Transgenic Expression of Single-Chain Anti-CTLA-4 Fv on β Cells Protects Nonobese Diabetic Mice from Autoimmune Diabetes[1]

Shing-Jia Shieh,* Feng-Cheng Chou,* Pei-Ning Yu,† Wen-Chi Lin,† Deh-Ming Chang,¶ Steve R. Roffler,‖ and Huey-Kang Sytwu[2]*†‡§

T cell-mediated immunodestruction of pancreatic β cells is the key process responsible for both the development of autoimmune diabetes and the induction of rejection during islet transplantation. In this study, we investigate the hypothesis that transgenic expression of an agonistic, membrane-bound single-chain anti-CTLA-4 Fv (anti-CTLA-4 scFv) on pancreatic β cells can inhibit autoimmune processes by selectively targeting CTLA-4 on pathogenic T cells. Strikingly, transgenic expression of anti-CTLA-4 scFv on pancreatic β cells significantly protected NOD mice from spontaneous autoimmune diabetes. Interestingly, local expression of this CTLA-4 agonist did not alter the diabetogenic properties of systemic lymphocytes, because splenocytes from transgenic mice or their nontransgenic littermates equally transferred diabetes in NOD/SCID recipients. By analyzing the T cell development in anti-CTLA-4 scFv/Th1/Th2 triple transgenic mice, we found that β cell-specific expression of CTLA-4 agonist did not affect the development of Th1/Th2 or $CD4^+CD25^+$ regulatory T cells. Most strikingly, islets from transgenic mice inhibited T cell response to immobilized anti-CD3 in a T cell-islet coculture system, suggesting a trans-mediated inhibition provided by transgenic islets. Finally, transgenic islets implanted in diabetic recipients survived much longer than did wild-type islets, indicating a therapeutic potential of this genetically modified islet graft in autoimmune diabetes. *The Journal of Immunology,* **2009, 183: 2277–2285.**

Insulin-dependent diabetes mellitus (IDDM)[3] is a T cell-mediated autoimmune disease caused by a progressive destruction of the insulin-producing β cells in the pancreatic islets of Langerhans. Both genetic and environmental factors have been linked to the pathogenesis of this disease. The NOD mouse, an inbred strain that spontaneously develops autoimmune diabetes resembling human IDDM, is a widely used animal model for dissecting immunopathological mechanisms and developing preventive and/or therapeutic strategies in IDDM (1–3).

It has been well characterized that costimulatory pathways regulate the activation and tolerance of T cells. CTLA-4, also known as CD152 and mainly expressed on activated T cells, is a member of the Ig superfamily and has been defined as a negative regulator of T cell responses (4). By contrast, CD28, expressed on resting and activated T cells, is a well-known positive regulator for T cell activation (5). CTLA-4 and CD28 share the same ligands, B7-1 and B7-2, but CTLA-4, with higher binding affinity to B7 molecules, transduces an inhibitory signal to T cells and maintains the tolerance of self-reactive T cells (6). CTLA-4 knockout mice develop severe lymphoproliferative disorders and die at the age of 3–4 wk (7–9), confirming the negative regulatory role of CTLA-4 in T cell activation and effector function. In the BDC2.5 TCR transgenic NOD mouse model, blocking CTLA-4 signaling by anti-CTLA-4 mAb or deficiency in CTLA-4 leads to early onset of diabetes, indicating a critical role of CTLA-4 in the initiation and progression of autoimmune diabetes (7, 10). Genetic studies also indicate that the *CTLA-4* locus is associated with both human IDDM and diabetic susceptibility in NOD mice (11). Recently, a lentivirus-transduced RNAi-based CTLA-4 knockdown NOD mouse with a partial reduction in CTLA-4 expression revealed severe lymphocyte infiltrations primarily in islets and acceleration in diabetic development (12), further confirming that CTLA-4 plays an important role in the negative regulation of lymphocyte activation and is involved in controlling the progression of diabetes in NOD mice. Therefore, augmentation of inhibitory signals by CTLA-4 engagement could be a potential preventive or therapeutic approach to ameliorating autoimmunity in NOD mice.

Membrane-bound single-chain anti-CTLA-4 Fv (anti-CTLA-4 scFv), a chimeric molecule that specifically binds CTLA-4 and provides an agonistic signal, has been demonstrated to down-regulate T cell activation in vitro (13). Moreover, overexpression of this anti-CTLA-4 scFv on allogeneic tumor cells significantly inhibits allogeneic rejection, indicating an immunosuppressive effect of this molecule in vivo (14). A recent report nicely demonstrates that transgenic expression of anti-CTLA-4 scFv on B cells can inhibit T cell activation and suppress autoimmune diabetes in NOD mice (15). However, it is still not known whether expression of membrane-bound agonistic anti-CTLA-4 scFv on pancreatic β cells provides protection in situ in the NOD model by directly targeting CTLA-4 on islet-infiltrating T cells, or whether such

*Graduate Institute of Life Sciences, †Department of Microbiology and Immunology, ‡Graduate Institute of Medical Sciences, §Department of Medical Research, ¶Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, ‖Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

Received for publication March 6, 2009. Accepted for publication June 9, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Science Council, Taiwan, Republic of China (NSC-94-3112-B-016-005, NSC-95-3112-B-016-001, NSC-96-3112-B-016-001, and NSC-96-2628-B-016-002-MY3 to H.-K.S.), and in part by the C.Y. Foundation for Advancement of Education, Sciences, and Medicine.

[2] Address correspondence and reprint requests to Dr. Huey-Kang Sytwu, Graduate Institute of Medical Sciences, National Defense Medical Center, 161, Section 6, Min-Chuan East Road, Neihu, Taipei, Taiwan. E-mail address: sytwu@ndmctsgh.edu.tw

[3] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; anti-CTLA-4 scFv, single-chain anti-CTLA-4 Fv; PLN, pancreatic lymph node; TM, transmembrane; Treg, regulatory T cell; DC, dendritic cell; pINS, human insulin promoter; CT, cytosolic tail.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900679

genetically modified islets survive better and/or longer in diabetic recipients in a transplantation model.

In this study, we generated a transgenic NOD mouse overexpressing anti-CTLA-4 scFv on pancreatic β cells that could directly bind CTLA-4 molecules on islet-infiltrating self-reactive T cells. Our results show that transgenic expression of anti-CTLA-4 scFv on pancreatic β cells significantly mitigates the severity of insulitis and decreases the incidence of spontaneous diabetes in NOD mice. The protection mediated by this transgenic anti-CTLA-4 scFv is a local effect, because the development of lymphocytes and the diabetogenic properties of systemic T cells were not affected in these transgenic mice. Pancreatic β cells expressing this membrane-bound agonistic anti-CTLA-4 scFv inhibit anti-CD3-stimulated T cell proliferation in a T cell-islet coculture system. Moreover, islet grafts carrying anti-CTLA-4 scFv transgene survive longer in diabetic recipients than do control islets, and significantly attenuate the infiltration of Th1 cells to the implanted area.

Materials and Methods

Mice

NOD/Sytwu (K^d, D^b, L^d, I-A^{g7}) and NOD/SCID mice were initially purchased from The Jackson Laboratory and subsequently bred at the Animal Center of the National Defense Medical Center (NDMC) in Taipei, Taiwan. NOD-Th1/2 transgenic mice were generated in our laboratory as previously described (16). All mice were maintained in specific pathogen-free facilities at NDMC. Experiments were conducted in accord with institutional guidelines and were approved by NDMC Institutional Animal Care and Use Committee (IACUC). The spontaneous incidence of diabetes in the NOD colony was 80–90% in females and 20–30% in males by 25 wk of age.

Construction and expression of single-chain anti-CTLA-4 Fv gene

The anti-CTLA-4 scFv was constructed as previously described (17). Briefly, the anti-CTLA-4 scFv was cloned from total RNA isolated from UC10–4F10–11 (4F10) hybridoma cells (American Type Culture Collection) (4) by the method described by Gilliland et al. (18). Leucine residues at positions 43 and 89 in the 4F10 L chain variable region were mutated to methionine and glutamine, respectively, to increase scFv expression and to enhance the stability and affinity of 4F10 expressed on the surface of cell membranes (19). The anti-4–1BB scFv in pLNCX-anti-4–1BB scFv (20) was removed by digestion with *Sfi*I and *Sal*I restriction enzymes and replaced by anti-CTLA-4 scFv to generate pLNCX-4F10m2-myc-eB7-B7TM. 3T3 fibroblasts were cultured overnight before transfection with 5 μg pLNCX-4F10m2-myc-eB7-B7TM and Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Expression of anti-CTLA-4 scFv on the cell surface of transfectants was detected by staining the cells with rat-anti-HA (3F10, Roche) followed by FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories). The functional activity of anti-CTLA-4 scFv on these transfected cells was measured by adding recombinant CTLA-4-Ig followed by FITC-conjugated goat anti-human IgG Fc (MP Biomedicals).

