with 5 μM CFSE (Invitrogen, Carlsbad, CA) for 10 min at room temperature, then washed and coincubated at a 2:1 ratio with irradiated allogeneic CD2-depleted APCs. ILT3-Fc–induced $CD8^+$ Ts cells or alloactivated $CD8^+$ T cells transfected with BCL6 or empty vector were added to the cultures at a 1:1 CD8 to CD4 T cell ratio. On day 6, cells were stained with anti-CD4 mAb, and cell division patterns, reflected by CFSE dilution, were examined on a FACSCalibur flow cytometry instrument (BD Biosciences).

To study modulation of ILT3 and costimulatory molecule expression on APCs, monocytes were incubated with $CD8^+$ Ts, with and without CD40L-transfected Jurkat cells from the D1.1 line. After 24 h, cells were washed and stained for FACS analysis (13, 20).

Proliferation assays

Primary MLCs were performed using responding $CD4^+CD25^-$ T cells (5×10^4 cells/well) and allogeneic CD2-depleted stimulating APCs (2.5×10^4 cells/well) irradiated with 3000 rad.

For suppressor cell assays, $CD8^+$ Ts or control $CD8^+$ T cells were added to primary MLCs containing unprimed, autologous $CD4^+$ T cells and APCs from the same stimulator used to generate Ts cells. Cultures were labeled with [^{3}H]thymidine on day 5 and harvested 18 h later. The percentage of inhibition of CD4 T cell proliferation by $CD8^+$ Ts cells was calculated according to the following formula: $(1 - \text{cpm [Th + Ts + APCs]}/\text{cpm [Th + APCs]}) \times 100$.

Induction of Ts cells by ILT3-Fc treatment

$CD3^+CD25^-$ T cells (1×10^6/ml) were cultured with irradiated CD2-depleted PBMCs (0.5×10^6/ml) in the presence or absence of ILT3-Fc protein (50 μg/ml). On day 7, $CD8^+$ T cells were negatively selected and tested for suppressor activity.

Induction of Ts cells by BCL6 transfection

Adenoviral vector encoding BCL6 was constructed by first cloning full-length BCL6 cDNA into pENTR/D-TOPO vector of Gateway system (Invitrogen) using 5′-CACCATGGCCTCGCCGGCTGAC-3′ and 5′-TCCGCAGGCTTTGGGGAGCTCCG-3′. The recombinant intermediate vector was then recombined with an adenoviral vector pAd/CMV/V5-DEST (Invitrogen). The stop codon (TGA) of BCL6 was replaced with a glycine codon (GGA) to allow BCL6 transcription in frame with a C-terminal V5 tag. All intermediate vectors and adenoviral vectors containing BCL6 DNA inserts were confirmed by sequencing.

Recombinant adenoviral vectors were transfected into 293A cells using lipofectin 2000 (Invitrogen). Adenoviral viruses collected from culture supernatant were amplified and added to cultured cells at 1:4 ratio (v/v). Expression of exogenous BCL6 was confirmed by Western blot analysis using both BCL6 and V5 Abs.

$CD3^+CD25^-$ T cells were allostimulated as above in primary MLCs without ILT3-Fc. On day 7, adenoviral vector encoding BCL6 or vector alone was added to the cultures. $CD8^+$ T cells were negatively sorted from these cultures after 48 h of incubation and tested for suppression of primary MLCs, as above.

Prevention of Ts generation by knockdown of BCL6

To generate BCL6 short hairpin RNA (shRNA) viral vectors, a nucleotide sequence (5′-GCAGTAAGAATGCCTGCATCC-3′) corresponding to position of 1046–1066 of BCL6 cDNA was selected according to BLOCK-iT RNAi Designer (Invitrogen). A double-stranded oligonucleotide containing this sequence was first cloned into a shuttle vector pENTR/U6 (Invitrogen). Efficiency of BCL6 knockdown was determined in 293T cells cotransfected with this vector (pENTR/U6-BCL6^{kd1046}) and pMT2T-BCL6 (21) by quantitative RT-PCR. This vector was subsequently recombined

with an adenoviral vector, Adeno/block-it-DEST (Invitrogen), according to manufacturer's protocol. As a control, we used the adenoviral empty vector pAd-RNAi^{con}, which contains a U6 RNA polymerase promoter and nucleotide sequence of AAAAA (18) and, as an off-target control, the adenoviral vector pAd-RNAi^{LacZ}, which comprises the sample RNA polymerase promoter and the nucleotides of bacterial lacZ gene (Invitrogen). All intermediate vectors and the adenoviral vector containing the hairpin oligomer were confirmed by sequencing. Production and use of shRNA adenoviral vectors were previously described (18).

PBMCs were cultured with BCL6 shRNA viral vectors for 48 h. Efficiency of knockdown was tested by RT-PCR. CD8$^+$ T cells were isolated from the cultures and coincubated with unprimed autologous CD4$^+$ T cells and allogeneic APCs at a 1:1:0.5 ratio, in the presence or absence of ILT3-Fc (50 μg/ml). After 7 d, CD8$^+$ T cells were negatively sorted from these cultures and tested for Ts activity in primary MLCs, as described above.

Animals

NOD/SCID female mice purchased from Charles River Laboratories (Wilmington, MA) were used at 6–10 wk of age. All protocols involving animal care procedures were approved by the Columbia University Institutional Animal Care and Use Committee. The animals were kept in microisolator cages and were fed autoclaved food and water. Diabetes was induced by i.v. injection of streptozotocin (Sigma-Aldrich, St. Louis, MO) at a dose of 180 mg/kg. Blood glucose level was measured twice per week using Ascensia Elite XL Blood Glucose Meter (Bayer AG, Leverkusen, Germany). Diabetes was diagnosed after two consecutive glucose measurements >350 mg/dl.

