



## Anti-4-1BB-based immunotherapy for autoimmune diabetes: lessons from a transgenic non-obese diabetic (NOD) model

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### Abstract

Various therapeutic strategies have been developed to tolerize autoreactive T cells and prevent autoimmune pathology in type 1 diabetes. 4-1BB, a member of the tumor necrosis factor receptor (TNFR) superfamily, is a costimulatory receptor primarily expressed on activated T cells. The administration of an agonistic anti-4-1BB antibody (2A) dramatically reduced the incidence and severity of experimental autoimmune encephalomyelitis (EAE). Treatment with the same antibody in Fas-deficient MRL/lpr mice blocked lymphadenopathy and lupus-like autoimmune processes. Paradoxically, transgenic non-obese diabetic (NOD) mice overexpressing membrane-bound agonistic single-chain anti-4-1BB Fv in pancreatic  $\beta$  cells developed more severe diabetes than their non-transgenic littermates, with earlier onset, faster diabetic processes, and higher mortality. Forty percent of transgenic mice developed diabetes by 4 weeks of age, compared with their control littermates, which first exhibited diabetes at 14 weeks. The frequency of diabetes in female transgenics reached 70% by 8 weeks of age. Most female transgenic mice died around 12 weeks. Consistent with this, transgenic mice developed earlier and more severe insulinitis and showed stronger GAD-specific T-cell responses, compared with age-matched control littermates. Our results indicate an adverse effect of transgenic anti-4-1BB scFv in NOD mice and suggest a potential risk of this anti-4-1BB-based immunotherapy for autoimmune diseases.

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### 1. Introduction

Insulin-dependent diabetes mellitus (IDDM) is caused by progressive autoimmune destruction of the insulin-producing  $\beta$  cells in the pancreatic islets of Langerhan. Both genetic predisposition and environmental factors contribute to its pathogenesis. A widely used animal model for dissecting the immunopathologi-

cal mechanisms in IDDM and for developing preventive or therapeutic strategies is the NOD mouse, an inbred strain that spontaneously develops autoimmune diabetes resembling human IDDM [1–3]. Obviously, the amelioration of the autoreactive T-cell responses or the induction of autoantigen-specific tolerance will be the key to preventing or curing IDDM. Recently, some newly identified molecules or approaches have been reported that regulate T-cell functions *in vitro*. 4-1BB (CDw137), a member of the tumor necrosis factor receptor (TNFR) superfamily, mediates both CD28-dependent and -independent T-cell costimulation [4], and 4-1BB-based

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immunomodulation has been applied in tumor rejection [5], allograft transplantation [6], viral infection [7], experimental autoimmune encephalomyelitis (EAE) [8], and lupus autoimmune disease [9].

4-1BB is mainly expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells and natural killer (NK) cells. Its natural ligand, the 4-1BB ligand (4-1BBL), a member of the TNF superfamily, has been detected on activated macrophages, dendritic cells, and T and B cells [4]. 4-1BB signaling augments T-cell proliferation and cytokine production through both CD28-dependent and -independent mechanisms [10]. In addition, agonistic anti-4-1BB monoclonal antibodies (mAbs) preferentially stimulate proliferation of, and interferon (IFN)- $\gamma$  production by, CD8<sup>+</sup> T cells [11]. Consistent with this, in vivo experiments using agonistic 4-1BB mAbs or 4-1BBL-transfected tumor cells have shown that signaling through 4-1BB can induce the preferential expansion of CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) that recognize and reject tumors and allograft transplants [5,6,11,12]. This antibody also prevents superantigen-induced T-cell death, preferentially of CD8<sup>+</sup> T cells [13]. Furthermore, a study of immune responses in 4-1BBL-knockout mice indicated that the CD8<sup>+</sup> T-cell response to viral infections is impaired [6,7]. It is therefore obvious that activation via 4-1BB is required for the induction and maintenance of optimal CD8<sup>+</sup> T-cell-mediated immune responses in vivo. Similarly, the role of 4-1BB in CD4<sup>+</sup> T-cell function has been studied. A previous study demonstrated that 4-1BB can transduce a costimulatory signal for T-cell activation in the absence of CD28/B7 interactions by using an MHC class II-restricted autoreactive T-cell hybridoma or purified CD4<sup>+</sup> T cells [14]. Another study, using pigeon cytochrome-c-presenting fibroblasts transfected with 4-1BBL, demonstrated that 4-1BB engagement on naive CD4<sup>+</sup> T cells promotes proliferation, cell-cycle progression, and interleukin (IL)-2 secretion [15].