Generation of β cell-specific anti-CTLA-4 scFv/eB7/B7TM transgenic NOD mice

The pLNCX-4F10m2-myc-eB7-B7TM was digested with *Hin*dIII and *Cla*I and the anti-CTLA-4 scFv/eB7/B7TM were inserted into the human insulin promoter (pINS) plasmid (21) to generate a pINS-anti-CTLA-4 scFv/eB7/B7TM construct. A modified human *insulin* promoter was provided by Dr. Miyazaki at Osaka University, Japan. The linearized DNA fragment with the human *insulin* promoter and anti-CTLA-4 scFv/eB7/B7TM was purified and microinjected into the pronuclei of one-cell NOD embryos. These injected embryos were then implanted into pseudopregnant (BALB/c $\times$ FVB) F_1 females. The presence of transgene was detected by PCR and Southern blot. All transgenic mice used in our study were heterozygous for the anti-CTLA-4 transgene.

Assessment of diabetes and insulitis

Urine glucose concentration was measured weekly or every second day using the Medi-Test Glucose Kit (Macherey-Nagel). Mice with glycosuria >500 mg/dl on two consecutive tests were classified as diabetic. For histological analysis, pancreata from female mice at the age of 13–14 wk were fixed in 10% buffered formalin. The severity of insulitis was scored blindly on H&E stained sections as previously described (22). Each islet was scored as follows: intact, islet without infiltrating cells; peri-insulitis, <25% of the area of an islet was occupied by infiltrating cells; intrainsulitis, >25% but <50% of the area of an islet was occupied by infiltrating cells; severe insulitis, >50% of the area of an islet was occupied by infiltrating cells.

Abs and flow cytometry

Splenocytes and lymphocytes of pancreatic lymph nodes (PLN) of transgenic mice or control littermates or lymphocytes isolated from islet grafts implanted under kidney capsule of diabetic NOD-Th1/2 transgenic recipients were stained with mAbs to surface markers. Abs to murine CD4 (RM4–5) and CD8a (53–6.7) were purchased from eBioscience. Abs to murine CD19 (1D3), CD25 (PC61), CD90.1 (mThy1.1, OX-7), or to human CD90 (hThy1, 5E10) were purchased from BD Biosciences. Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences).

Isolation and flow cytometric analysis of islet-infiltrating lymphocytes

Pancreata were collected from 12- to 14-wk-old transgenic or control NOD mice. Islets were isolated by collagenase digestion and Histopaque gradient purification. Marginal cells were collected and incubated with Cell Dissociation Buffer (Life Technologies). After passing over a cell strainer, suspended single cells were counted and stimulated with PMA (Sigma-Aldrich) and Ionomycin (Sigma-Aldrich) in the presence of monensin (Sigma-Aldrich) for 4–5 h. The percentage of IFN-γ-producing Th1 cells was analyzed by intracellular cytokine staining gating on $CD4^+$ lymphocytes.

T cell proliferation assay

Splenocytes from 8-wk-old mice were treated with Tris-buffered ammonium chloride to eliminate the erythrocytes, washed, and resuspended at a cell concentration of 2.5×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin G, 0.1 mg/ml streptomycin (Life Technologies), and 10 mM HEPES (Life Technologies). Cells were cultured in triplicate in 96-well microtiter plates (5×10^5 cells/200 μl per well) in the presence of synthetic glutamic acid decarboxylase 65 (GAD65) peptides (p247–266) (23). For functional assay, NIT-1 cells (American Type Culture Collection) transfected with anti-CTLA-4 scFv were treated with 50 μg/ml mitomycin C (Sigma-Aldrich) for 2 h, and then cultured with immobilized anti-CD3-stimulated splenocytes. For coculture experiments, isolated islets were treated with trypsin-EDTA, and dissociated into single cells. Before adding single islet cells, splenocytes were prestimulated with immobilized anti-CD3 (145–2C11, BD Biosciences) for 4–5 h. After 48 h, the cultured cells were pulsed with 1 μCi of [^{3}H]methyl thymidine/per well (PerkinElmer) and harvested 16 h later. The plates were harvested onto UniFilter-96, GF/C (PerkinElmer), and the incorporated [^{3}H]methyl thymidine was detected with a Packard TopCount Microplate Scintillation Counter (Packard).

Adoptive transfer

Splenocytes (2×10^7 cells) isolated from 12-wk-old female mice were injected into 8-wk-old female NOD/SCID via the retro-orbital plexus. Diabetes of NOD/SCID recipients was assessed as described above.

Islet isolation and transplantation

Islets were isolated from male mice (<8-wk-old) by the collagenase digestion method as previously described (24). In brief, after digestion with collagenase XI (Sigma-Aldrich), islets were separated by density gradient centrifugation. Islets with a diameter between 75 and 250 μm were handpicked under a dissecting microscope and confirmed by dithizone (Sigma-Aldrich) staining (data not shown). For islet transplantation, 700 islets were implanted to the left kidney capsule of newly diabetic female NOD mice or NOD-Th1/2 transgenic mice with blood glucose between 300 and 500 mg/dl. Primary graft function was defined as blood glucose <200 mg/dl 2 days after transplantation, and graft loss was defined as blood glucose >300 mg/dl at two consecutive tests.

Statistic

The statistical significance of diabetic incidence and survival of islet grafts were calculated using the Kaplan-Meier method, and the log-rank test was used for the evaluation of significance. In addition, when needed, results were analyzed using Student's *t* test. Values of $p < 0.05$ were considered significant.

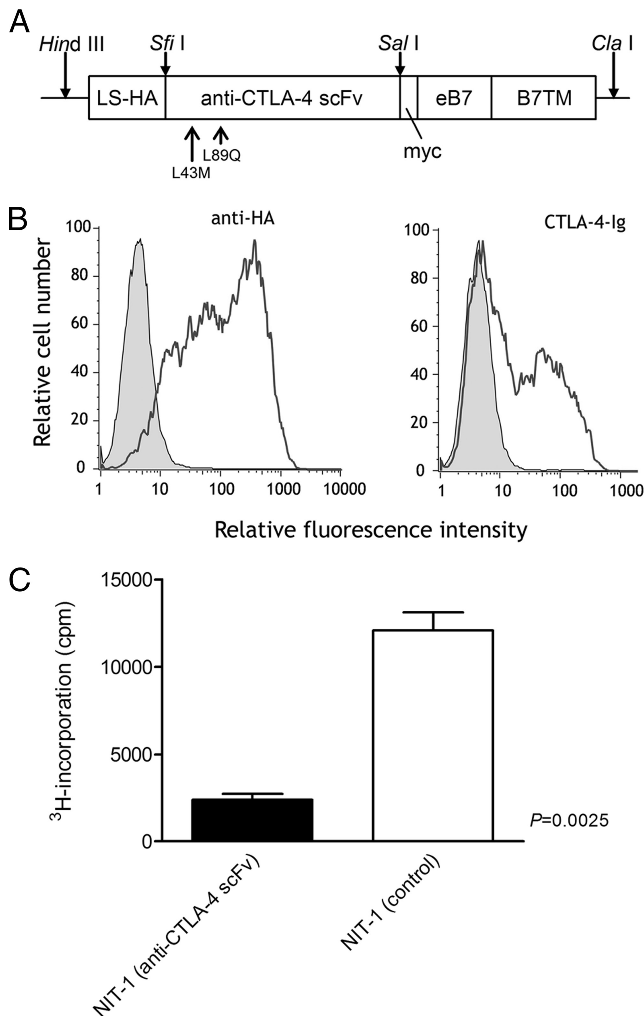

FIGURE 1. Construction and expression of anti-CTLA-4 scFv gene. *A*, The pLNCX-4F10m2-myc-eB7-B7TM construct encodes a membrane-bound agonistic anti-CTLA-4 scFv from 4F10 hybridoma. This chimeric receptor consists of a murine Ig κ-chain signal peptide and an HA epitope (indicated as LS-HA), the anti-CTLA-4 scFv containing two mutations at the L chain variable region (indicated as anti-CTLA-4 scFv), a myc epitope (indicated as myc), the extracellular portion (indicated as eB7) and the transmembrane domain (indicated as B7TM) of murine B7-1. *B*, 3T3 fibroblasts transfected with pLNCX-4F10m2-myc-eB7-B7TM were stained with rat anti-HA followed by FITC-conjugated goat anti-rat IgG (*left*, open histogram) or stained with CTLA-4-Ig followed by FITC-conjugated goat anti-human IgG Fc (*right*, open histogram). Shaded histograms indicate the results of staining with secondary Abs only. *C*, Mitomycin C-treated NIT-1 transfectants (5×10^5) were cultured with immobilized anti-CD3 (0.1 μg)-stimulated splenocytes (5×10^5) of 8-wk-old female NOD mice. Proliferation was determined by [^{3}H]thymidine incorporation.