Generation, transplantation, and treatment of hu-NOD/SCID mice

Aliquots of 1500 human pancreatic islet equivalents with >70% purity and >90% viability were transplanted under the kidney capsule of NOD/SCID mice rendered diabetic by streptozotocin injection, as described before (10). Seven to 10 d posttransplantation, mice that were restored to euglycemia (glucose level <100 mg/dl) were humanized by i.p. injection of 50 × 10^6 PBMCs, isolated from fresh buffy coats purchased from the New York Blood Center. Treatment with ILT3-Fc was initiated on days 0, 15, or 20 after PBMC injection and consisted of 10 consecutive i.p. injections of ILT3-Fc at 250 μg/day. An additional control group of NOD/SCID mice was humanized and transplanted, as above, but received no treatment (i.e., saline only). Ten days after humanization, circulating human T cells were evaluated by flow cytometry using heparinized retro-orbital venous samples. Animals were sacrificed when they rejected the graft, becoming diabetic, or if normoglycemic, on day 100 posthumanization, at the termination of the experiment. Animals failing to reconstitute (<5% human CD45$^+$ PBMCs in the circulation) or developing graft-versus-host disease (hunched back, lethargy, weight loss, and tachypnea) were excluded from analysis by design and sacrificed.

HLA typing

HLA genotypes of human PBMCs and pancreatic islets were determined by PCR with sequence-specific primers from One Lambda (Canoga Park, CA).

Histology and immunochemistry

Twenty serial paraffin sections of islet-transplanted kidneys were cut at 4 μm thickness. Levels 1, 10, and 20 were stained for light-microscopic evaluation (H&E). The remaining sections were used for immunostains, including insulin and CD8 (DakoCytomation, Carpinteria, CA). Islet quantity and islet inflammatory infiltration (insulitis) were graded semiquantitatively in blinded fashion by a renal pathologist on a scale of 0 to 3$^+$. The degree of islet inflammation by CD8$^+$ T cells was graded according to the number of CD8 per ×40 high-power field: 0 (none), 1$^+$ (1–10), 2$^+$ (11–25), and 3$^+$ (>25). The results were averaged over at least five high-power fields per slide.

Measurement of suppressor activity of human T cells from islet-transplanted animals

Human CD8$^+$ or CD4$^+$ T cells obtained from the spleens of islet-trans planted, hu-NOD/SCID mice, treated or nontreated with ILT3-Fc, were incubated ex vivo at an 8:1 ratio with autologous unprimed CD4$^+$ T cells (1 × 10^4/well) and APCs (0.5 × 10^4/well), sharing at least one HLA class I and class II Ag with the transplanted islets. Supernatants were collected after 48 h and tested for cytokine contents using the human Th1/Th2 cytometric bead array kit (BD Biosciences). Following incubation with supernatants or cytokine standards, capture beads were washed and analyzed on a FACSCalibur flow cytometry instrument. T cell proliferation was measured on day 6 by tritiated thymidine incorporation, as described above.

Statistical analysis

Graft survival curves were computed using the Kaplan-Meier method. Differences between groups were compared by the log-rank test. Parametric and nonparametric data were statistically compared using the two-tailed Student *t* test and Mann-Whitney *U* test, respectively.

Results

ILT3-Fc–induced Ts cells express BCL6

To identify molecular changes underlying the acquisition of suppressor function by T cells that have been primed in the presence of ILT3-Fc, we stimulated CD3$^+$CD25$^-$ T cells, from five different responders, in individual MLC, with APCs from allogeneic stimulators. ILT3-Fc (50 μg/ml) was added at the initiation of the cultures, whereas the replicate control cultures from the same responder/stimulator pair contained no ILT3-Fc. On day 7, T cells were harvested, washed, and restimulated with APCs from the priming stimulator. CD8$^+$ T cells were sorted from these five cultures after 4 h for gene profile analysis. Differences >2-fold between mRNA expression in ILT3-Fc–treated cultures and their untreated counterparts were analyzed. Gene ontology categories in which ILT3-Fc treatment induced significant modulation of gene expression in CD8$^+$ T cells are shown in Supplemental Table I. ILT3-Fc was found to inhibit expression of IFN-γ, IL-2, IL-5, and granzyme B, consistent with our previous studies (8–10). There was a striking increase in the expression of transcription factors belonging to a class of zinc finger transcriptional repressors, including BCL6, and of the chemokine receptor CXCR4, involved in migration and homing of lymphocytes (22, 23). Expression of these molecules was confirmed by real-time PCR analyses (Fig. 1). There was no difference between ILT3-Fc–induced Ts and control cultures in the expression of Blimp-1, a transcriptional antagonist of BCL6.

Previous reports showed that BCL6 represses the expression of IFN-γ (24), IL-17 (25, 26), IL-5 (27, 28), and granzyme B (29) and promotes the expression of CXCR4 (24, 26).