A new exploration of the use of anti-4-1BB mAb in autoimmune therapy has been reported recently. Treatment with an agonistic 4-1BB-specific mAb (2A) initially promoted the activation and proliferation of antigen-specific CD4<sup>+</sup> T cells and subsequently increased their probability of undergoing activation-induced cell death, thereby inhibiting both effector and relapsing T-cell responses in an experimental autoimmune encephalomyelitis (EAE) model [8]. Moreover, 2A treatment blocked spontaneous autoimmune lymphadenopathy and lupus-like disease in Fas-deficient MRL/lpr mice [9]. In this study, anti-4-1BB treatment rapidly enhanced IFN- $\gamma$  production and triggered the depletion of autoreactive B cells and abnormal double-negative T cells. The 2A-induced apoptosis of these autoreactive cells may act through Fas- and TNFR-independent mechanisms. These results indicate that the agonistic 2A mAb specific for the 4-1BB costimulatory molecule can be used as a

novel therapeutic agent to delete autoreactive lymphocytes and inhibit autoimmune disease progression. To further address the preventive/therapeutic potential of this antibody in autoimmune diabetes, we generated transgenic NOD mice overexpressing a single-chain anti-4-1BB Fv (anti-4-1BB scFv) on pancreatic  $\beta$  cells. Unexpectedly, we found that transgenic mice developed more severe diabetes than their non-transgenic littermates, in terms of earlier onset, faster diabetic process, and higher mortality rate. Our results indicate an adverse effect of transgenic anti-4-1BB scFv in NOD mice and suggest a potential risk with this anti-4-1BB-based immunotherapy for autoimmune diseases.

## 2. Materials and methods

### 2.1. Mice

Female NOD/Sytwu (K<sup>d</sup>, D<sup>b</sup>, L<sup>d</sup>, I-A<sup>g7</sup>) mice were initially purchased from Jackson Laboratory (Bar Harbor, ME, USA), and were subsequently bred and raised at the Animal Center of the National Defence Medical Center (Taipei, Taiwan) under specific pathogen-free conditions. The spontaneous incidence of diabetes in the colony is currently 80–90% in females and 20–30% in males by 25 weeks of age. The mice used to provide single-cell embryos after superovulation were aged from 4 to 6 weeks.

### 2.2. Construction and expression of single-chain anti-4-1BB Fv gene

The IgG-like C-type domain, the transmembrane domain, and the cytoplasmic tail of murine CD80 were PCR-amplified from pCD80/zeo with primers 5'-AAAGTCGACGCTGACTTCTCTACCCCCAACATAACT-3' and 5'-AACTCGAGCTAAAGGAAGACGGTCTGTTCAGC-3'. The PCR product was cut with *SalI* and *XhoI* and inserted into p2C11-B7 [16] to replace the B7 transmembrane domain and cytoplasmic tail, to generate p2C11-eB7. The anti-4-1BB single-chain Fv (scFv) was cloned from total RNA isolated from 2A hybridoma cells (a gift from Dr Chen Lieping, the Mayo Clinic, Minnesota, USA) following the method of Gilliland et al. [17,18]. Briefly, total RNA was isolated from 2A hybridoma cells as described previously [19]. cDNA was generated by reverse transcription of total RNA with the primers 5'-GTYTTRTGNGTNACYTCRCA-3' for the light chain and 5'-WRTCRCANGCNGGNGCNARNGG-3' for the heavy chain (note: Y=C+T; R=A+G; N=A+C+T+G; W=A+T; D=A+T+G). PolyG was added to the 3' end of the first-strand cDNA by the addition of dGTP in the presence of terminal transferase. The reaction product was amplified by touchdown PCR [20] using primers