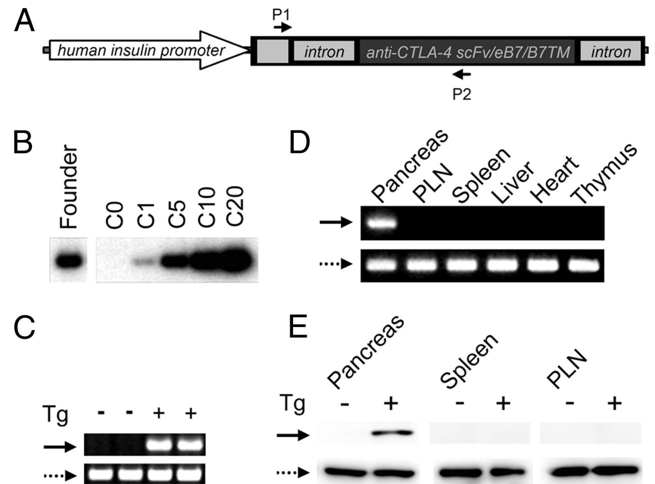

FIGURE 2. Generation of β cell-specific anti-CTLA-4 scFv/eB7/B7TM transgenic NOD mice. *A*, Schematic diagram of the whole transgene construct. The black areas represent exons and the gray areas represent introns. The entire first noncoding exon followed by the first intron and 16 bp of the second exon of the human *insulin* gene, which are not translated into protein, were preserved to ensure the stringency of the insulin promoter. A forward primer located in the second exon of *insulin* gene (P1) and a reverse primer in the coding region of *anti-CTLA-4 scFv/eB7/B7TM* (P2) were designed for genotyping and to evaluate the transcription of transgene. *B*, Southern blot analysis verified the transgenic anti-CTLA-4 scFv signal in the Founder and provided the relative copy number of the transgene. *C*, Genotyping of tail genomic DNA by PCR to test for the existence of the transgene is indicated by the solid arrow and the internal control of *IL-12 p35* is indicated by the dashed arrow. *D*, RNA isolated from multiple organs of mice at the age of 14 wk was used to detect the transcription of the transgene by RT-PCR. The *anti-CTLA-4 scFv/eB7/B7TM* transcript was only detected in pancreas confirming tissue-specific expression (solid arrow). Transcripts of *hgprt* were the internal control (dashed arrow). *E*, Protein extracts of pancreas, spleen, and PLN from a transgenic mouse or a nontransgenic control at the age of 14 wk were analyzed by Western blot to detect the expression of anti-CTLA-4 scFv (solid arrow). β-actin (indicated by the dashed arrow) served as a control.

Results

Construction and expression of single-chain anti-CTLA-4 Fv gene

To generate an agonistic membrane-bound single-chain Ab which can directly target on CTLA-4, we molecularly engineered the pLNCX-4F10m2-myc-eB7-B7TM construct. This chimeric construct consists of a murine Ig κ-chain signal peptide, an HA epitope, the anti-CTLA-4 scFv from 4F10 hybridoma with two mutations in the L chain variable region, a myc epitope, an extracellular portion, a transmembrane (TM) domain and the cytosolic tail (CT) of murine B7-1 (Fig. 1*A*). Cell surface expression and target molecule binding activity of anti-CTLA-4 scFv on pLNCX-4F10m2-myc-eB7-B7TM-transfected 3T3 fibroblasts were measured by flow cytometric analysis (Fig. 1*B*). Transfected cells were stained with rat anti-HA followed by FITC-conjugated goat anti-rat IgG (Fig. 1*B*, *left*), and binding activity of anti-CTLA-4 scFv was detected by adding recombinant CTLA-4-human IgG Fc fusion protein followed by FITC-conjugated goat anti-human IgG Fc (Fig. 1*B*, *right*). Our results clearly indicated that the membrane-bound anti-CTLA-4 scFv encoded by the chimeric gene was expressed on the surface of transfected cells and could specifically bind CTLA-4. Transfectants expressing anti-CTLA-4 scFv were able to inhibit T cell proliferation induced by immobilized anti-CD3 (Fig. 1*C*). These results not only demonstrate the expressional availability and functionality of this chimeric *anti-CTLA-4 scFv/eB7/B7TM* gene but also suggest that this membrane-anchored single-chain Ab selectively targeting CTLA-4 functions as a negative regulator for primary T cell activation.

Generation of β cell-specific anti-CTLA-4 scFv/eB7/B7TM transgenic NOD mice

To directly investigate the immunomodulatory effect of an organ-specific anti-CTLA-4 scFv on the autoimmune process in NOD mice, we established a transgenic mouse model by injecting the pINS-anti-CTLA-4 scFv/eB7/B7TM construct (Fig. 2*A*) into fertilized NOD eggs. This construct consists of the human *insulin*

promoter, the first and part of the second noncoding exons of *insulin*, an intron from rabbit *betaglobin*, membrane-bound agonistic *anti-CTLA-4 scFv/eB7/B7TM*, and a poly-A signal from rabbit *betaglobin*. The *insulin* promoter used in this construct is highly specific to pancreatic β cells and guides significant production of the transgene (21, 25). One transgenic line denoted as Founder was obtained from three independent pronucleus microinjections. Southern blot analysis of tail genomic DNA indicated that Founder carries 5–10 copies of the chimeric transgene (Fig. 2*B*). To determine the existence of the transgene by genomic PCR and to evaluate the transcription of the transgene by RT-PCR, a forward primer located in the second exon of the *insulin* gene (P1) and a reverse primer in the coding region of *anti-CTLA-4 scFv/eB7/B7TM* (P2), as indicated in Fig. 2*A*, were designed. Transgenic mice can be easily distinguished from nontransgenic littermates by PCR genotyping with P1 and P2 primers (Fig. 2*C*). Our RT-PCR results also demonstrated that the expression of transgene was islet-specific, because the spliced *anti-CTLA-4 scFv/eB7/B7TM* transcript was only detected in pancreas, not in PLN, spleen, liver, heart, or thymus (Fig. 2*D*). The expression level of anti-CTLA-4 scFv protein was measured by Western blot using rat anti-HA Ab. Consistent with the RNA level expression, anti-CTLA-4 scFv protein was also detected in pancreas of transgenic mice, not in PLN or spleen (Fig. 2*E*). These results demonstrate that the transgene expression driven by *insulin* promoter is highly stringent and strongly supports the availability and functionality of our transgenic model system.

Diabetes and insulitis in transgenic mice

To investigate the protective potential of this organ-specific and membrane-bound agonistic anti-CTLA-4 scFv on the development of autoimmune diabetes, we first compared the kinetics and incidence of spontaneous diabetes between transgenic mice and their nontransgenic littermates. Compared with control littermates that started to develop diabetes at 14 wk of age, the first transgenic mouse became diabetic after 17 wk, indicating a delay in disease onset. At around 21 wk of age, the incidence of diabetes in control mice increased rapidly to 59%, but the incidence in transgenic mice was only 7.4%. After 35 wk, more than 88% of control mice had developed diabetes, but the incidence in transgenic mice was around 40% (Fig. 3*A*), demonstrating that transgenic expression of anti-CTLA-4 scFv on pancreatic β cells significantly inhibits the development of autoimmune diabetes in NOD mice ($p < 0.0001$).

