

# DcR3/TR6 Effectively Prevents Islet Primary Nonfunction After Transplantation

Yulian Wu,<sup>1,2</sup> Bing Han,<sup>2</sup> Hongyu Luo,<sup>1</sup> Raphael Roduit,<sup>3</sup> Theodora W. Salcedo,<sup>4</sup> Paul A. Moore,<sup>4</sup> Jun Zhang,<sup>4</sup> and Jiangping Wu<sup>1,5</sup>

**Islet primary nonfunction (PNF) is defined as the loss of islet function after transplantation for reasons other than graft rejection. It is a major obstacle to successful and efficient islet transplantation. DcR3/TR6 is a soluble death decoy receptor belonging to the tumor necrosis factor (TNF) receptor family, and it can block apoptosis mediated by several TNF receptor family members such as Fas and LTβR. In this study, we used TR6 to protect islets from PNF after transplantation. Untreated isogeneic or allogeneic islet transplantation had PNF incidence of 25 and 26.5%, respectively. Administration of TR6 totally prevented PNF in allogeneic islet transplantation. In vitro experiments showed an increased apoptosis among islets that were treated with FasL and γ-interferon (IFN-γ) in combination. TR6 significantly reduced such apoptosis. Functional study showed that insulin release was compromised after FasL and IFN-γ treatment, and the compromise could be prevented with TR6-Fc. This indicates that TR6 indeed protected β-cells from damage caused by FasL and IFN-γ. Further in vivo experiments showed that syngeneic islet transplantation between *lpr/lpr* and *gld/gld* mice was significantly more efficacious than that conducted between wild-type mice. These results suggest that Fas-mediated apoptosis plays an important role in PNF, and use of TR6 may be a novel strategy to prevent PNF in clinical islet transplantation. *Diabetes* 52: 2279–2286, 2003**

From the <sup>1</sup>Laboratory of Transplantation Immunology, Centre hospitalier de l'Université de Montreal, Montreal, Quebec, Canada; the <sup>2</sup>Department of Surgery, the Second Affiliated Hospital of the Zhejiang Medical College, Zhejiang University, Zhejiang, People's Republic of China; the <sup>3</sup>Research Center, Centre hospitalier de l'Université de Montreal, Montreal, Quebec, Canada; the <sup>4</sup>Human Genome Sciences, Rockville, Maryland; and the <sup>5</sup>Nephrology Service of the Notre Dame Hospital, Centre hospitalier de l'Université de Montreal, Montreal, Quebec, Canada.

Address correspondence and reprint requests to Dr. Jiangping Wu, Laboratory of Transplantation Immunology, Research Center, CHUM-Notre Dame Hospital, Pavilion DeSève, Room Y-5616, 1560 Sherbrooke Street East, Montreal, Quebec H2L 4M1, Canada. E-mail: jianping.wu@umontreal.ca.

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R.R. is currently affiliated with the Division of Medical Genetics, CHUV-University Hospital, Lausanne, Switzerland.

AO, acridine orange; DcR3, decoy receptor 3; EB, ethidium bromide; HBSS, Hank's balanced salt solution; mAb, monoclonal antibodies; PNF, primary nonfunction; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TR6, tumor necrosis factor family receptor 6; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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**I**slet transplantation is an effective treatment for insulin-dependent diabetes. There are two major obstacles to successful islet transplantation: islet primary nonfunction (PNF) and graft rejection. PNF is defined as the loss of islet function after transplantation for reasons other than graft rejection. PNF frequency can be very high in islet transplant models (1). In humans, pancreas transplantation has produced better results [70–80% insulin independence at 3 years (2)] in reversing the diabetic state than islet transplantation conducted several years ago, suggesting that PNF of islets significantly contributes to the high failure rate of the procedure. The recent high success rate of the Edmonton group (3) with islet transplantation probably reflects better islet isolation and immunosuppression management. It is to be noted, however, that all patients fail to achieve insulin independence after islet transplantation from the first donor, and on average  $8 \times 10^5$  islets (from two or more donors) are transplanted, even though  $3 \times 10^5$  islets are sufficient for insulin independence (4). This suggests that PNF remains a problem in current islet transplantation.