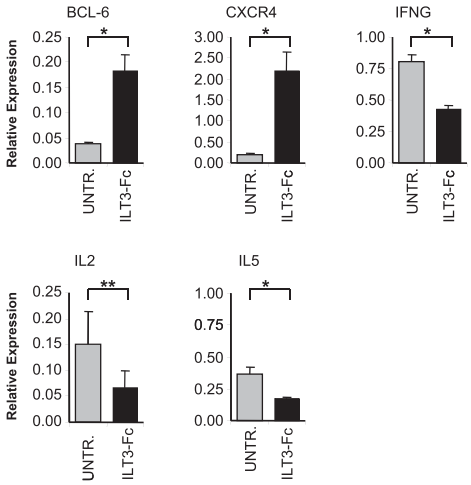

FIGURE 1. Real-time PCR analysis of selected genes in CD8$^+$ T cells allostimulated in the presence or absence of ILT3-Fc. Expression of each gene was assessed by the crossing threshold method and was normalized by GAPDH expression. Means ± SEM obtained from five independent experiments are represented. Statistical significance is indicated by $*p < 0.001$; $**p < 0.01$ (Student *t* test). ILT3-Fc, ILT3-Fc–treated cultures. UNTR, untreated.

Our finding that ILT3-Fc induces upregulation of BCL6 in conjunction with upregulation of CXCR4 and repression of IFN-γ, IL-5, and granzyme B in allospecific $CD8^+$ Ts cells suggested that BCL6 is involved in Ts-mediated suppression.

Alloactivated $CD8^+$ T cells transfected with BCL6 acquire suppressor function

To determine when ILT3-Fc affects the expression of BCL6 in $CD8^+$ T cells, we performed a time-point analysis. T cells were allostimulated for 7 d in MLCs in the presence or absence of ILT3-Fc. In $CD8^+$ T cells, BCL6 was downregulated for the first 5 d and increased slightly thereafter in the absence of ILT3-Fc. In $CD8^+$ T cells from cultures with ILT3-Fc, the expression of BCL6 decreased for 24 h and then increased by day 7 to levels 7 times higher than those seen in cultures without ILT3-Fc (Fig. 2*A*).

Hence, the maximal increase occurred on day 7 at a time known to correspond to that required for $CD8^+$ Ts differentiation from T cells allostimulated in the presence of ILT3-Fc (8). Kinetic studies of Blimp-1 mRNA expression showed no differences between ILT3-Fc–treated and nontreated $CD8^+$ T cells (data not shown).

To determine whether BCL6 is essential to the suppressor function acquired by allostimulated $CD8^+$ T cells, we primed $CD3^+$ T cells to allogeneic, CD2-depleted APCs. On day 7, cultures were infected with an adenovirus vector containing BCL6 or an empty vector. $CD8^+$ T cells sorted from these cultures after 48 h were transferred to primary MLCs containing autologous $CD4^+$ T cells and APCs from the original stimulator. T cell proliferation measured 6 d later was inhibited by 56 ± 8% in the presence of BCL6-transfected $CD8^+$ T cells and 5 ± 3% when control-alloactivated (untransfected) or vector-transfected $CD8^+$ T cells were added to the cultures. ILT3-Fc–induced $CD8^+$ Ts cells from the same responder/stimulator combination, tested concomitantly in separate cultures, inhibited the primary MLC response by 85 ± 7% (Fig. 2*B*, 2*C*). The capacity of ILT3-Fc–induced and BCL6-transfected $CD8^+$ T cells to suppress autologous $CD4^+$ T cell responses in MLCs was confirmed in five different experiments. Fig. 2*C* shows the cpm in one representative experiment. Comparison of BCL6 expression in ILT3-Fc–induced and BCL6-transfected T cells indicated relatively similar levels of expression (Fig. 2*D*).

Similar results revealing inhibition of proliferation were obtained in three experiments when unprimed $CD4^+$ T cells were labeled with CFSE and stimulated with allogeneic APCs in the presence of $CD8^+$ Ts cells generated as above. One representative experiment is shown in Fig. 2*E*.

These experiments support the concept that overexpression of BCL6 accounts, at least in part, for the suppressor function acquired by ILT3-Fc–treated $CD8^+$ T cells.

BCL6-transfected $CD8^+$ T cells induce tolerogenic APCs

A characteristic feature of allospecific $CD8^+$ Ts cells resides in their capacity to render APCs tolerogenic, inducing the upregulation of ILT3 (13) and downregulation of costimulatory molecules, upon cell-to-cell contact (20, 30, 31).

To further substantiate the hypothesis that BCL6 is involved in the acquisition of Ts function, we tested the capacity of $CD8^+$ T cells, primed under the conditions described above (i.e., before or after ILT3-Fc induction or BCL6 transfection), to modulate the expression of ILT3, CD40, CD80, and CD86 on the membrane of