To investigate the modulatory effect of transgenic anti-CTLA-4 scFv on the development of insulitis, we used histological analysis to evaluate the severity of insulitis in transgenic and control mice at the age of 13–14 wk. Our results indicate that >41% of islets isolated from transgenic mice were free from mononuclear cell infiltration, while only 16% of islets were intact in their control littermates ($p < 0.01$). Moreover, around 20% of transgenic islets showed severe insulitis, compared with 40% of control islets ($p < 0.01$, Fig. 3*B*). These data demonstrate that local expression of transgenic anti-CTLA-4 scFv efficiently attenuates the infiltration of mononuclear cells to islets in NOD mice.

It has been well characterized that an imbalance between Th1 and Th2 responses predisposes NOD mice to developing autoimmune diabetes and the pathogenic T cells inside islets are mostly IFN-γ-producing Th1 cells. To investigate whether the transgenic expression of anti-CTLA-4 scFv down-regulates islet-infiltrating Th1 cells, we collected pancreata and isolated the islet-infiltrating T cells. Results from intracellular cytokine analyses clearly indicated that the percentage of IFN-γ-producing Th1 cells infiltrating islets from each transgenic mouse is markedly lower than that of control islets from its paired nontransgenic littermate ($p = 0.0011$,

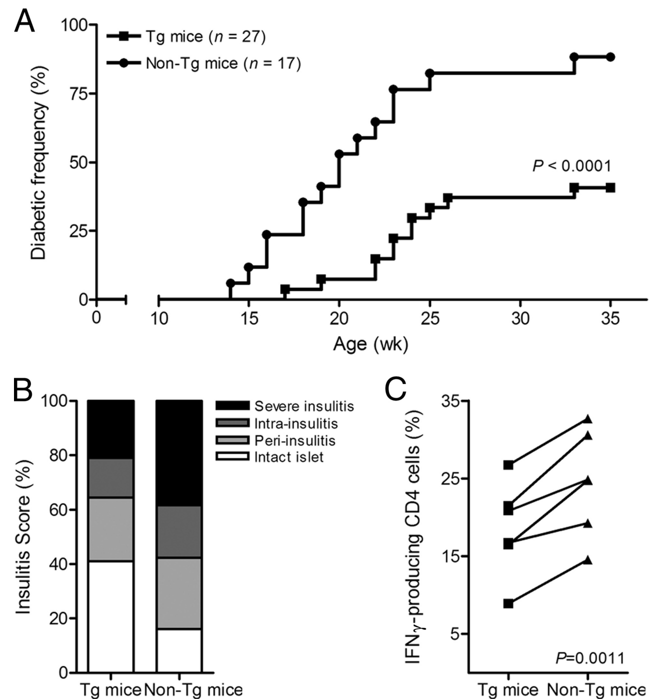

FIGURE 3. Characterization of the diabetogenic process in anti-CTLA-4 scFv transgenic mice. *A*, Urine glucose concentrations of female transgenic mice and control littermates were monitored weekly for spontaneous diabetes incidence. Diabetes was defined as glycosuria >500 mg/dl at two consecutive tests. *B*, The severity of insulitis was scored for the degree of mononuclear cell infiltration. Cumulative results in each group were determined from six mice at the age of 13–14 wk and at least 100 islets in total. *C*, The islet-infiltrating lymphocytes were isolated and stimulated with PMA and Ionomycin in the presence of monensin for 4–5 h. The IFN-γ-producing CD4 cells were analyzed by flow cytometry.

Fig. 3*C*), suggesting that β cell-specific anti-CTLA-4 scFv significantly mitigates the pathogenic Th1 cell infiltration in transgenic islets and thus protects the NOD mice from the development of diabetes.

Lymphocyte development in transgenic mice

It has been well characterized that Th1 cells play an important role in the pathogenesis of autoimmune diabetes, and Th2 cells are believed to suppress the development of diabetes (26). To study whether anti-CTLA-4 scFv-mediated protection in transgenic mice is through the regulation of Th1 and Th2 cell development, we crossed anti-CTLA-4 scFv transgenic mice to NOD-Th1/2 double transgenic mice and monitored the development of Th1 and Th2 cells in these anti-CTLA-4 scFv/Th1/2 triple transgenic mice. NOD-Th1/2 double transgenic mice carry two transgenes: human *Thy1* (*hThy1*, *hCD90*) and murine *Thy1.1* (*mThy1.1*, *mCD90.1*) driven by the murine *IFN-γ* and *IL-4* promoters, respectively (16). Based on this double transgenic NOD mouse model, we can kinetically monitor and reliably distinguish Th1 ($CD4^+hCD90^+$) and Th2 ($CD4^+mCD90.1^+$) cells by cell surface staining in vivo or in vitro. The total cell numbers and proportion of each lymphocyte subpopulation (CD4, CD8, and CD19) in the spleen (Fig. 4*A*, *left*) or PLN (Fig. 4*B*, *left*) were indistinguishable in anti-CTLA-4 scFv/Th/1/2 triple transgenic and their Th1/2 double transgenic littermates at the age of 8 wk, indicating that islet-specific expression of anti-CTLA-4 scFv does not interfere with the systemic development of lymphocytes in NOD mice. Additionally, the absolute cell numbers and percentages of Th1 and Th2 cells in spleen (Fig. 4*A*, *right*) or PLN (Fig. 4*B*, *right*) were not different between