During isolation and transplantation, islets experience extreme conditions, such as enzyme digestion, mechanical shear, endotoxins present in various solutions, osmotic shock, deprivation of obligatory trophic support, inflammatory cytokines, and free radicals (4). This may cause the β-cells to undergo substantial apoptosis when cultured in vitro after isolation (5,6). It is consistent with this finding that immunoisolated, microencapsulated islets that are injected into the dog peritoneal cavity fail within 6 months and that islets that are placed in a vascularized, biohybrid pancreas also fail at 1 year (7,8). Histologic evaluation shows a major loss of islet mass in these devices. Therefore, PNF is probably one of the major reasons for the large number of islets needed to achieve euglycemia. Consequently, prevention of PNF will likely improve outcomes from islet transplantation.

Decoy receptor 3 (DcR3), also known as tumor necrosis factor (TNF) family receptor 6 (TR6), is a new member of the TNFR family (9). TR6 lacks an apparent transmembrane domain in its sequence and is a secreted protein (10,11). The mRNA of TR6 is expressed at high levels in several normal human tissues such as the stomach, spinal cord, colon, lymph node, and spleen (9,12), whereas its mRNA expression in the thymus and peripheral blood lymphocytes is weak and undetectable, respectively. TR6 can bind to a TNF family member FasL and prevent

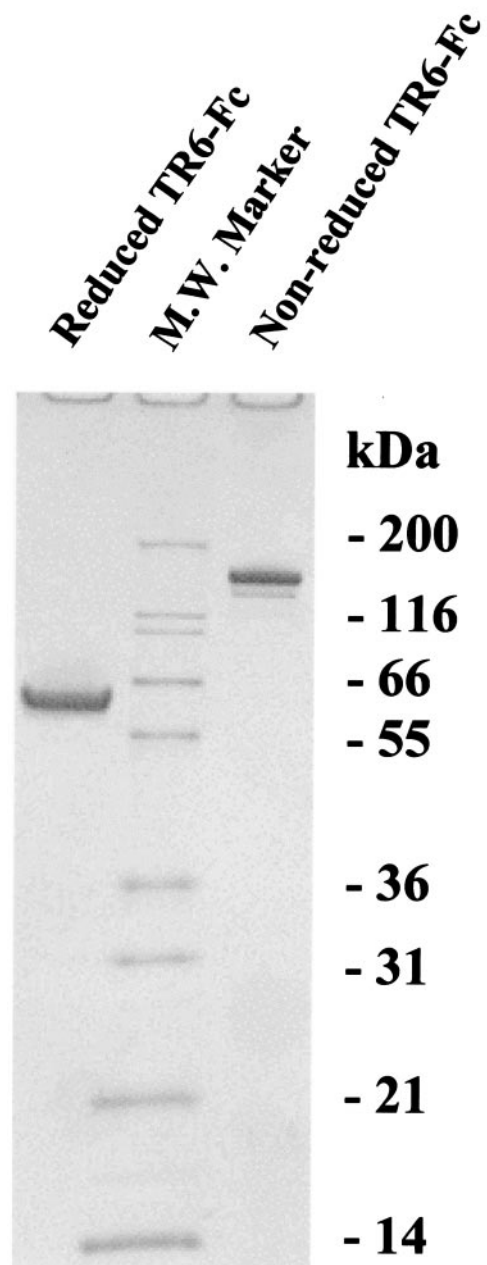
FasL-induced apoptosis in lymphocytes and several tumor cell lines (9). TR6 can also bind to LIGHT, another member of the TNF family (11). LIGHT, a ligand for both TR2/HVEM and LT $\beta$ R (13), is expressed on activated T-cells (13) and immature dendritic cells (14). LIGHT is found to induce apoptosis in cells that express both TR2 and LT $\beta$ R (15). However, Rooney et al. (16) showed that LT $\beta$ R is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. As TR6 is a soluble receptor that interacts with several ligands, one of the physiologic functions of TR6 may be to act as a death decoy to prevent apoptosis mediated by TNF receptor family members such as Fas and LT $\beta$ R. In connection with islet transplantation, DcR3/TR6 might exert beneficial effects by functioning as a death decoy to reduce islet  $\beta$ -cell apoptosis mediated by Fas and/or LT $\beta$ R, hence ameliorating PNF. TR6 can also bind to a third TNF family member, TL1A, and block its apoptosis-inducing effect via DR3 (17). As DR3 is mainly expressed on cells of hematopoietic origin, its relevance to  $\beta$ -cell apoptosis and PNF is not clear. In the present study, we demonstrated that TR6-Fc reduced PNF from  $\sim$ 26.5% to nil in a mouse islet transplantation model. We also demonstrated that the mechanism of this beneficial effect was, at least in part, due to blockage of Fas-mediated islet apoptosis by TR6.