5'-CGTCGATGAGCTCTAGAATTCGCATGTGCAA GTCCGATGGTCCCCCCCCCCCCCCC-3' and 5'-CGT CATGTCGACGGATCCAAGCTTACYTTCCAYTTN ACRTTDAATRTC-3' for the light chain variable region, and 5'-CGTCGATGAGCTCTAGAATTCGCATGTGC AAGTCCGATGGTCCCCCCCCCCCCCCC-3' and 5'-CGTCATGTCGACGGATCCAAGCTTRCANGCN GGNGCNARNGGRTANAC-3' for the heavy chain variable region. The PCR products were cloned into pBLUNT and three independent clones were sequenced. The variable light ( $V_L$ ) chain cDNA sequences of 2A were amplified with primers 5'-TGTGCTGGGGCC CAGCCGGCCGACATCATGATGACCCAGTCTCC T-3' and 5'-GCCTCCACCTGATCCACCACCACCT TTG ATTTCCACCTTGGTCCCAGC-3' to introduce a *SfiI* restriction site at the 5' end and half of the linker at the 3' end of the  $V_L$  cDNA. Similarly, primers 5'-GGAAGTGGAGGTGGCGGATCCATCCAGCT TCAGGAGTCAGGACCT-3' and 5'-TGTTGTGTGC ACTGTGGCTGAGGAGACGGTGACCAT-3' were used to introduce the other half of the linker to the 5' end and a *SalI* restriction site at the 3' end of the amplified  $V_H$  cDNA. The  $V_L$  and  $V_H$  PCR products were assembled in a second round of PCR with primers 5'-TGTGCTGGGGCCAGCCGGCCGACATCATG ATGACCCAGTCTCT-3' and 5'-TGTTGTGTGC ACTGTGGCTGAGGAGACGGTGACCAT-3', and a linker primer 5'-GGTGGTGGTGGATCGGGGAGG CGGAAGTGGAGGTGGCGGATCC-3' encoding a 15 amino acid residue linker peptide. The PCR product was digested with *SfiI* and *SalI* restriction enzymes and subcloned into p2C11-eB7 to replace 2C11 scFv, to generate p2A-eB7. The transgene was excised from p2A-eB7 by digestion with *EcoRV* and *XhoI*, treated with Klenow polymerase, and blunt-end ligated into pLNCX (Invitrogen, CA, USA) at the unique *HpaI* site.

BALB/3T3 cells were generously provided by Dr Chu Hsin of the National Health Research Institute, Taipei, Taiwan. Cells were cultured in Dulbecco's minimal essential medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C in an atmosphere of 5% CO<sub>2</sub>. All cells were free of mycoplasma as determined by a PCR-based mycoplasma detection kit (American Type Culture Collection). The plasmid pGC3-m4-1BB-IgG2a, encoding murine 4-1BB fused to murine IgG2a Fc, was generously provided by Dr Chen Lieping of the Mayo Clinic, Minnesota, USA. Stable BALB/3T3 fibroblasts were generated by cotransfection with pGC3-m4-1BB-IgG2a and pcDNA3 and selection in G418. A single clone was isolated by limiting dilutions without antibiotic selection. 4-1BB-IgG2a was further purified from the supernatant of confluent fibroblast transfectants on protein A Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden).

BALB/3T3 cells were cultured overnight in six-well plates before transfection with 3  $\mu$ g plasmid (pLNCX-anti-4-1BB scFv) and 10  $\mu$ l Lipofectamine according to the manufacturer's instructions (Gibco Laboratories, Grand Island, NY, USA). Transfected cells ( $5 \times 10^5$ ) were washed and suspended in 50  $\mu$ l DMEM containing 0.5% bovine serum. Expression of anti-4-1BB scFv on cells was measured by staining the cells with rat anti-HA (5  $\mu$ g/ml) followed by goat anti-rat IgG-FITC (4  $\mu$ g/ml) at 4 °C. To measure the functional activity of anti-4-1BB scFv on cells, recombinant 4-1BB-IgG2a was added, the cells were washed, and goat anti-mouse IgG-FITC added. The cells were washed and suspended in phosphate-buffered saline (PBS) containing 5  $\mu$ g/ml propidium iodide. The surface immunofluorescence of 5000–10,000 viable cells was measured with a FACS-Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Dead cells, identified by red propidium-iodide fluorescence, were gated out. Fluorescence intensities were analyzed with Flowjo v3.2 (Tree Star Inc., San Carlos, CA, USA).

### 2.3. Generation and screening of *pIns-anti-4-1BB Fv transgenic NOD mice*

A modified human insulin promoter, linked to the rabbit  $\beta$ -globin gene sequence from the second exon to the polyadenylation signal [21], was generously provided by Dr J. Miyazaki of Osaka University, Japan. The DNA fragment containing the anti-4-1BB scFv gene, constructed as described in the Materials and methods above, was cut with *HindIII* and *ClaI* and inserted into the pINS-1 plasmid to produce pINS-1-anti-4-1BB scFv. The DNA fragment with the human insulin promoter fused to the anti-4-1BB scFv gene was purified and microinjected into the pronuclei of one-cell NOD embryos, which were then implanted into pseudopregnant (Balb/c  $\times$  FVB) F1 females. Tail DNA of the offspring was digested with *BamHI*, resolved on agarose gels, transferred to nylon membranes, and hybridized with pINS-1-anti-4-1BB scFv DNA. Transgenic mice were crossed with NOD mice, and all analyses were performed with mice heterozygous for the transgene.