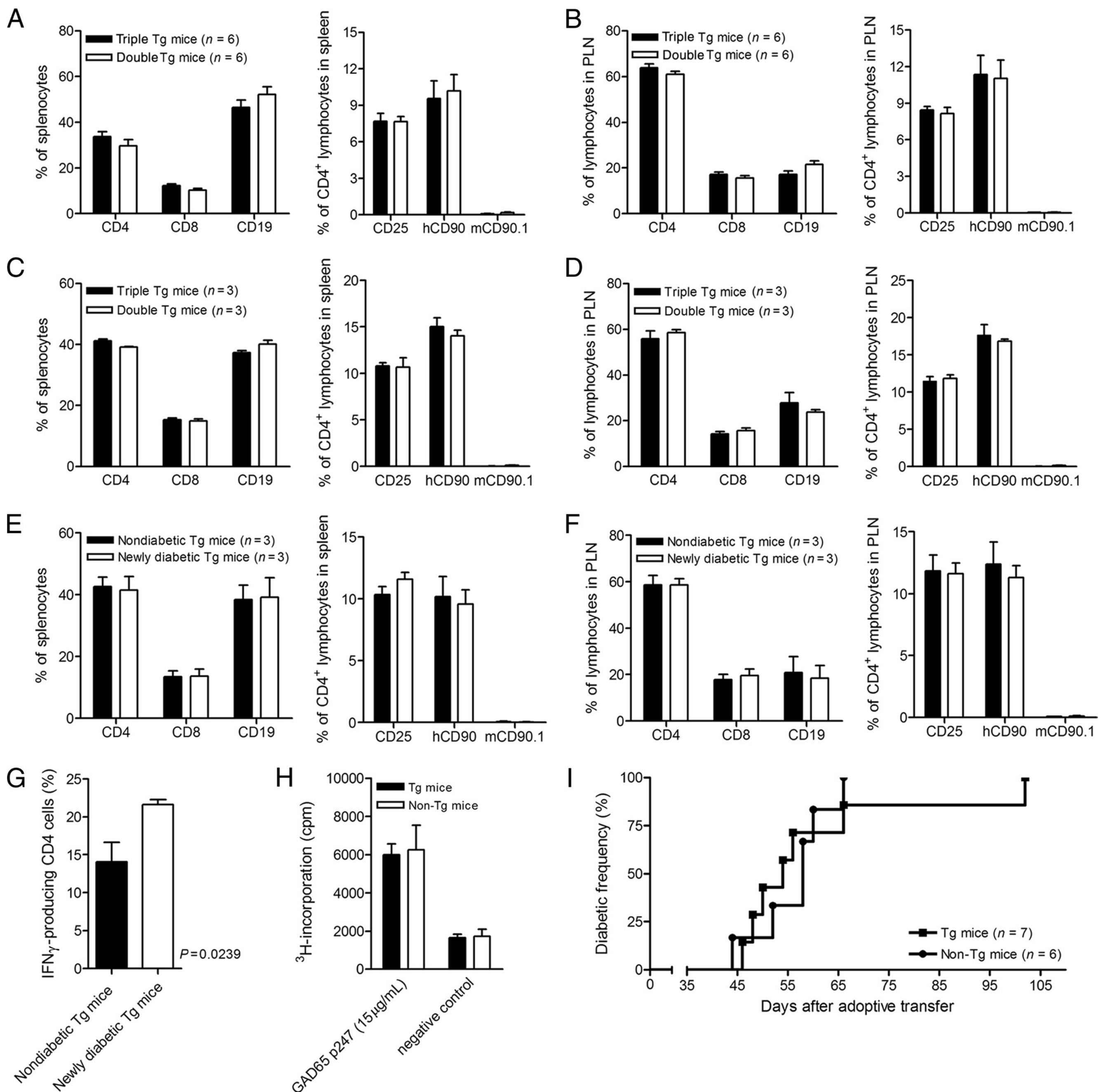

FIGURE 4. Lymphocyte development and diabetogenic properties of T cells in anti-CTLA-4 scFv transgenic mice. The percentage of $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells in spleen and PLN from 8-wk-old (*A* and *B*, *left*) or 13- to 14-wk-old (*C* and *D*, *left*) female triple and double transgenic mice were analyzed by flow cytometry. The percentage of Th1 ($CD4^+hCD90^+$), Th2 ($CD4^+mCD90.1^+$), and Treg ($CD4^+CD25^+$) cells in spleen and PLN from 8-wk-old (*A* and *B*, *right*) or 13- to 14-wk-old (*C* and *D*, *right*) were analyzed. *E* and *F*, The lymphocyte composition in spleen (*E*, *left*) and PLN (*F*, *left*) from nondiabetic or newly diabetic female triple transgenic mice were analyzed by flow cytometry. The percentage of Th1, Th2, and Treg cells in spleen (*E*, *right*) and PLN (*F*, *right*) were analyzed. *G*, The islet-infiltrating lymphocytes isolated from nondiabetic or newly diabetic transgenic mice ($n = 3$) were stimulated with PMA and Ionomycin in the presence of monensin for 4–5 h. The IFN-γ-producing CD4 cells were analyzed by flow cytometry. *H*, Splenocytes from 8-wk-old female mice were stimulated with synthetic GAD65 peptide p247–266 in vitro. The GAD65-specific T cell response was measured by the incorporation of [^{3}H]thymidine. *I*, Adoptive transfer of splenocytes from 12-wk-old female transgenic mice or nontransgenic littermates to NOD/SCID female recipients by i.v. injection. Glycosuria of recipients was measured every second day. Diabetes was defined as glycosuria >500 mg/ml at two consecutive tests.

triple and double transgenic mice, suggesting that anti-CTLA-4 scFv-mediated protection in NOD mice is not through suppressing the development of Th1 cells or inducing Th2 cells.

It has been well characterized that $CD4^+CD25^+$ regulatory T cells (Treg) can inhibit the activity of self-reactive T cells and maintain immune tolerance (27, 28). CTLA-4, which is constitutively expressed on Treg cells, has been reported to be crucial for the immunosuppressive function of these cells (29, 30). Previous studies have demonstrated that blocking CTLA-4 can affect the suppressive function of Treg cells and abolish the Treg-mediated protection against autoimmune colitis and transplant rejection (29, 31, 32). Moreover, previous reports indicated that NOD mice have

a deficiency in $CD4^+CD25^+$ Treg cells (33). To investigate whether the protection in NOD mice transgenic for anti-CTLA-4 scFv works through enhancing the development or activity of Treg cells, we first analyzed the number and percentage of $CD4^+$ $CD25^+$ Treg cells in transgenic mice. Our results indicated that the numbers and percentages of $CD4^+CD25^+$ Treg cells in spleen (Fig. 4*A*, *right*) or PLN (Fig. 4*B*, *right*) were indistinguishable between anti-CTLA-4 scFv transgenic and nontransgenic littermates, suggesting that the β cell-specific expression of anti-CTLA-4 scFv is not able to increase the number of Treg cells.

To investigate whether the anti-CTLA-4 scFv influences lymphocyte development in the transgenic mice at the elder age, we performed the experiment to analyze the lymphocyte development in spleen and PLN from 13- to 14-wk-old or 19-wk-old triple and double transgenic mice. The total cell numbers and proportion of each lymphocyte subpopulation in the spleen or PLN were indistinguishable in triple and double transgenic mice at the age of 13–14 wk (Fig. 4, *C* and *D*, *left*) or 19 wk (data not shown). Besides, the absolute cell numbers and percentages of Th1/Th2 or Treg in spleen or PLN were also indistinguishable between triple and double transgenic mice at the age of 13–14 wk (Fig. 4, *C* and *D*, *right*) or 19 wk (data not shown). These results indicated that the lymphocyte development was not affected by transgene in different age.

To further investigate whether transgenic mice developing diabetes differed from transgenic mice protected from disease with regard to lymphocyte composition in spleen, PLN and islets, we analyzed the lymphocyte composition in spleen, PLN, and pancreatic islets from nondiabetic or newly diabetic triple transgenic mice. The lymphocyte compositions in spleen (Fig. 4*E*) or PLN (Fig. 4*F*) were indistinguishable between nondiabetic and newly diabetic transgenic littermates. However, the percentage of IFN-γ-producing CD4 cells in islets from nondiabetic transgenic mice is lower than that of islets from newly diabetic transgenic mice (Fig. 4*G*), indicating the pathogenic role of Th1 cells in the development of autoimmune diabetes.

Diabetogenic properties of T cells in transgenic mice

To investigate whether local expression of anti-CTLA-4 scFv affects the diabetogenicity of systemic T cells, we performed in vitro T cell proliferation assays to examine the GAD65-specific T cell response in transgenic mice. GAD65 is one of the key autoantigens in NOD mice and GAD65-specific T cell activity is responsible for the diabetogenic process in IDDM. The levels of T cell proliferative responses to synthetic GAD65 peptide (247–266) of 8-wk-old transgenic mice were similar to those observed from age-matched control littermates (Fig. 4*H*). This result indicates that anti-CTLA-4 scFv transgenic mice protected from autoimmune diabetes retain a significant autoantigen-specific T cell response. To further analyze the diabetogenic properties of lymphocytes in vivo, we adoptively transferred splenocytes from 12-wk-old transgenic mice or their nontransgenic controls to 8-wk-old NOD/SCID recipients. The two groups of recipients developed diabetes with similar time of onset, diabetic process, and severity (Fig. 4*I*), strongly suggesting that transgenic anti-CTLA-4 scFv protects islets in situ instead of down-regulating the diabetogenicity of lymphocytes by systemically altering T cell function.

Transgenic expression of anti-CTLA-4 scFv on pancreatic β cells inhibits T cell response to immobilized anti-CD3

To evaluate whether transgenic expression of anti-CTLA-4 scFv on pancreatic β cells alters T cell function, we performed T cell proliferation assays in an islet-T cell coculture system. Islets isolated from transgenic mice or their nontransgenic littermates were dissociated into single cells and then verified for the expression of anti-CTLA-4 scFv by FITC-conjugated anti-HA Ab. The expression of anti-CTLA-4 scFv was significant and could only be detected on transgenic islet cells (80–85% positive in transgenic islet cells vs 0% in nontransgenic islet cells, Fig. 5*A*). For coculture experiments, T cells isolated from 7- to 9-wk-old nontransgenic NOD mice were first stimulated with anti-CD3 Ab then coincubated with islet cells either from transgenic or control mice. Islet cells are well-differentiated and almost nondividing cells, so the [^{3}H]thymidine incorporation in islet cells is very low and can be ignored (data not shown). Data from the coculture experiment demonstrated that T cell proliferation in response to immobilized anti-CD3 was significantly inhibited by coincubation with islet cells expressing anti-CTLA-4 scFv compared with control islet cells ($p < 0.005$, Fig. 5*B*). This result indicates that transgenic anti-CTLA-4 scFv on pancreatic β cells acts as a CTLA-4 agonist to down-regulate the response of T cells.