## RESEARCH DESIGN AND METHODS

**Preparation of recombinant human TR6-Fc.** The mouse counterpart of human TR6 might not exist, because a BLAST search of the mouse genome using the human TR6 cDNA sequence as a query failed to yield significant homologues. We therefore used human TR6 to study the role of TR6 in mouse PNF. Full-length TR6 fused with human IgG Fc (TR6-Fc) was prepared for this purpose, as described in our previous publication (10). The purified TR6-Fc protein was analyzed with 10% SDS-PAGE under reducing or nonreducing conditions, showing that the protein is a disulfide-linked dimer under non-reducing conditions (Fig. 1). Light-scattering analysis also confirmed that it behaves as a disulfide-linked dimer in solution (data not shown). NH<sub>2</sub>-terminal sequencing revealed that the mature secreted TR6-Fc had the predicted sequence of VAETP (single-letter code of amino acids), starting at amino acid 30. The estimated purity of the protein preparation was  $>$ 95% according to SDS-PAGE.

**Flow cytometry.** Murine L929 cells ( $1 \times 10^6$ ) with or without murine FasL transfection were incubated with 1  $\mu$ g of Fas-Fc, 10 ng of TR6-Fc, or 1  $\mu$ g of phycoerythrin-conjugated anti-murine FasL (clone Kay-10; BD Pharmingen, San Diego, CA) in 100  $\mu$ l of buffer for 20 min at room temperature. In certain samples, unlabeled soluble TR6 without Fc was added (1  $\mu$ g/sample) as a competitor at this step. The cells were washed twice. For detection of the human-Fc fusion proteins, cells were reacted with goat F(ab)<sub>2</sub> anti-human IgG (Southern Biotechnology, Birmingham, AL) for 15 min at room temperature. After washing, the cells were resuspended in 0.5  $\mu$ g/ml propidium iodide, and live cells were gated and analyzed by flow cytometry.