### 2.4. Insulinitis assessment

The pancreases were removed from female NOD transgenic mice of different ages and their transgene-negative littermates, and fixed with 20% formalin. After paraffin-embedded fixation, 3- $\mu$ m slices of each pancreas were prepared for hematoxylin and eosin staining. Twenty-five islets from each pancreas were randomly chosen for microscopic examination for insulinitis. According to the classification system of Kurasawa et al. [22], each islet was scored as follows: 0, an islet without

infiltration; 1, <25% of the area of an islet was occupied by immunocytes; 2, >25% but <50%; 3, >50%.

### 2.5. Diabetes monitoring

All mice (female transgenics from 3 weeks of age and non-transgenic littermates from 10 weeks of age) were checked for blood sugar levels and glycosuria every week using an Optium detection kit (Abbott Laboratories, MediSense Products, MA, USA) and Chemstrip UG (Boehringer Mannheim, Indianapolis, IN, USA), respectively. When animals were positive (>250 mg/dl) in two consecutive blood tests, they were scored as diabetic.

### 2.6. T-cell proliferation assay

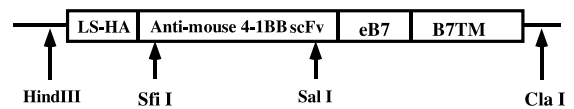
The glutamic acid decarboxylase (GAD)-specific lymphoproliferative responses of transgenic and control NOD mice were determined as described elsewhere [23]. Briefly, spleens were prepared from 5- and 7-week-old female transgenic and control mice. Splenic cell suspensions were treated with Tris-buffered ammonium chloride to eliminate the red blood cells, washed, and resuspended at a cell concentration of  $2.5 \times 10^6$ /ml in RPMI 1640 supplemented with 1% Nutridoma-SP (Boehringer Mannheim),  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol (Gibco BRL, Life Technologies, USA), 1 mM HEPES buffer (Gibco BRL, Life Technologies, USA), 1 mM sodium pyruvate (Gibco BRL, Life Technologies, USA),  $1 \times$  nonessential amino acids (Gibco BRL, Life Technologies, USA), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin (Merck, USA). Cells were cultured in triplicate in 96-well flat-bottomed microtiter plates ( $5 \times 10^5$  cells/200  $\mu$ l per well) in the presence of synthetic GAD peptides (p247–266; 30  $\mu$ g/ml), control ovalbumin peptide (p323–339; 30  $\mu$ g/ml), or concanavalin A (1  $\mu$ g/ml). After 48 h, the cultures were pulsed with 1  $\mu$ Ci of  $^3$ H-methyl thymidine (Amersham Pharmacia Biotech) and harvested 16 h later. The plates were harvested on to glass fiber, and the incorporated  $^3$ H-methyl thymidine was detected with TopCount (Packard, USA). Standard errors of the mean (SEM) were less than 15% of the mean.

## 3. Results

### 3.1. Construction and expression of single-chain anti-4-1BB Fv gene

Costimulatory-molecule-targeted antibody therapy has been applied to several autoimmune diseases. To create a chimeric gene encoding an antibody structure that manipulates the immune system in the same way as the efficacious monoclonal anti-4-1BB antibody, we constructed a vector, pLNCX-anti-4-1BB scFv, encod-

A



B

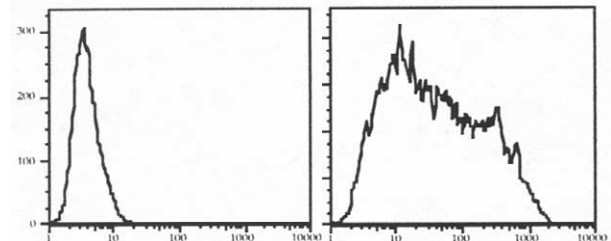
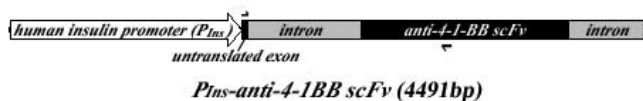