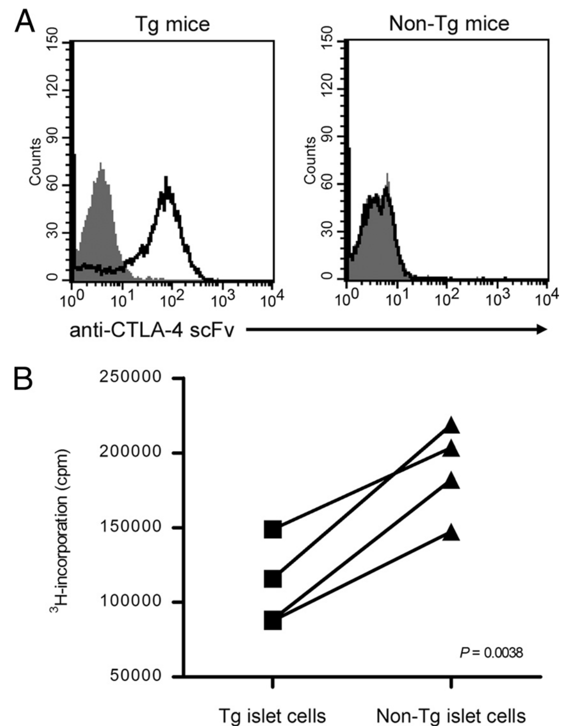

FIGURE 5. Transgenic expression of anti-CTLA-4 scFv on pancreatic β cells inhibits the T cell response to immobilized anti-CD3. Islets isolated from 6- to 8-wk-old female mice were treated with trypsin-EDTA, and dissociated into single cells. *A*, Anti-CTLA-4 scFv expression on single islet cells was measured by staining the cells with FITC-conjugated anti-HA Ab (shown as open histograms). Shaded histograms indicate the results for the unstained control. *B*, Splenocytes (5×10^5) of 7- to 9-wk-old female NOD mice were prestimulated with immobilized anti-CD3 (0.1 μg), and then the single islet cells (1×10^4) were added. Proliferation was determined by [^{3}H]thymidine incorporation. The T cell response to immobilized anti-CD3 was significantly inhibited by islet cells isolated from anti-CTLA-4 scFv mice ($p < 0.005$).

Transgenic anti-CTLA-4 scFv prolongs islet graft survival and attenuates the infiltration of Th1 cells in the implanted area

Transplantation of pancreatic islets into a diabetic recipient is a potential strategy to cure IDDM. To investigate whether transgenic expression of anti-CTLA-4 scFv in transplanted islets could reverse diabetes in recent-onset recipients and protect β cells against immune attack, we performed islet transplantation. We isolated islets from transgenic or control mice and implanted them into the

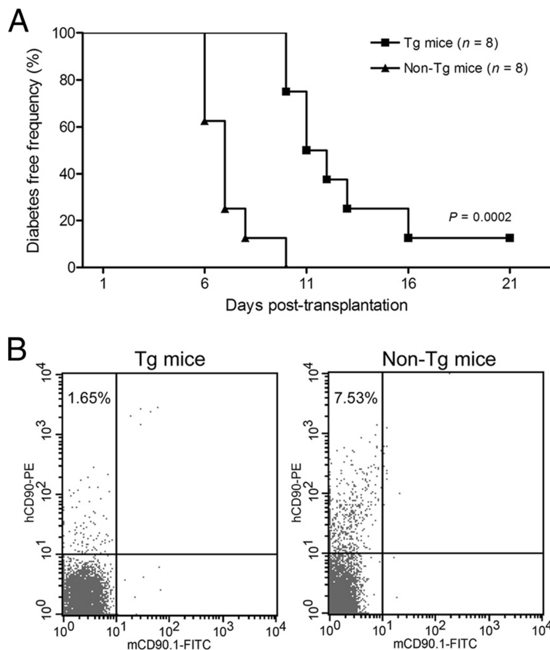

FIGURE 6. Transgenic anti-CTLA-4 scFv prolongs islet graft survival and attenuates the infiltration of Th1 cells to the implanted area. *A*, Islets isolated from young (<8-wk-old) male mice were transplanted into newly diabetic female NOD mice. Blood glucose of recipients was monitored daily after islet transplantation. A successful transplantation procedure was defined as the nonfasting blood glucose returning to normal (<200 mg/dl) for two consecutive days after transplantation, and loss of graft function was defined as blood glucose >300 mg/dl at two consecutive evaluations. *B*, Lymphocytes isolated from islet grafts of anti-CTLA-4 scFv transgenic (*left*) or control (*right*) donors implanted to diabetic NOD-Th1/2 transgenic mice were analyzed by flow cytometry. $CD4^+$ lymphocytes were further separated into Th1 ($CD4^+hCD90^+$) or Th2 ($CD4^+mCD90.1^+$) cells. The percentage of $CD4^+$ lymphocytes is as indicated.

left kidney capsule of newly diabetic female NOD recipients. In most recipients implanted with control islets hyperglycemia recurred within 7 days of transplantation, and the mean graft survival time was 6.125 days. All recipients grafted with transgenic islets maintained normoglycemia for at least nine days, and the mean graft survival time was >12.125 days ($p < 0.0005$; Fig. 6*A*). One recipient with grafted transgenic islets remained normoglycemic at day 21, and nephrectomy was performed to confirm that the normoglycemia in this recipient was due to a functional islet graft. These results indicate that transgenic expression of anti-CTLA-4 scFv in grafted islets can significantly prolong the survival of β cells in diabetic recipients.

It has been demonstrated that Th1 cytokines such as IFN-γ, IL-2, and TNF-α are involved in the destruction of syngeneic islet grafts and result in diabetes recurrence in NOD mice (34, 35). To investigate whether transgenic expression of anti-CTLA-4 scFv in transplanted islets further modulates the infiltration of Th1 cells, we analyzed the number and percentage of Th1 cells ($CD4^+hCD90^+$) infiltrating implanted sites by using NOD-Th1/2 mice as diabetic recipients. Data from flow cytometric analysis clearly showed a significant decrease in the number and percentage of Th1 cells in transgenic islet-implanted sites, compared with control islet-implanted sites (1.65 vs 7.53%, respectively; Fig. 6*B*). These results suggested that the longer survival of transgenic islet grafts may be, at least in part, due to the down-regulation of Th1 cell infiltration to the graft area.

Discussion

CTLA-4 or CD152 is an essential receptor involved in the negative regulation of T cell activation and effector function. Because of its profound inhibitory role, manipulation of CTLA-4 signaling by blockage or augmentation has been considered a sound strategy for cancer immunotherapy or in prevention/therapy of autoimmunity, respectively, for over a decade. Because CTLA-4 shares its ligands, B7-1 and B7-2, with CD28, it has been difficult to develop a strategy of selective engagement of this molecule in vivo. A new exploration of the use of surface-linked, anti-CTLA-4 scFv in autoimmune therapy has been recently reported (15). The long-term goal of immunotherapy for autoimmune diseases is to selectively inactivate and/or delete self-reactive lymphocytes, while avoiding systemic immunosuppression. Extending from this idea, we tested the hypothesis that islet-specific expression of an agonistic, membrane-bound, anti-CTLA-4 scFv could down-regulate the β cell-reactive pathogenic T lymphocytes and protect NOD mice from autoimmune diabetes. In the present study, we successfully generated transgenic NOD mice overexpressing anti-CTLA-4 scFv on the surface of β cells under the control of human *insulin* promoter. Transgene expression in these mice is highly β cell-specific, and mice are well protected from diabetes. Interestingly, our results demonstrated that β cell-specific expression of anti-CTLA-4 scFv did not alter the regular development of $CD4^+$, $CD8^+$, Th1, Th2, Treg, and B cells in spleen and PLN of transgenic mice. Moreover, local expression of membrane-bound anti-CTLA-4 scFv did not influence the diabetogenic properties of systemic lymphocytes, because splenocytes from transgenic mice responded equally to in vitro GAD65 peptide stimulation and had equal capacity to transfer diabetes in NOD/SCID recipients as did splenocytes from nontransgenic littermates. Nevertheless, expression of transgenic anti-CTLA-4 scFv on pancreatic β cells significantly inhibited the development of autoimmune diabetes in NOD mice, consistent with the result that transgene expression on pancreatic β cells significantly suppressed mononuclear cell infiltration in pancreatic islets. Based on these results, we concluded that pancreatic β cell-specific anti-CTLA-4 scFv protects NOD mice from autoimmune diabetes in an in situ manner, providing a theoretical basis for genetic manipulations in autoimmune prevention and/or therapy using an organ-specific strategy.