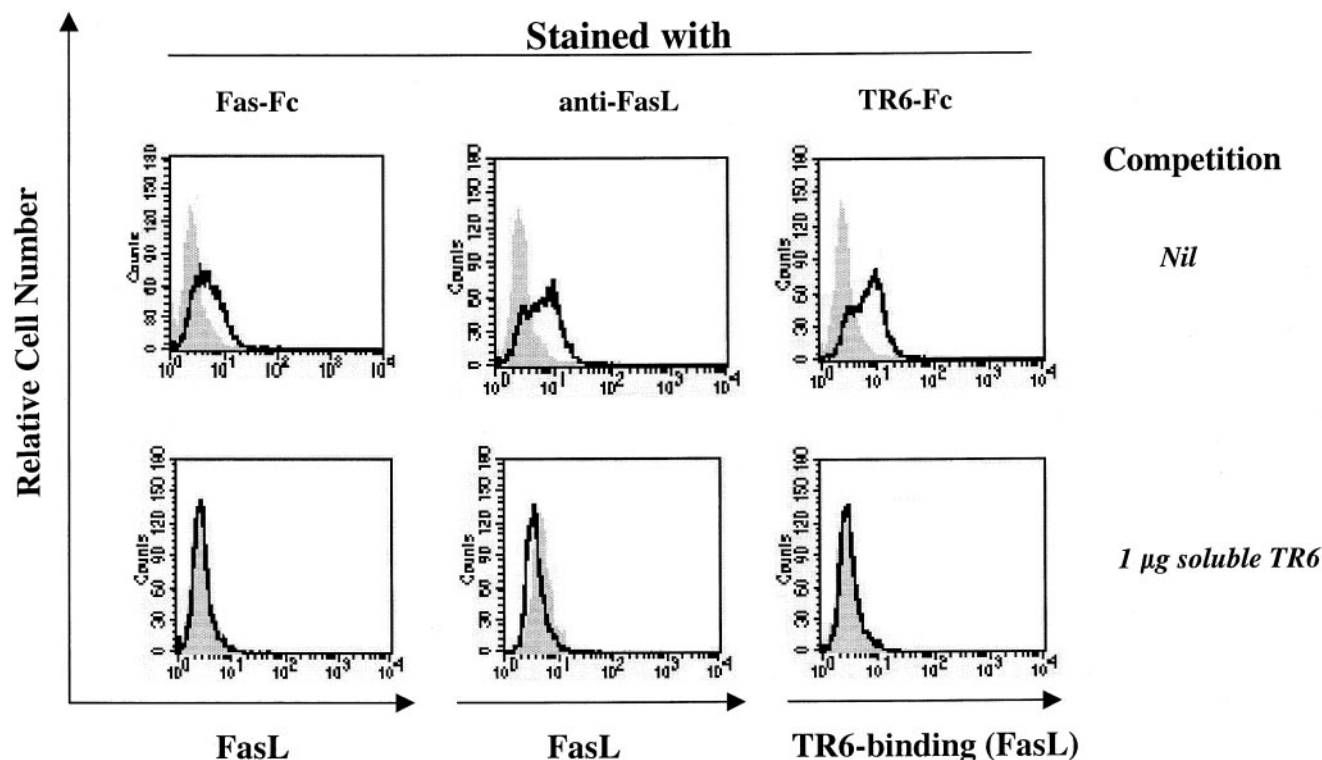
**Islet purification.** Two milliliters of digestion solution (Hanks' balanced salt solution [HBSS] containing 20 mmol/l HEPES and 2 mg/ml collagenase IV; Worthington Biochemical, Lakewood, NJ) were injected into the common bile duct of BALB/c, C57BL/6, or *lpr/lpr* mice (20–24 g) after the distal end of the duct was ligated. The distended pancreas was isolated and put into a 15-ml tube containing an additional 0.5 ml of digestion solution. The pancreas was digested at 37°C for exactly 38 min, and the digestion process was stopped by the addition of 10 ml of cold HBSS containing 20 mmol/l HEPES. The islet suspension was filtered through No. 7880 cheesecloth gauze (Tyco Healthcare, Mansfield, MA) and centrifuged at 500g for 1–2 min. The pellet was washed with cold HBSS once at 500g for 1–2 min, and the supernatant was removed completely. The pellet was then resuspended in 3 ml of 25% Ficoll, and 2-ml layers of 23, 20, and 11% Ficoll were added sequentially. The Ficoll gradient was centrifuged at 700g for 5 min. Most of the islets were in the interface between the 20 and 23% Ficoll layers and were handpicked with Pasteur pipettes. The islets were then washed twice with cold HBSS. The islets were cultured overnight in RPMI 1640 containing 10% FCS on wells precoated with 0.5% BSA in PBS, and then used for transplantation or in vitro study.



**FIG. 1.** Human TR6-Fc analyzed by SDS-PAGE. TR6-Fc under reducing and nonreducing conditions was analyzed by 10% SDS-PAGE.

**Islet transplantation.** Diabetes was induced in recipient C57BL/6 or *gld/gld* mice by intraperitoneal injection of streptozotocin in PBS (Sigma, Oakville, ON, Canada) at 220 mg/kg body wt. The diabetes status was defined as nonfasting blood glucose measuring over 20 mmol/l for two consecutive days. Approximately 400 islets from C57BL/6 or from BALB/c mice were injected intraperitoneally into the diabetic C57BL/6 mice. TR6-Fc was given to the recipients intraperitoneally twice a day at 15 mg  $\cdot$  kg<sup>-1</sup>  $\cdot$  day<sup>-1</sup>, starting right after islet transplantation. PNF was defined as blood glucose remaining above 18 mmol/l 48 h after islet transplantation. For *lpr/lpr* (in C57BL/6 background) to *gld/gld* (also in C57BL/6 background) islet transplantation, the *gld/gld* recipients with streptozotocin-induced diabetes received a transplant of  $\sim$ 250–270 *lpr/lpr* islets, and recipient blood glucose was monitored for 14 days; for the controls of this experiment, C57BL/6 to C57BL/6 islet transplantation was conducted under similar conditions.