Fig. 1. Construction and expression of single-chain anti-4-1BB Fv gene. A vector, pLNCX-anti-4-1BB scFv, encoding an agonistic, cell-bound single-chain Fv fragment from 2A hybridoma was constructed. This chimeric receptor consists of a murine immunoglobulin  $\kappa$ -chain signal peptide and an HA epitope (indicated as LS-HA), the 2A anti-4-1BB scFv followed by the Ig-like C2-type and Ig hinge-like domains (indicated as anti-mouse 4-1BB scFv), extracellular portion (indicated as eB7), and the transmembrane domain (indicated as B7TM) of murine B7-1 (Fig. 1A). BALB/3T3 cells transfected with 3  $\mu$ g plasmid (pLNCX-anti-4-1BB scFv) and 10  $\mu$ l Lipofectamine were first stained with recombinant 4-1BB-murine IgG2a, washed, and subsequently with goat anti-mouse IgG-FITC (Fig. 1B, right panel). In the control group, the cells were first incubated with recombinant CD28-hamster IgG2a, washed, and then stained with goat anti-hamster IgG-FITC (Fig. 1B, left panel).

ing an agonistic, cell-bound single-chain Fv fragment from 2A. This chimeric receptor consists of a murine immunoglobulin  $\kappa$ -chain signal peptide, an HA epitope, the 2A anti-4-1BB scFv followed by the Ig-like C2-type and Ig hinge-like domains, and the transmembrane domain and cytosolic tail of murine B7-1 (Fig. 1A). Expression of this chimeric receptor on pLNCX-anti-4-1BB scFv-transfected cells was confirmed by flow cytometric analysis of cells stained with rat anti-HA followed by goat anti-rat IgG-FITC (data not shown). To further measure the functional activity of anti-4-1BB scFv on transfected cells, recombinant 4-1BB-murine IgG2a was added, the cells were washed, and then goat anti-mouse IgG-FITC was added (Fig. 1B, right panel). In the control group, the cells were first incubated with recombinant CD28-hamster IgG2a, then washed; and goat anti-hamster IgG-FITC was then added (Fig. 1B, left panel). This result clearly indicated that membrane-bound anti-4-1BB scFv encoded by the transgene bound specifically to the 4-1BB molecule. These transfectants were also shown functionally to augment T-cell activation and proliferation, indicating the availability of

A



B

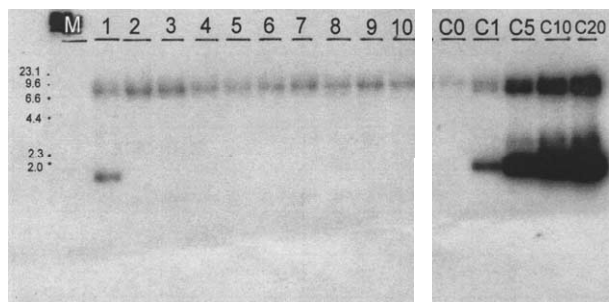


Fig. 2. Production of pIns-anti-4-1BB scFv transgenic mice. The schematic construction of pIns-anti-4-1BB scFv transgene is indicated (Fig. 2A). The transgene contains human insulin promoter (pIns), an intron from the rabbit  $\beta$  globin gene, chimeric anti-4-1BB scFv, and a poly A signal from the rabbit  $\beta$  globin gene. The DNA positions to which the primers for PCR and RT-PCR analysis are shown. Southern blot analysis of tail DNA from ten F1 mice (Fig. 2B, left panel) showed that the copy number of the transgene, quantitatively compared to equivalent DNA fragment, in one of the representative founders was one (Fig. 2B, right panel).

this chimeric molecule for manipulating the immune system (data not shown).

### 3.2. Production of pIns-anti-4-1BB scFv transgenic mice

To further investigate the effect of this engineered anti-4-1BB scFv on the autoimmune processes in NOD mice, the pIns-anti-4-1BB scFv transgene, containing an engineered single-chain anti-4-1BB Fv, the IgG-like C-type domain, the transmembrane domain, and the cytoplasmic tail of murine CD80, under the human insulin promoter (Fig. 2A), was microinjected into the fertilized eggs of NOD mice to generate NOD transgenic mice. In the F1 generation, Southern blot analysis of tail DNA showed that the copy number of the transgene in one of the representative founders was one (Fig. 2B). PCR analysis of tail DNA with specific primers, 5'-GGATCCTGAGAACTTCAGG-3' (forward primer; sequence located at the 3' end of the human insulin promoter) and 5'-AGAGACAGTGCAGGTGAGGGA-3' (reverse primer; sequence located in the variable region of the anti-4-1BB heavy chain), revealed results completely compatible with those of Southern blotting, and was subsequently used for transgenic screening (data not shown). The expression of the transgene was detected almost exclusively in the pancreas, but

also unexpectedly in the colon, of transgenic mice using RT-PCR analysis and Northern blotting (data not shown).