It has been well characterized that NOD mice gradually lose tolerance to β cell Ags and that this leads to an aggressive peripheral immunity to pancreatic islets. Previous studies have demonstrated that CTLA-4 engagement is required for the induction of peripheral T cell tolerance in vivo (36, 37). CTLA-4 signaling transduced by an agonistic, membrane-bound anti-CTLA-4 scFv has been reported to down-regulate T cell responses both in vitro and in vivo (13–15, 19). It has been proposed that CTLA-4 signaling requires cross-linking in conjunction with TCR engagement in an in *cis* manner. Thus, the effective negative signal through CTLA-4 engagement depends on the molecule being engaged together with the TCR signal from the same APC. Interestingly, our result demonstrated that β cells with anti-CTLA-4 scFv on their surface significantly inhibited the T cell response to plate-bound anti-CD3 Ab, suggesting an in *trans* signal provided by these transgenic islet cells. This result also suggests that anti-CTLA-4 scFv on β cells may directly interact with CTLA-4 on activated self-reactive T cells infiltrating islets and subsequently inhibit their pathogenicity. This is supported by our observation that both the severity of insulitis and β cell destruction in transgenic mice are markedly alleviated.

For generating agonistic single-chain Ab which can directly target on CTLA-4, membrane-bound form was the best choice (38, 39). In previous study, Chou et al. (38) and Liao et al. (39) analyzed the ability of different TM domains to target proteins (e.g., anti-CD3 scFv) to the plasma membrane of cells. They demonstrated that high surface expression was achieved with chimeric

proteins composed of target protein and the TM and CT of murine B7-1 as well as with target protein containing a GPI-anchor from decay-accelerating factor (38, 39). Griffin et al. (13) generated mem4F10 scFv (anti-CTLA-4 scFv) with B7 TM/CT or GPI anchor and tested its expression respectively. They demonstrated that high levels of surface expression were more consistently achieved using the B7 TM/CT. We therefore chose B7 TM/CT to target anti-CTLA-4 scFv to express on the plasma membrane of pancreatic β cells.

The study published by Grohmann et al. (40) indicated that B7 can transmit suppressive signals into dendritic cells (DCs), following engagement of CTLA-4. Their data showed that engagement of B7-1/B7-2 on murine DCs by CTLA-4-Ig stimulates DCs to produce IFN-γ in a STAT-1-dependent pathway and subsequently induce IDO, which degrades tryptophan to byproducts that inhibit T cell proliferation. Apparently, the presence of IFN-γ itself is absolutely essential for this B7-mediated reversed signaling because genetic deficiency of IFN-γ completely blocked the ability of CTLA-4-Ig to generate kynurenine in vitro. To our knowledge, not much evidence supports that STAT-1/IFN-γ pathway is active in pancreatic β cells, therefore, it is not clear the B7-1 CT in our transgenic construct would transmit suppressive signals into pancreatic β cells. Nevertheless, this interesting point needs to be further addressed.

A stringent way to test whether transgenic expression of anti-CTLA-4 scFv could protect β cells against immune attack is to examine whether transgenic islet grafts could reverse diabetes in spontaneously diabetic mice. Based on our results, transgenic anti-CTLA-4 scFv significantly prolonged the survival of islet grafts in newly diabetic recipients, demonstrating the therapeutic potential of these genetically modified islet grafts. However, transgenic expression of anti-CTLA-4 scFv in islet grafts did not provide permanent protection from diabetes recurrence, indicating that other mechanisms such as inflammatory cytokine-induced β cell stress (41, 42), perforin-mediated cytotoxicity (43), or elevated oxidative stress (25, 44) are involved in islet graft destruction, and that these are not able to be prevented by CTLA-4-mediated inhibition. Our laboratory previously demonstrated that islet-specific expression of transgenic decoy receptor 3 and PD-L1 protected mice from the development of autoimmune diabetes, and islet grafts from these transgenic mice survived better and longer in diabetic recipients, compared with islet grafts from their nontransgenic littermates (21, 45). Based on our findings and the fact that various immune cells and cytokine effectors are involved in the destruction of islet cells in autoimmune diabetes, we hypothesize that a "mixture" expression of various transgenes, such as transgenic decoy receptor 3, PD-L1, and anti-CTLA-4 scFv, with different protective effects and mechanisms can synergistically or additively make islet cells better and stronger in their resistance to immune attack. We are currently testing this hypothesis.

Rabinovitch and colleagues have demonstrated that mononuclear cells such as $CD4^+$, $CD8^+$, macrophages, and B cells infiltrating islet grafts and Th1 cytokines such as IL-2, IFN-γ, and TNF-α were involved in syngeneic islet graft rejection and diabetes recurrence in NOD mice (34, 35). Our flow cytometric data indicated that the major lymphocyte population infiltrating both transgenic and control islet grafts was $CD4^+$ T cells (data not shown), consistent with previous reports by other groups. To further dissect whether these graft-infiltrating $CD4^+$ T cells are IFN-γ-producing Th1 cells, we used newly diabetic NOD-Th1/2 double transgenic mice as recipients to directly analyze infiltrating $CD4^+$ T cells in islet grafts. Consistent with previous reports, we found that IFN-γ-producing Th1 cells were critically involved in islet graft destruction (35). Moreover, we observed that the number of IFN-γ-producing Th1 cells inside transgenic islet grafts was significantly attenuated compared with that infiltrating control islet grafts. Although transgenic islet grafts were infiltrated with fewer IFN-γ-producing Th1 cells, they were eventually destroyed. This may suggest two possibilities. One possible explanation may be that the *insulin* promoter-driven expression of anti-CTLA-4 scFv was disturbed by primary nonfunction of transplanted islets. Another possible explanation is that transplantation processes trigger strong oxidative stress- or cytokine-induced apoptosis of transplanted islets, which cannot be blocked by CTLA-4 agonist. Nevertheless, targeting CTLA-4 negative costimulatory pathways with membrane-bound anti-CTLA-4 scFv in an organ-specific manner is a potential strategy for preventing or curing autoimmune diabetes. Transgenic expression of surface-linked anti-CTLA-4 scFv on B cells nicely protects NOD mice from autoimmune diabetes (15), confirming the potential of CTLA-4 agonists in autoimmune diabetes. However, we take full advantage of this transgenic anti-CTLA-4 scFv in our animal model, because transgenic expression of anti-CTLA-4 scFv on pancreatic β cells not only provides better islet grafts for transplantation but also avoids systemic immunosuppression, providing a theoretical basis for an organ-specific immunotherapy for autoimmune diabetes.

Disclosures

The authors have no financial conflict of interest.

References

1. Bach, J.-F. 1994. Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr. Rev.* 15: 516–542.
2. Tisch, R., and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell* 85: 291–297.
3. Wicker, L. S., J. A. Todd, and L. B. Peterson. 1995. Genetic control of autoimmune diabetes in the NOD mouse. *Annu. Rev. Immunol.* 13: 179–200.
4. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1: 405–413.
5. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 356: 607–609.
6. Krummel, M. F., and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med.* 182: 459–465.
7. Luhder, F., C. Chambers, J. P. Allison, C. Benoist, and D. Mathis. 2000. Pinpointing when T cell costimulatory receptor CTLA-4 must be engaged to dampen diabetogenic T cells. *Proc. Natl. Acad. Sci. USA* 97: 12204–12209.
8. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3: 541–547.
9. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270: 985–988.
10. Luhder, F., P. Hoglund, J. P. Allison, C. Benoist, and D. Mathis. 1998. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes. *J. Exp. Med.* 187: 427–432.
11. Kristiansen, O. P., Z. M. Larsen, and F. Pociot. 2000. CTLA-4 in autoimmune diseases–a general susceptibility gene to autoimmunity? *Genes Immun.* 1: 170–184.
12. Chen, Z., J. Stockton, D. Mathis, and C. Benoist. 2006. Modeling CTLA4-linked autoimmunity with RNA interference in mice. *Proc. Natl. Acad. Sci. USA* 103: 16400–16405.
13. Griffin, M. D., D. K. Hong, P. O. Holman, K.-M. Lee, M. J. Whitters, S. M. O'Herrin, F. Fallarino, M. Collins, D. M. Segal, T. F. Gajewski, et al. 2000. Blockade of T cell activation using a surface-linked single-chain antibody to CTLA-4 (CD152). *J. Immunol.* 164: 4433–4442.
14. Hwang, K. W., W. B. Sweatt, I. E. Brown, C. Blank, T. F. Gajewski, J. A. Bluestone, and M.-L. Alegre. 2002. Cutting edge: targeted ligation of CTLA-4 in vivo by membrane-bound anti-CTLA-4 antibody prevents rejection of allogeneic cells. *J. Immunol.* 169: 633–637.
15. Fife, B. T., M. D. Griffin, A. K. Abbas, R. M. Locksley, and J. A. Bluestone. 2006. Inhibition of T cell activation and autoimmune diabetes using a B cell surface-linked CTLA-4 agonist. *J. Clin. Invest.* 116: 2252–2261.
16. Hung, J.-T., J.-H. Liao, Y.-C. Lin, H.-Y. Chang, S.-F. Wu, T.-H. Chang, J. T. Kung, S.-L. Hsieh, H. McDevitt, and H.-K. Sytwu. 2005. Immunopathogenic role of T_H1 cells in autoimmune diabetes: evidence from a T1 and T2 doubly transgenic non-obese diabetic mouse model. *J. Autoimmun.* 25: 181–192.