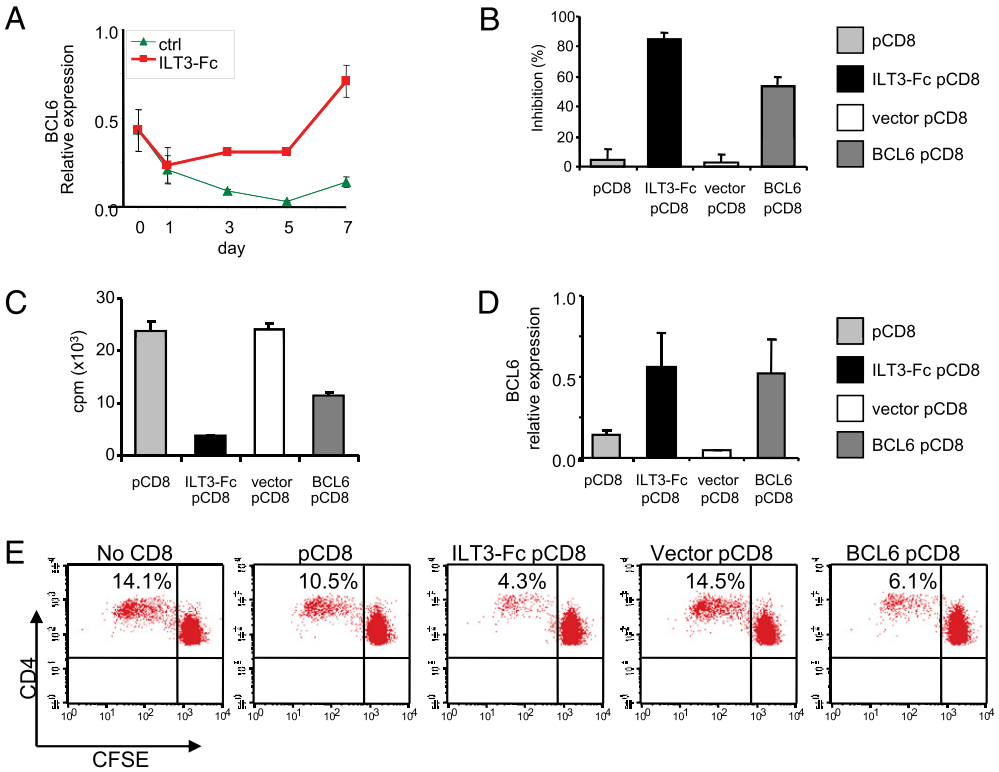

FIGURE 2. $BCL6^{high}$ $CD8^+$ T cells display suppressor function. *A*, Kinetics of BCL6 expression in CD8 T cells from MLCs with and without ILT3-Fc, measured by real-time PCR. *B*, Inhibition of CD4 T cell proliferation in the presence of primed CD8 T cells (pCD8), ILT3-Fc–primed CD8 (ILT3-Fc pCD8), empty vector-transfected primed CD8 (vector pCD8), and BCL6-transfected primed CD8 (BCL6 pCD8). *C*, [^{3}H]thymidine incorporation in CD4 T cells from an MLC to which pCD8, ILT3-Fc pCD8, vector pCD8, or BCL6 pCD8 was added (cpm ± SD of triplicate reaction). *D*, Level of BCL6 expression in pCD8, ILT3-Fc pCD8, vector pCD8, and BCL6 pCD8, measured by real-time PCR. *E*, Flow cytometric analysis of CFSE-labeled CD4-gated T cells stimulated in 6-d cultures with priming APCs in the presence of pCD8, ILT3-Fc pCD8, vector pCD8, and BCL6 pCD8, or without CD8 T cells.

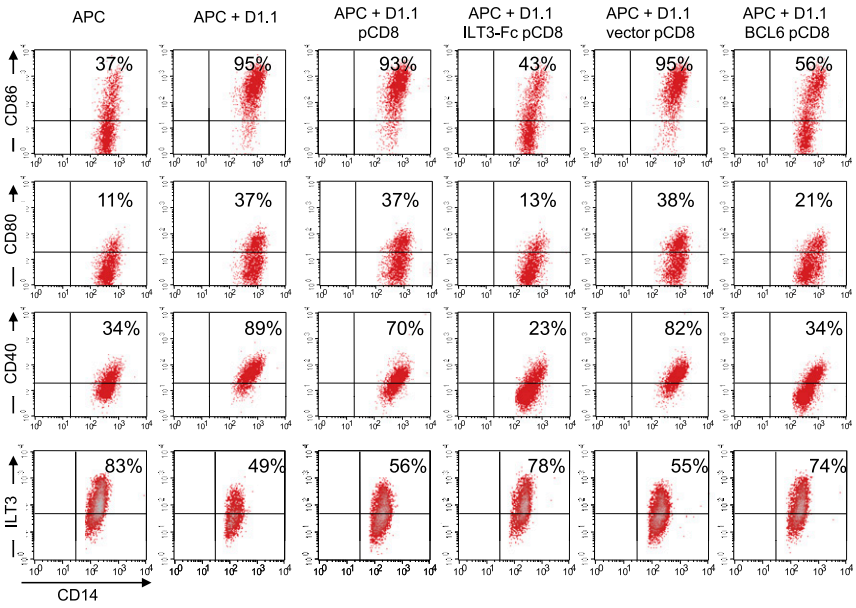

FIGURE 3. Modulation of CD86, CD80, CD40, and ILT3 expression on CD40L-triggered monocytes by CD8 Ts cells. Monocytes were incubated with CD40L-transfected D1.1 cells and pCD8, ILT3-Fc pCD8, vector pCD8, BCL6 pCD8, or no CD8 T cells for 24 h. Expression of cell surface molecules was analyzed by flow cytometry on CD14$^+$ gated cells; data shown are representative of three experiments performed.

APCs from the stimulating donor or from a HLA different individual.

For these experiments, we used as targets CD14$^+$ monocytes from the priming stimulator or a HLA-mismatched control. To induce the upregulation of costimulatory molecules and downregulation of ILT3, APCs were incubated with CD40L-transfected Jurkat cells from the D1.1 cell line (20). When ILT3-Fc–induced or BCL6-transfected CD8$^+$ Ts cells were added to the cultures, they promoted high expression of ILT3 and inhibited upregulation of CD40, CD80, and CD86 in CD40L-triggered APCs from the priming donor (Fig. 3), but not in APCs from the HLA-mismatched control (data not shown). Control CD8$^+$ T cells transfected with an empty vector, or nontransfected CD8$^+$ T cells primed in the absence of ILT3-Fc had no significant effect on the expression of ILT3 and costimulatory molecules on target APCs (Fig. 3).