### 3.3. Diabetes in pIns-anti-4-1BB Fv transgenic mice

To evaluate the potential impact of the transgenic expression of membrane-bound agonistic anti-4-1BB scFv in pancreatic islets on the development of diabetes, transgenic NOD mice, both male and female, and their female control littermates were monitored for blood sugar and glycosuria every week. Surprisingly, transgenic NOD mice developed more severe diabetes than their non-transgenic littermates, in terms of earlier onset, faster diabetic process, and higher mortality rate (Fig. 3). Forty percent of transgenic mice developed diabetes by 4 weeks of age, whereas their control littermates first displayed diabetes at 14 weeks of age. The frequency of diabetes in female transgenics quickly reached 70% by 8 weeks of age. Most female transgenic mice died around 12 weeks (Fig. 3). The acceleration of diabetogenic processing and the augmentation of disease severity was similarly observed in all other transgenic lines, indicating a common adverse effect induced by the overexpression of transgenic anti-4-1BB scFv in NOD pancreatic  $\beta$  cells (data not shown).

### 3.4. Insulinitis and GAD-specific T-cell response in transgenic mice

To determine whether anti-4-1BB scFv overexpression affects the development of insulinitis in pancreatic islets, we used histological analyses to evaluate the degree of insulinitis in transgenic NOD mice and their transgenic-negative littermates at different ages. Obviously, the transgenic NOD mice developed earlier and more severe insulinitis, compared with that of age-matched control littermates. Female transgenic mice around 5 weeks of age developed significant peri-insulinitis (one representative islet is shown in Fig. 4A, left panel, 200 $\times$ ), and female transgenic mice around 7 weeks of age developed severe intra-insulinitis with disruption of the islet architecture (one representative islet is shown in Fig. 4A, right panel, 200 $\times$ ). The average insulinitis scores for 7-week-old female transgenic NOD mice and their female transgenic-negative littermates were  $1.71 \pm 0.35$  and  $0.81 \pm 0.19$  (mean  $\pm$  SD;  $P \leq 0.01$ ), respectively, indicating that transgenic anti-4-1BB scFv overexpression augmented the severity of insulinitis.

To investigate the potential mechanism involved in the transgene-expression-induced acceleration of the autoimmune process, we examined the GAD-specific T-cell response in pIns-anti-4-1BB scFv transgenic NOD mice. Splenic cells from 5- and 7-week-old transgenic and non-transgenic females were incubated with the GAD65 antigenic peptide p247–266, for 3 days. The

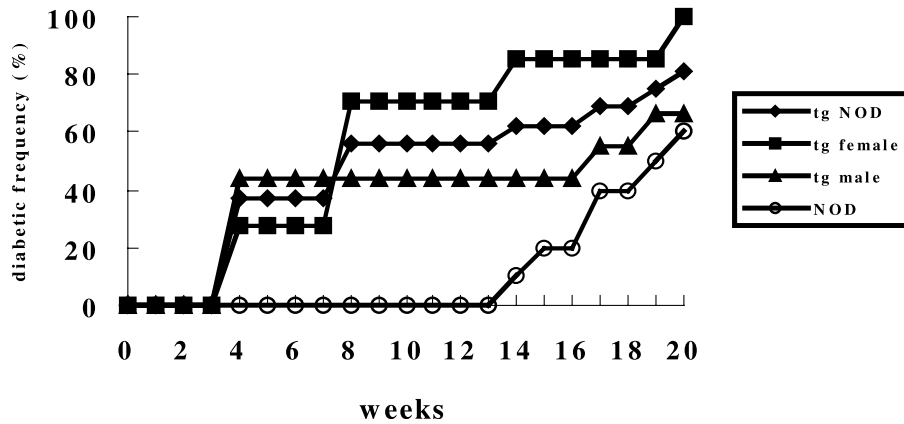


Fig. 3. Cumulative incidence of diabetes in pIns-anti-4-1BB Fv transgenic NOD mice. Mice were monitored by weekly measurements of glycosuria and blood sugar. The number of animals tested in female transgenic mice, male transgenic mice, and control littermates, is 24, 18, and 16, respectively. Statistical analysis demonstrated a significant difference between transgenic mice (either male or female) and their non-transgenic littermates (Kaplan–Meier method,  $P \leq 0.01$  for both male and female transgenics).