**Islet viability staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.** Islet viability was assessed by ethidium bromide and acridine orange (EB/AO) staining. Islets were stained with 0.04  $\mu$ g/ml ethidium bromide and 4  $\mu$ g/ml acridine orange in PBS for 15 min at room temperature. After being washed once with PBS, they were examined



**FIG. 2.** Human TR6 binds to mouse FasL. Mouse L929 cells were stably transfected with a mouse FasL expression construct. The cells were stained with mouse Fas-Fc, anti-mouse FasL, or human TR6-Fc (top). Soluble TR6 (1  $\mu\text{g}$ ) without the Fc tag was used as a competitor during the staining procedure (bottom). The shaded areas and solid lines represent wild-type and FasL-transfected L929 cells, respectively.

under a fluorescent microscope. Islets from triplicate wells were counted for AO-positive ones (defined as having >50% EB/AO-positive cells in the islet mass) among total islets. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was used to detect islet apoptosis with apoTACS kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Apoptotic islets were quantified by one-way blinded examination of TUNEL-labeled islets, with the examiner unaware of the islet treatment. Islets whose mass was >50% TUNEL-positive were considered apoptotic.

**Insulin release assay.** After 48 h of culture in complete RPMI 1640 medium in the presence of various reagents, the islets were transferred to 12-well plates at a density of 10 islets/well. The islets were gently washed twice with 1 ml of Krebs's buffer (135 mmol/l NaCl, 3.6 mmol/l KCl, 5 mmol/l  $\text{NaH}_2\text{PO}_4$ , 0.5 mmol/l  $\text{MgCl}_2$ , 1.5 mmol/l  $\text{CaCl}_2$ , 2 mmol/l  $\text{NaHCO}_3$ , 10 mmol/l HEPES [pH 7.4], 0.07% BSA) and then incubated in Krebs's buffer containing 2.8 mmol/l glucose for 5 min at 37°C. A total of 200  $\mu\text{l}$  of supernatant was removed for determination of basal insulin levels. The islets were cultured for an additional 40 min, and all of the supernatants were harvested. The islets were then cultured in Krebs's buffer containing 16.7 mmol/l glucose for 45 min at 37°C, and the supernatants were harvested. The harvested supernatants were assayed for insulin by enzyme-linked immunosorbent assay. The basal insulin levels were deducted from the 2.8 and 16.7 mmol/l glucose-stimulated levels in final data presentation.

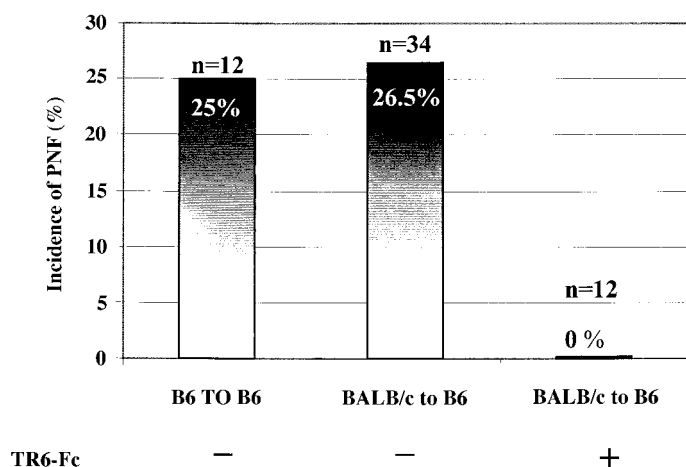
## RESULTS

**Human TR6-Fc binds to mouse FasL.** As we were to use human TR6 in a mouse model, it is essential to establish whether human TR6 could associate with mouse FasL, which might be involved in PNF. As shown in Fig. 2, both Fas-Fc and anti-mouse FasL monoclonal antibodies (mAb) bound to the mouse FasL-transfected L929 cells (solid lines) but not to untransfected wild-type cells (shaded areas). TR6-Fc presented similar binding to transfected L929 cells as Fas-Fc and anti-FasL mAb. Binding by Fas-Fc, anti-FasL mAb, and TR6-Fc to the transfected cells could be inhibited by soluble TR6 at 1  $\mu\text{g}/\text{ml}$ , demonstrat-

ing the binding specificity. This result proved for the first time that human TR6 could associate with mouse FasL and established a molecular basis for inhibition of mouse Fas-mediated apoptosis by human TR6-Fc.