These data demonstrate that allostimulated CD8$^+$ T cells transfected with BCL6 or treated with ILT3-Fc share the capacity of modulating the phenotype of priming APCs upon cell-to-cell contact.

Knockdown of BCL6 prevents the differentiation of Ts cells

Having established that BCL6 transfection endows primed CD8$^+$ T cells with Ts function, we sought to determine whether the reverse also holds true (i.e., whether the acquisition of Ts function is impaired following knockdown of this gene).

Unprimed PBMCs were incubated with an adenovirus encoding BCL6 small interfering RNA or an empty vector control. After 48 h, CD8$^+$ T cells from these cultures were sorted, tested for lack of BCL6 expression, mixed with autologous CD4$^+$ T cells, and stimulated with allogeneic CD2-depleted APCs in the presence or absence of ILT3-Fc. After 7 d, CD8$^+$ T cells were sorted and tested for suppression function.

ILT3-Fc–treated CD8$^+$ T cells transfected with an empty vector inhibited proliferation by >85%, whereas ILT3-Fc–treated CD8$^+$ T cells that had been transfected with BCL6 small interfering RNA induced <20 ± 5% inhibition of T cell proliferation in four repeat experiments. The finding that ILT3-Fc treatment did not result in the generation of CD8$^+$ Ts cells from BCL6KD cells demonstrates that the BCL6 signaling pathway is involved in the development of CD8$^+$ Ts cells (Fig. 4*A*). The cpm obtained in one of these four experiments is shown in Fig. 4*B*.

ILT3-Fc treatment reverses rejection

Because there is no murine ortholog of ILT3, the tolerogenic effect of this molecule can be studied only in humanized SCID mice. Pancreatic islet transplants provide a suitable model particularly because rejection is reminiscent of events occurring during onset of T1D.

In previous studies, we demonstrated that tolerance to allogeneic islets can be induced in hu-NOD/SCID mice by initiating treatment with ILT3-Fc immediately upon injection of allogeneic human PBMCs. In non–ILT3-Fc–treated mice, rejection started 2–3 wk after humanization and was completed in all animals by the seventh week (10).

Control IgG has been used in all the in vitro and in vivo experiments described in our previous papers (8, 10). There was absolutely no difference between the results obtained in control mice treated with human IgG or saline (10). For this reason, in the current study, human IgG was not used as a control for the effect of ILT3-Fc on graft survival. Instead, control mice received no treatment.

To determine whether islet allograft rejection could be reversed after its onset, we initiated ILT3-Fc treatment 15 or 20 d after humanization. The actuarial 100-d islet allograft survival was 100%

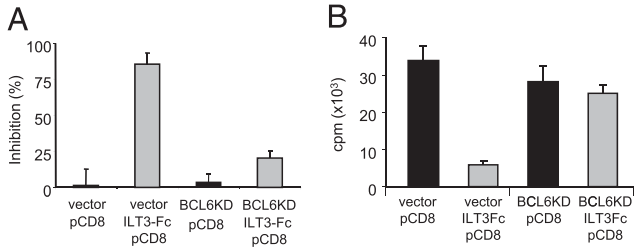

FIGURE 4. Silencing of BCL6 inhibits the ILT3-Fc–induced differentiation of CD8$^+$ Ts cells. *A*, Unprimed BCL6KD or vector-transfected CD8$^+$ T cells were incubated with autologous CD4$^+$ T cells in MLCs in the presence or absence of ILT3. After 7 d, CD8$^+$ T cells were sorted and tested in suppressor assays for their capacity to inhibit the proliferation of autologous CD4$^+$ T cells in response to the original stimulator. Percentage of inhibition induced by BCL6KD pCD8, BCL6KD ILT3-Fc pCD8, vector pCD8, and vector ILT3-Fc pCD8 in four independent experiments is shown. *B*, A representative [^{3}H]thymidine incorporation assay (mean cpm ± SD of triplicate reactions) is shown.

in animals receiving 10 daily injections of ILT3-Fc immediately after humanization, whereas control mice that received no treatment rejected the islets in <50 d, as also reported before (10). All allograft recipients whose treatment was initiated 20 d after humanization rejected the islets in <80 d. In contrast, 70% of the animals that received ILT3-Fc treatment 15 d after humanization tolerated the graft for the 100 d of observation (Fig. 5*A*). The time of initiation of ILT3-Fc treatment had a highly significant effect on islet allograft survival as rejection could be reversed in animals treated at the onset of this process ($p < 0.0001$, log-rank test).

Histological comparison of islet-transplanted kidneys showed that the quantity of islets was greater in ILT3-Fc–treated tolerant animals sacrificed on day 100 (mean 2.8 ± 0.4, $n = 5$) than in control animals sacrificed at the time when they rejected (mean 1.4 ± 0.5, $n = 5$, $p < 0.05$, Mann-Whitney U test). Insulitis by $CD8^+$ T cells was markedly reduced in treated (mean 0.5 ± 0.5) versus control animals (mean 2.6 ± 0.5, $p < 0.01$). ILT3-Fc–treated animals showed strong and diffuse expression of insulin (mean 2.8 ± 0.4), indicating that the human islets were functionally active and well tolerated, whereas islets undergoing rejection in untreated controls showed markedly reduced expression of insulin (mean 0.7 ± 0.4, $p < 0.01$) (Fig. 5*B–G*). We previously reported that there were no histological signs of rejection in ILT3-Fc–treated mice at the time when this process was already completed in control, non–ILT3-Fc–treated animals (10). Similarly, in this study, mice in which ILT3-Fc treatment was initiated on day 15 showed no histologic signs of rejection on day 44 when rejection was completed in control mice (data not shown).