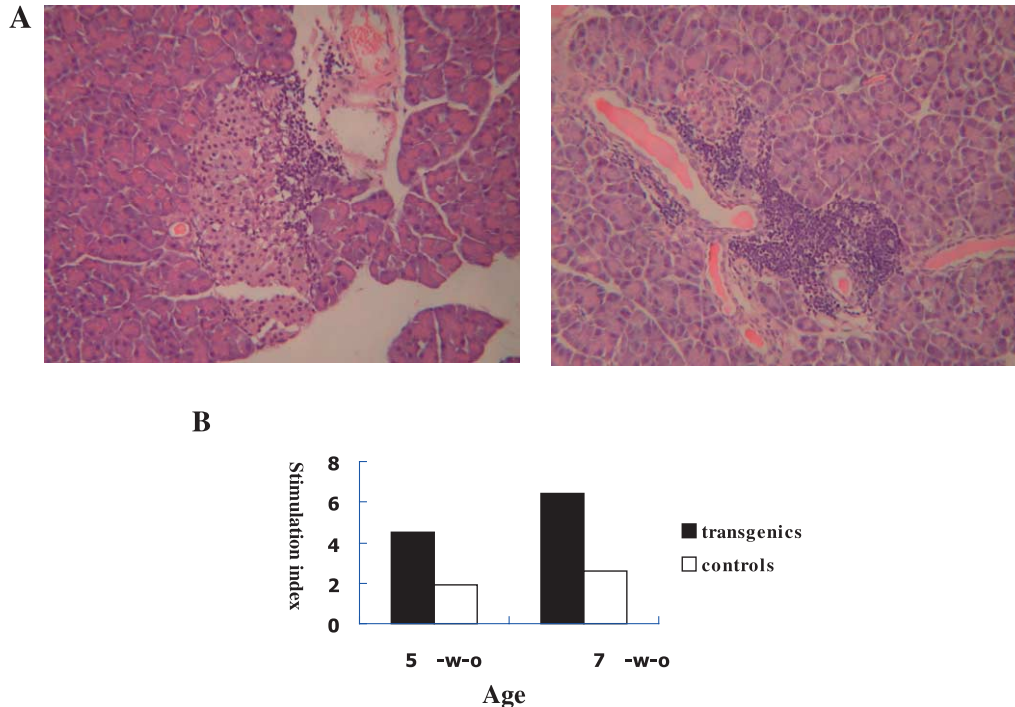


Fig. 4. Insulinitis and GAD-specific T-cell response in transgenic mice. Female transgenic mice around 5 weeks of age developed significant peri-insulinitis (one representative islet in Fig. 4A, left panel,  $200\times$ ), and female transgenic mice around 7 weeks of age developed severe intra-insulinitis with disruption of the islet architecture (one representative islet in Fig. 4A, right panel,  $200\times$ ). Splenic cells from 5- and 7-week-old transgenic and non-transgenic females were incubated with the GAD65 antigenic peptide p247–266, for 3 days. The stimulation index (mean counts per min with antigen/mean counts per min with media alone) was significantly higher in transgenic mice than in their non-transgenic littermates (4.5 versus 1.9 in 5-week-old mice; 6.4 versus 2.6 in 7-week-old mice,  $P \leq 0.01$ ) (Fig. 4B).

stimulation index (mean counts per min with antigen/mean counts per min with media alone) was significantly higher in transgenic mice than in their non-transgenic littermates (4.5 versus 1.9 in 5-week-old mice; 6.4 versus 2.6 in 7-week-old mice,  $P \leq 0.01$ ) (Fig. 4B). These results demonstrate that overexpression of anti-4-1BB scFv in transgenic islets promotes an autoantigen-specific T-cell

response, and may thus accelerate the autoimmune destruction of  $\beta$  cells. Consistent with this, the secretion of IFN- $\gamma$  from transgenic splenic cells stimulated with GAD peptide was much higher than that from the cells of non-transgenic littermates (data not shown). The overproduction of IFN- $\gamma$  in transgenic mice may also contribute to the damage of  $\beta$  cells.

#### 4. Discussion

Costimulatory-molecule-targeted antibody therapy has been applied to several autoimmune disease models. Unlike conventional T-cell costimulation blockage, an agonistic mAb (2A) directed against the costimulatory molecule CD137 has been demonstrated to effectively ameliorate autoreactive T cells in mice with EAE [8], and to reduce both lymphadenopathy and spontaneous autoimmune diseases in the MRL/lpr strain [9], a murine model of human systemic lupus erythematosus (SLE). Moreover, 2A treatment prolongs the long-term survival of these mice, suggesting a potential therapeutic strategy for patients with SLE. Lymphocyte depletion induced by 2A has been shown to be Fas- and TNFR-independent [9], suggesting that an agonistic antibody may deliver a strong costimulatory signal that promotes the activation-induced cell death of active lymphocytes, circumventing the need for Fas- or TNFR-mediated apoptosis. In this study, we investigated the preventive/therapeutic potential of this antibody in autoimmune diabetes by generating and analyzing an insulin-promoter-controlled single-chain anti-4-1BB Fv transgene in NOD mice.