**TR6 prevents PNF in mouse islet transplantation.** PNF, one of the major problems occurring during islet transplantation, can be easily demonstrated in animal models. When transplanted with a lower-than-optimal number of BALB/c islets (~400), 26.5% diabetic C57BL/6 (H-2<sup>b</sup>) recipient mice failed to achieve a blood glucose level <18 mmol/l within 48 h after transplantation (Fig. 3), indicative of PNF. This was not due to allograft rejection responses, in which cytotoxic T-cells need 4–5 days to complete processes of activation, proliferation, and differentiation; our test confirmed that within 48 h of mixed lymphocyte culture, cytotoxic lymphocyte activity was not detectable (data not shown). In support of this notion, the control isogeneic islet transplantation from C57BL/6 donors to C57BL/6 diabetic recipients with 400 islets per recipient had a similar incidence (25%) of PNF 48 h after transplantation. When TR6-Fc was administered at 15  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  intraperitoneally twice a day to the allograft recipients, PNF was completely prevented. This result showed that TR6 could prevent islet PNF in vivo.

**TR6 protects islets from IFN- $\gamma$  plus FasL-induced damage.** We next explored mechanisms with which TR6 protected islets from damage. Islets were cultured in complete medium for 24 h after isolation and then treated with different apoptosis-inducing factors. After an additional 48-h culture, they were stained with EB/AO for viability assessment. As shown in Fig. 4A, FasL (Super-FasL; Q-Biogene, Carlsbad, CA) at 60 ng/ml or IFN- $\gamma$  at



**FIG. 3.** TR6 prevents PNF in mouse islet transplantation. C57BL/6 mice, which had streptozotocin (220 mg/kg)-induced diabetes with blood glucose >20 mmol/l, were injected intraperitoneally with 400 islets. PNF was defined as blood glucose remaining >18 mmol/l after 48 h from islet transplantation. TR6-Fc was given intraperitoneally at 15 mg · kg<sup>-1</sup> · day<sup>-1</sup> twice a day.

1,000 units/ml alone had no apparent effect on viability of the islets after 48 h. However, when these two factors were combined, they significantly decreased islet viability. This was apparent as yellow staining on the islets was increased. TR6-Fc at 10 μg/ml protected the islets from death caused by the combination of FasL and IFN-γ, as the yellow staining in TR6-Fc-treated islets was reduced. Semiquantitative assessment confirmed the impression above. As shown in Fig. 4B, islets that were treated with FasL or IFN-γ alone had no statistical difference from islets that were cultured in medium in the EB/AO staining (89.6, 82.8, and 89.9% viable, respectively). When FasL and IFN-γ were combined, the viability of the islets decreased to 58.4%, and this was statistically different from all of the above-mentioned three treatments ( $P < 0.01$  in all three comparisons, i.e., medium versus FasL/IFN-γ, FasL versus FasL/IFN-γ, and IFN-γ versus FasL/IFN-γ). When TR6-Fc was added to the culture, it significantly reversed the FasL plus IFN-γ-induced islet death with the viability raised to 82.8% ( $P < 0.01$ , FasL/IFN-γ versus FasL/IFN-γ/TR6-Fc). This showed that TR6 had a protective effect against islet death induced by a combination of FasL and IFN-γ.

The TUNEL assay was performed after islet culture to assess whether islet death was due to apoptosis and whether TR6 could prevent the apoptosis. IFN-γ (1,000 units/ml) plus FasL (60 ng/ml)-treated islets showed significantly increased apoptosis as seen in the photos and bar graph of Fig. 5 ( $P < 0.01$ , control versus IFN-γ/FasL). TR6-Fc (10 μg/ml) significantly protected the islets from IFN-γ plus FasL-induced apoptosis ( $P < 0.01$ , IFN-γ/FasL versus IFN-γ/FasL/TR6-Fc). The results of this section disclosed that a possible mechanism of the protective effect of TR6 on PNF was inhibition of islet apoptosis.