17. Tuve, S., B.-M. Chen, Y. Liu, T.-L. Cheng, P. Toure, P. S. Sow, Q. Feng, N. Kiviat, R. Strauss, S. Ni, et al. 2007. Combination of tumor site-located CTL-associated antigen-4 blockade and systemic regulatory T-cell depletion induces tumor-destructive immune responses. *Cancer Res.* 67: 5929–5939.
18. Gilliland, L. K., N. A. Norris, H. Marquardt, T. T. Tsu, M. S. Hayden, M. G. Neubauer, D. E. Yelton, R. S. Mittler, and J. A. Ledbetter. 1996. Rapid and reliable cloning of antibody variable regions and generation of recombinant single chain antibody fragments. *Tissue. Antigens* 47: 1–20.
19. Griffin, M. D., P. O. Holman, Q. Tang, N. Ashourian, U. Korthauer, D. M. Kranz, and J. A. Bluestone. 2001. Development and applications of surface-linked single chain antibodies against T-cell antigens. *J. Immunol. Methods* 248: 77–90.
20. Sytwu, H.-K., W.-D. Lin, S. R. Roffler, J.-T. Hung, H.-S. Sung, C.-H. Wang, T.-L. Cheng, S.-C. Tsou, S.-C. Hsi, and K.-L. Shen. 2003. Anti-4-1BB-based immunotherapy for autoimmune diabetes: lessons from a transgenic non-obese diabetic (NOD) model. *J. Autoimmun.* 21: 247–254.
21. Sung, H.-H., J.-H. Juang, Y.-C. Lin, C.-H. Kuo, J.-T. Hung, A. Chen, D.-M. Chang, S.-Y. Chang, S.-L. Hsieh, and H.-K. Sytwu. 2004. Transgenic expression of decoy receptor 3 protects islets from spontaneous and chemical-induced autoimmune destruction in nonobese diabetic mice. *J. Exp. Med.* 199: 1143–1151.
22. Kurasawa, K., T. Koike, R. Matsumura, K. Takabayashi, H. Tomioka, I. Ito, and S. Yoshida. 1990. The immunosuppressant FK-506 prevents progression of diabetes in nonobese diabetic mice. *Clin. Immunol. Immunopathol.* 57: 274–279.
23. Kaufman, D. L., M. Clare-Salzler, J. Tian, T. Forsthuber, G. S. P. Ting, P. Robinson, M. A. Atkinson, E. E. Sercarz, A. J. Tobin, and P. V. Lehmann. 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 366: 69–72.
24. Juang, J.-H., C.-H. Kuo, and B. R.-S. Hsu. 2002. Effects of multiple site implantation on islet transplantation. *Transplant. Proc.* 34: 2698–2699.
25. Hotta, M., F. Tashiro, H. Ikegami, H. Niwa, T. Ogihara, J. Yodoi, and J.-i. Miyazaki. 1998. Pancreatic β cell-specific expression of thioredoxin, an antioxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes. *J. Exp. Med.* 188: 1445–1451.
26. Liblau, R. S., S. M. Singer, and H. O. McDevitt. 1995. Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today* 16: 34–38.
27. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184: 387–396.
28. Suri-Payer, E., A. Z. Amar, A. M. Thornton, and E. M. Shevach. 1998. $CD4^+CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* 160: 1212–1218.
29. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
30. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192: 303–310.
31. Kingsley, C. I., M. Karim, A. R. Bushell, and K. J. Wood. 2002. $CD25^+CD4^+$ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J. Immunol.* 168: 1080–1086.
32. Tang, Q., E. K. Boden, K. J. Henriksen, H. Bour-Jordan, M. Bi, and J. A. Bluestone. 2004. Distinct roles of CTLA-4 and TGF-β in $CD4^+CD25^+$ regulatory T cell function. *Eur. J. Immunol.* 34: 2996–3005.
33. Kukreja, A., G. Cost, J. Marker, C. Zhang, Z. Sun, K. Lin-Su, S. Ten, M. Sanz, M. Exley, B. Wilson, et al. 2002. Multiple immuno-regulatory defects in type-1 diabetes. *J. Clin. Invest.* 109: 131–140.
34. Rabinovitch, A., O. Sorensen, W. L. Suarez-Pinzon, R. F. Power, R. V. Rajotte, and R. C. Bleackley. 1994. Analysis of cytokine mRNA expression in syngeneic islet grafts of NOD mice: interleukin 2 and interferon γ mRNA expression correlate with graft rejection and interleukin 10 with graft survival. *Diabetologia* 37: 833–837.
35. Suarez-Pinzon, W., R. V. Rajotte, T. R. Mosmann, and A. Rabinovitch. 1996. Both $CD4^+$ and $CD8^+$ T-cells in syngeneic islet grafts in NOD mice produce interferon-γ during β-cell destruction. *Diabetes* 45: 1350–1357.
36. Greenwald, R. J., V. A. Boussiotis, R. B. Lorsbach, A. K. Abbas, and A. H. Sharpe. 2001. CTLA-4 regulates induction of anergy in vivo. *Immunity* 14: 145–155.
37. Perez, V. L., L. Van Parijs, A. Biuckians, X. X. Zheng, T. B. Strom, and A. K. Abbas. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* 6: 411–417.
38. Chou, W.-C., K.-W. Liao, Y.-C. Lo, S. Y. Jiang, M. Y. Yeh, and S. R. Roffler. 1999. Expression of chimeric monomer and dimer proteins on the plasma membrane of mammalian cells. *Biotechnol. Bioeng.* 65: 160–169.
39. Liao, K.-W., Y.-C. Lo, and S. R. Roffler. 2000. Activation of lymphocytes by anti-CD3 single-chain antibody dimers expressed on the plasma membrane of tumor cells. *Gene. Ther.* 7: 339–347.
40. Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat. Immunol.* 3: 1097–1101.
41. Augstein, P., G. Wachlin, S. Berg, J. Bahr, C. Salzsieder, B. Hehmke, P. Heinke, and E. Salzsieder. 2003. Surface and intracellular Fas expression associated with cytokine-induced apoptosis in rodent islet and insulinoma cells. *J. Mol. Endocrinol.* 30: 163–171.
42. Drage, M., P. Zaccone, J. M. Phillips, F. Nicoletti, J. Dawson, J. Andrew Bradley, and A. Cooke. 2002. Nondepleting anti-CD4 and soluble interleukin-1 receptor prevent autoimmune destruction of syngeneic islet grafts in diabetic NOD mice. *Transplantation* 74: 611–619.
43. Kagi, D., B. Odermatt, P. Seiler, R. M. Zinkernagel, T. W. Mak, and H. Hengartner. 1997. Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. *J. Exp. Med.* 186: 989–997.
44. Sandstrom, J., L. M. Jonsson, H. Edlund, D. Holmberg, and S. L. Marklund. 2002. Overexpression of extracellular-SOD in islets of nonobese diabetic mice and development of diabetes. *Free. Radical Biol. Med.* 33: 71–75.
45. Wang, C.-J., F.-C. Chou, C.-H. Chu, J.-C. Wu, S.-H. Lin, D.-M. Chang, and H.-K. Sytwu. 2008. Protective role of programmed death 1 ligand 1 (PD-L1) in nonobese diabetic mice: the paradox in transgenic models. *Diabetes* 57: 1861–1869.