The long-term goal of immunotherapies for autoimmune diseases is to selectively inactivate or delete self-reactive lymphocytes, while avoiding systemic immunosuppression. Extending this idea, we tested whether the organ-specific expression of agonistic membrane-bound anti-4-1BB scFv can deplete the  $\beta$ -cell-reactive autoimmune T lymphocytes and prevent diabetes in NOD mice. The strategy of using single-chain Fv fragments specific for 4-1BB has been demonstrated to successfully eradicate implanted tumors in a murine model [24,25]. Cases have been reported in which the administration of a monoclonal antibody, such as 1D8, specific for the T-cell-activation molecule 4-1BB, caused the regression of subcutaneously growing mastocytoma p815 or sarcoma Ag104 [11,12]. In some cases, immunization with tumor cells transfected with a gene encoding 4-1BBL can also be therapeutically effective [26]. However, vaccination with tumor cells expressing 4-1BBL was ineffective against sarcoma Ag104 unless they were cotransfected with CD80 [26]. This promising result suggested that a monoclonal antibody against 4-1BB engages the anti-tumor immune response more effectively than 4-1BBL. One alternative way to stimulate the immune system as a monoclonal antibody does is to construct a fusion gene that encodes single-chain Fv fragments for expression at the cell surface [24,25]. Using this method, we generated several transgenic lines of NOD mice overexpressing anti-4-1BB scFv under the control of the insulin promoter. These transgenic mice will provide a good model system with which to directly investigate the therapeutic potential and mechanisms induced

by single-chain anti-4-1BB Fv in autoimmune diabetes in vivo.

Paradoxically, transgenic NOD mice overexpressing membrane-bound agonistic single-chain anti-4-1BB Fv in pancreatic  $\beta$  cells developed more severe diabetes than their non-transgenic littermates, in terms of earlier onset, faster diabetic process, and higher mortality rate. Accordingly, the transgenic NOD mice developed a stronger autoantigen-specific T-cell response, higher levels of IFN- $\gamma$  secretion, and earlier and more severe insulinitis, compared with those of age-matched control littermates. These results indicate that the expression of anti-4-1BB scFv on  $\beta$  cells activates and expands autoreactive T cells in transgenic mice. However, these T cells do not undergo activation-induced cell death, which eventually causes accelerated damage to the  $\beta$  cells. This also demonstrates that the threshold for activation-induced cell death in activated T cells is extremely critical for the determination of this anti-4-1BB-based immunotherapy in various autoimmune diseases. The strong induction of the T helper 1 (Th1) immune response (e.g., IFN- $\gamma$  secretion) caused by anti-4-1BB treatment may be considered to detract from the therapy in this kind of Th1-mediated autoimmune diabetes. Moreover, in NOD mice, the disease progresses from insulinitis to islet destruction are highly related on sex hormone, and clinical diabetes is always in a high percentage of female mice [27,28]. However, in male anti-4-1BB scFv transgenic NOD mice, sex hormone (e.g., androgen)-mediated protection is apparently overridden by the anti-4-1BB-induced T-cell activation and subsequent  $\beta$  cell destruction. Both male and female transgenic mice developed diabetes in a similar kinetics (Fig. 3). Nevertheless, the acceleration of the diabetogenic processes and the augmentation of disease severity were similarly observed in all other transgenic lines, indicating a common adverse effect induced by the overexpression of transgenic anti-4-1BB scFv in NOD pancreatic  $\beta$  cells.

Autoimmune diabetes is becoming preventable in animal models and is gradually becoming predictable in humans. The massive accumulation of knowledge on basic immunology has allowed us to initiate large-scale clinical efforts to prevent this disease. The transgenic approach, in which engineered anti-4-1BB scFv is overexpressed in pancreatic  $\beta$  cells, has an advantage over the administration of monoclonal antibody in that it combines signals via islet-specific autoantigens and 4-1BB, whereas a monoclonal antibody expands or deletes many different lymphocytes that express 4-1BB and may create a greater risk for immunocompromised patients or be less efficacious. However, further studies extending to additional systems must be undertaken, and a better understanding must be sought of the mechanisms by which 4-1BB is engaged, using monoclonal antibodies, natural ligands, or scFv.

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