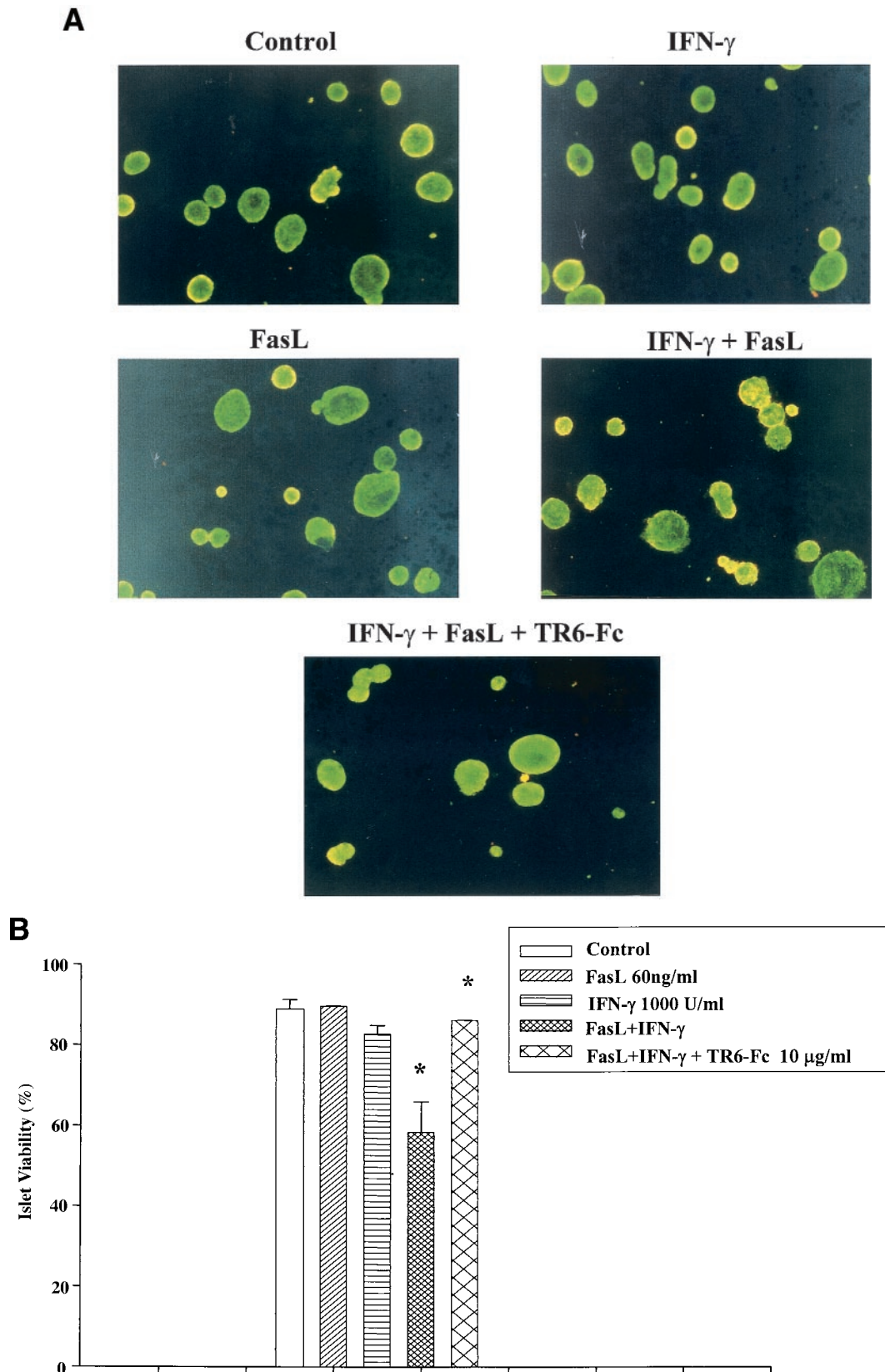
**β-cell function was protected by TR6-Fc in FasL- and IFN-γ-treated islets.** We next assessed β-cell function using islets treated with FasL and IFN-γ in the presence or absence of TR6-Fc. As shown in Fig. 6, as with the EB/AO staining, a combination of FasL and IFN-γ caused significant compromise in insulin release upon stimulation with 16.7 mmol/l glucose ( $P < 0.01$ , control versus FasL/IFN-γ),

and such compromise could be prevented in the presence of TR6-Fc ( $P < 0.01$ , FasL/IFN-γ versus FasL/IFN-γ/TR6-Fc). The result suggests that the morphologic damage on islets seen in EB/AO staining and in TUNEL assays occurred on β-cells. It is to be noted that we intentionally used a condition under which the damage caused by FasL and IFN-γ was moderate and was sensitive to the rescue effect of TR6. The compromise in insulin release and the rescue effect of TR6 was comparable in scale to the islet viability and apoptosis as shown in the previous section.