These data demonstrate that ILT3-Fc treatment prevented the progression of islet allograft rejection when administered at the time of its onset.

Tolerant islet recipients have $CD8^+$ Ts cells

To establish whether there were functional differences between human T cells from mice that tolerated and that rejected the graft, we sorted $CD4^+$ and $CD8^+$ T cells from their spleens and tested

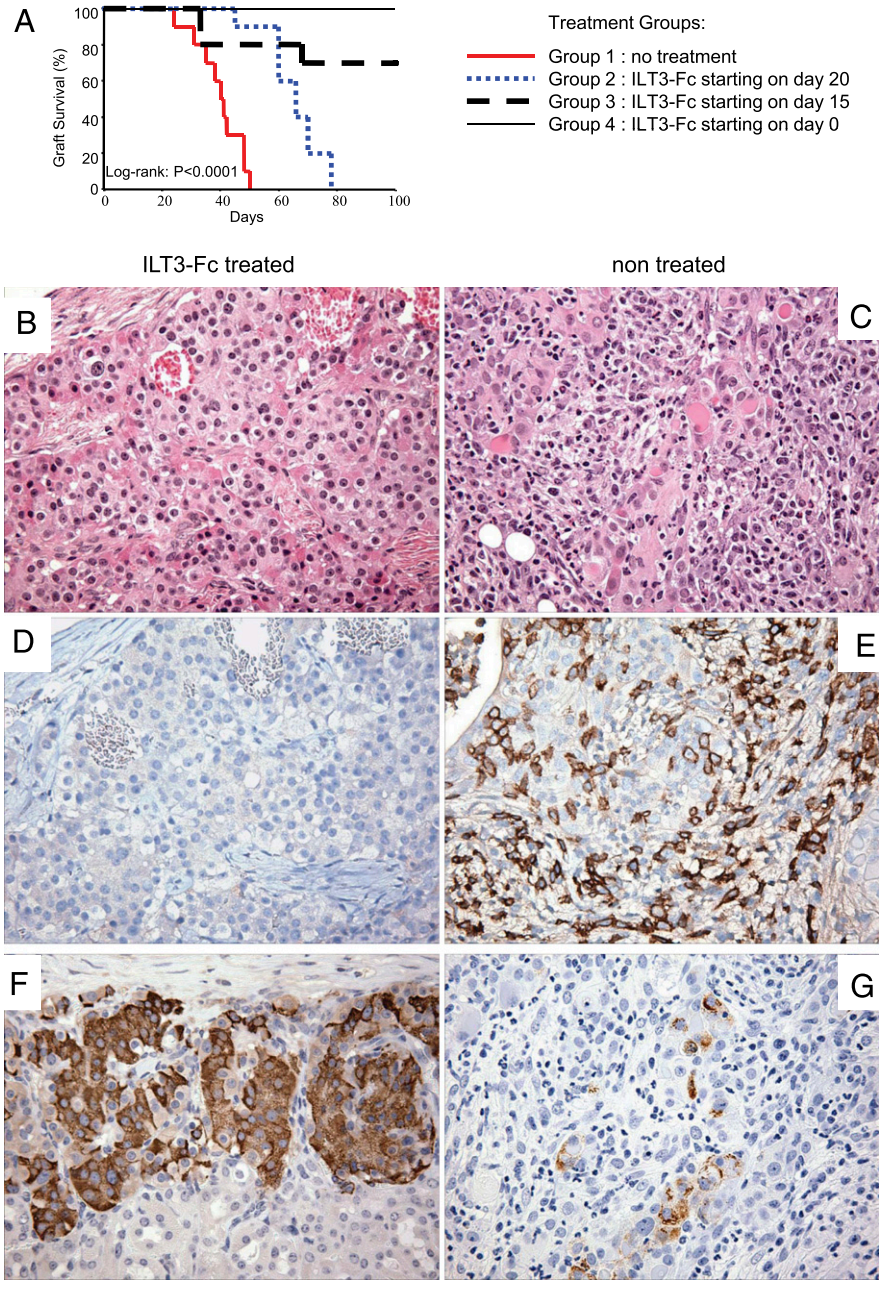

FIGURE 5. ILT3-Fc treatment reverses pancreatic islet allograft rejection in hu-NOD/SCID mice. *A*, Actuarial islet allograft survival in mice that received no treatment ($n = 10$), or were treated with ILT3-Fc at the time of humanization ($n = 5$), 15 d later ($n = 10$), or 20 d later ($n = 5$). H&E (*B*, *C*), CD8 (*D*, *E*), and insulin (*F*, *G*) staining of islet-engrafted kidneys from an euglycemic mouse treated with ILT3-Fc 15 d after humanization (*B*, *D*, *F*) and nontreated, hyperglycemic mouse (*C*, *E*, *G*), sacrificed on days 100 and 41, respectively.

them in parallel for suppressive activity. Putative Ts cells from individual animals were added to primary MLCs containing unprimed, autologous CD4$^+$ T cells. Allogeneic human APCs matched to the pancreatic islet allograft for at least one HLA class I and class II Ag were selected from a HLA reference panel and used as stimulators for these MLCs, as before (10).

CD8$^+$ T cells from the spleen of five mice tolerating the graft 100 d after humanization inhibited the response of autologous T cells in 5-d MLCs by 76–90% at an 8:1 suppressor to responder ratio. CD4$^+$ T cells from the same animals had no suppressive effect. Neither CD8$^+$ nor CD4$^+$ T cells from five untreated mice that rejected the graft between 40 and 50 d displayed any significant suppressive activity (Fig. 6*A–C*).

Supernatants from Ts cell assays were collected after 48 h of culture, and their cytokine content was compared with that of MLCs to which no CD8$^+$ T cells were added. In cultures with CD8$^+$ T cells from tolerant animals, the amount of IL-2, IL-5, IL-10, and IFN-γ was 50–90% lower than in cultures without CD8$^+$ T cells. There was no significant inhibition of cytokine production in supernatants from cultures in which CD4$^+$ T cells were allostimulated in the presence of CD8$^+$ T cells from animals that rejected (Fig. 6*D*). These results indicate that CD8$^+$ T cells from tolerant islet recipients inhibit the capacity of autologous CD4$^+$ T cells to produce Th1 and Th2 cytokines upon in vitro stimulation with priming, allogeneic APCs. Furthermore, our finding that the production of IL-10 was also suppressed reinforces our previous result indicating that CD8$^+$ Ts cells operate by contact-dependent, cytokine-independent mechanisms.

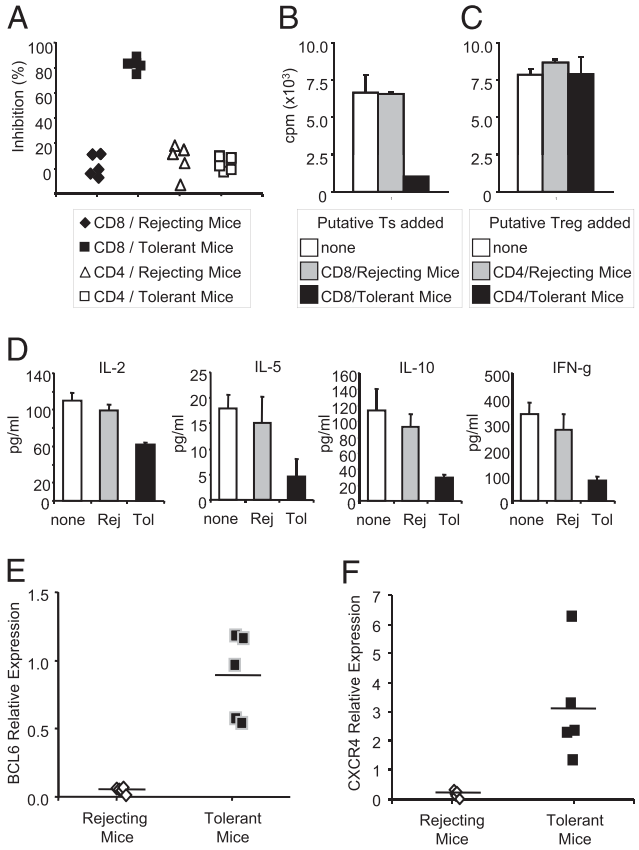

FIGURE 6. Inhibition of CD4$^+$ T cell proliferation and cytokine production by BCL6^{high} CD8$^+$ Ts cells. *A*, Human CD8$^+$ and CD4$^+$ T cells were sorted from the spleens of five mice that rejected the graft (on days 40, 41, 42, 48, and 50) and of five tolerant mice (sacrificed on day 100) and tested for their capacity to inhibit the proliferation of unprimed autologous CD4$^+$ T cells in response to allogeneic APCs, matched to the graft for at least one HLA class I and class II Ag. There was a statistically significant difference between the inhibitory capacity of CD8$^+$ T cells from mice that tolerated or rejected the graft ($p < 0.0001$, Student *t* test). *B*, A representative [^{3}H]thymidine incorporation assay (mean cpm ± SD of triplicate reactions) in which human CD8 T cells (8×10^4) obtained from the spleens of islet-transplanted hu-NOD/SCID mice treated or not treated with ILT3-Fc were incubated ex vivo with autologous CD4 T cells (1×10^4) and APCs (0.5×10^4) sharing one HLA class I and one HLA class II Ag with the transplanted islets is shown. *C*, CD4 T cells (8×10^4) from the same two animals shown in *B* were added to autologous CD4 T cells (1×10^4) and APCs (0.5×10^4), as described above. *D*, Supernatants collected from suppressor assays after 48 h were tested for their content of IL-2 ($p < 0.01$), IL-5 ($p < 0.001$), IL-10 ($p < 0.001$), and IFN-γ ($p < 0.001$). (Student *t* test). *E*, RT-PCR measurement of the relative expression of BCL6 in CD8 T cells from mice that tolerated or rejected the graft ($p = 0.004$, Student *t* test). *F*, RT-PCR measurement of the relative expression of CXCR4 in CD8 T cells from mice that tolerated or rejected the graft ($p = 0.001$, Student *t* test). Rej, Rejection; Tol, Tolerance.

BCL6 expression is increased in Ts cells from tolerant animals

Because BCL6 expression was significantly higher in ILT3-Fc–induced CD8$^+$ Ts cells compared with CD8$^+$ T cells from MLCs without ILT3-Fc, we studied the possibility that Ts cells induced in vivo by ILT3-Fc treatment of humanized mice display the same features. Real-time PCR demonstrated that the amount of BCL6 mRNA was significantly higher ($p = 0.004$) in tolerant animals ($n = 5$) compared with animals that rejected the graft ($n = 5$; Fig. 6*E*). These results are consistent with the notion that BCL6 is involved in suppression of the immune response to allogeneic islet cells. Fig. 6*F* shows the expression of CXCR4 in CD8$^+$ T cells from the same animals. Similar to the results obtained in vitro, we found that ILT3-Fc treatment enhanced the expression of CXCR4 in vivo.

Discussion

The induction of Ts cells capable of inhibiting T cell immune responses in an Ag-specific manner is an important focus of research on autoimmunity as well as allotransplantation.

In previous studies, we have demonstrated that Ag-specific, MHC class I-restricted CD8$^+$ Ts cells can be generated by multiple stimulation of T cells with allogeneic, xenogeneic, or autologous, Ag-pulsed APCs (20, 30–34). CD8$^+$ Ts cells act directly on APCs in a contact-dependent, cytokine-independent manner, inducing the upregulation of inhibitory receptors ILT3 and ILT4, and downregulation of costimulatory molecules (13, 20, 30–34). Tolerogenic priming of T cells with ILT3^{high} APCs induces anergy in CD4$^+$ Th cells and elicits the differentiation of CD8$^+$ Ts cells. CD8$^+$ Ts cells seem to derive from the same lineage like CD8$^+$ CTLs, as suggested by the fact that they recognize the same dominant epitopes of viral proteins (34).

The factors that drive the development of CD8$^+$ Ts cells versus CD8$^+$ CTLs may include the avidity of the TCR, the cytokine milieu, and the differentiation status of priming APCs (33). However, the transcription factors that dictate the fate of these cells are still unknown.

In the current study, we demonstrate that the transcriptional repressor BCL6 is crucial to the acquisition of Ts function by ILT3-Fc–treated CD8$^+$ T cells.

The inhibitory effect of ILT3-Fc was demonstrated in vitro using T cells allostimulated in primary or secondary MLCs (8) and in vivo in NOD/SCID mice humanized with PBMCs and transplanted with allogeneic human islets (10).

Although the ligand of ILT3 on activated T cells remains elusive, our in vitro studies showed that ILT3-Fc induces significant

upregulation of BCL6 and downregulation of the genes that it controls in CD8$^+$ T cells. Furthermore, transfection of BCL6 in alloactivated CD8$^+$ T cells converted them into suppressors, whereas silencing of BCL6 in unprimed CD8$^+$ T cells prevented their differentiation into Ts cells in the presence of ILT3-Fc. BCL6-transfected Ts, similar to ILT3-Fc–induced Ts, triggered enhanced expression of ILT3 and decreased expression of costimulatory molecules in priming APCs. These findings implicate BCL6 as an integral and indispensable part of the differentiation and function of CD8$^+$ Ts.

Kinetic studies showed that BCL6 mRNA was expressed in unprimed CD8$^+$ T cells, downregulated after allostimulation, yet progressively upregulated after 3 d in the presence of ILT3-Fc. This pattern is reminiscent of that which has been previously found after TCR activation of naive CD8$^+$ T cells in cultures with low IL-2 (35) and is consistent with the IL-2 inhibitory capacity of ILT3-Fc (8).

It has also been shown that overexpression of BCL6 in CD8$^+$ T cells results in lower killing activity and reduction of granzyme B expression (29, 35). In CD4$^+$ T cells, BCL6 was found to act as a transcriptional repressor that inhibits the differentiation of Th1, Th2, and Th17 cells binding directly to the promoters of human TBX21, GATA3, and RORC genes, respectively (6, 26). Forced BCL6 expression in human CD4$^+$ T cell represses IFN-γ and IL-17 production while enhancing expression of CXCR5 and CXCR4 (24, 26). Of interest, CXCL12 (the ligand of CXCR4) redirects the polarization of effector Th1 cells into IL-10–producing CD4$^+$ Treg cells, restraining autoimmune inflammatory processes (36, 37).

Our data demonstrate for the first time that in the presence of ILT3-Fc the repression of granzyme B, IFN-γ, IL-5, and enhancement of CXCR4 occur in conjunction with upregulation of BCL6 expression in CD8$^+$ T cells with suppressor activity. Hence, ILT3 may arbitrate T cell lineage fate through BCL6-mediated repression of Th1, Th2, Th17, and CTL and induction of Ts differentiation. The lack of effector molecules does not explain, however, the acquisition of Ts function by ILT3-Fc–treated CD8$^+$ T cells. It is likely that this treatment induces the upregulation of a still unidentified cell surface molecule that facilitates the tolerogenic interaction between Ts cells and APCs.

ILT3 may have important clinical implications for induction of allogeneic tolerance and treatment of islet allograft rejection, as well as for reversal of early-onset T1D, a disease known to be mediated by autoaggressive T cells (17, 38). We found that treatment with ILT3-Fc without any complementary immunosuppression induces tolerance and reverses rejection of allogeneic human islets transplanted in hu-NOD/SCID mice. Human CD8$^+$ T cells from tolerant, but not control mice exhibited suppressor activity and high expression of BCL6. These data are consistent with recent studies emphasizing the role of CD8$^+$ Ts cells in autoimmunity, transplantation, and infectious diseases (39–42).

Our finding that rejection can be reversed by treatment with ILT3-Fc suggests the plasticity of activated CD8$^+$ T cells that may be reprogrammed to become suppressor cells even after initiation of the inflammatory process. By inhibiting the production of T cell effector molecules, ILT3 may create a microenvironment that favors the differentiation of Ts cells capable of terminating the rejection process. ILT3-Fc treatment may hold the potential of reversing not only allograft rejection, but also autoimmune T cell responses against the pancreas.

Disclosures

The authors have no financial conflicts of interest.

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