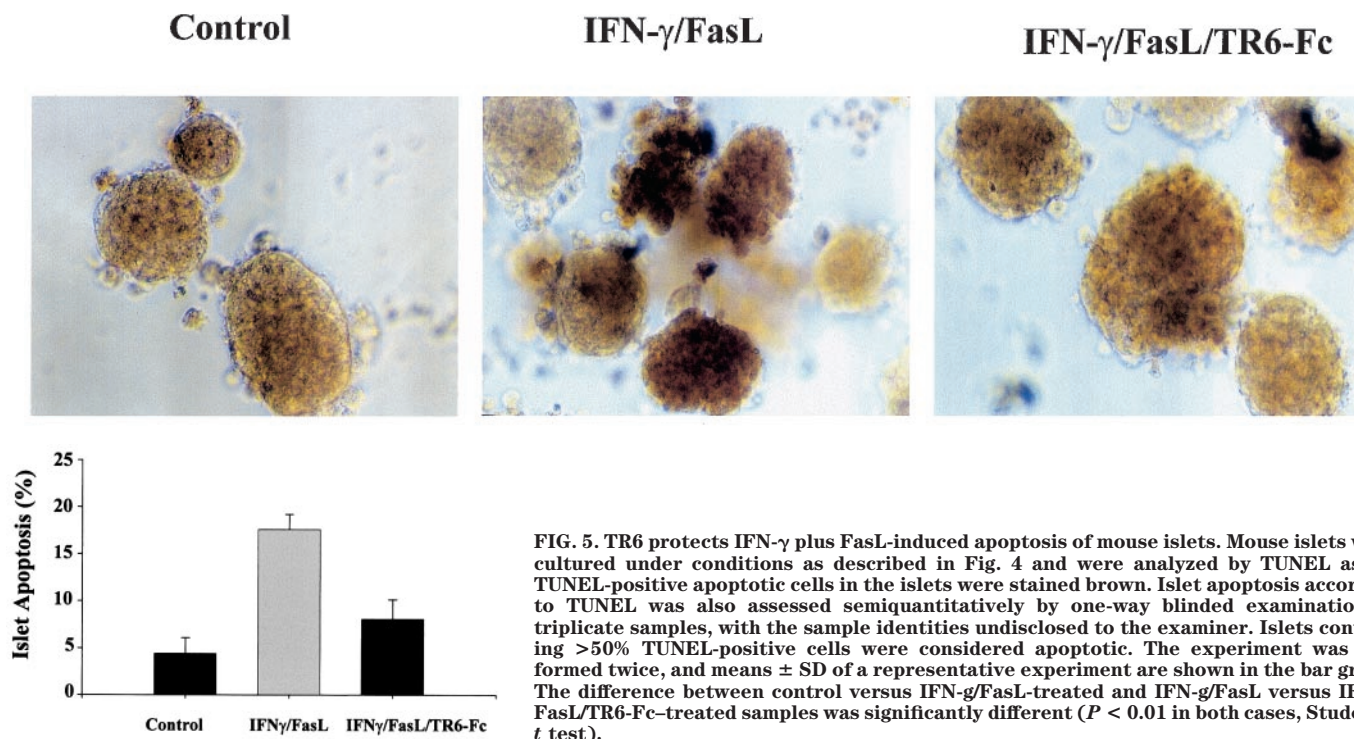
**The in vivo role of FasL and Fas in PNF.** To confirm the contribution of FasL and Fas to PNF in vivo, we conducted islet transplantation from *lpr/lpr* mice to diabetic *gld/gld* recipients. The former strain has mutation in FasL and the latter, in Fas. As a consequence, the FasL and Fas interaction is not functional in this in vivo model. As the *lpr/lpr* and *gld/gld* mice used both were in the C57BL/6 background, this model represents isogeneic islet transplantation with no allograft rejection involved. A marginal dose of 250–270 islets was used per recipient. The recipient mice had blood glucose levels between 20 and 30 mmol/l before islet transplantation (day 0). After islet transplantation, blood glucose was monitored daily. At such an islet dosage, normoglycemia was restored only in ~20% of the control C57BL/6-to-C57BL/6 (B6 to B6) group around day 13 posttransplantation. Therefore, this regimen was sensitive for the detection of the putative contribution of FasL and Fas to in vivo islet damage after transplantation. As shown Fig. 7A, starting from day 6 posttransplantation, the *lpr/lpr*-to-*gld/gld* group had significantly lower blood glucose than the B6-to-B6 group ( $P < 0.05$  at all points, starting from day 6; ANOVA). Figure 7B depicted the percentage of mice returned to normoglycemia (defined as ≤12 mmol/l) after the isogeneic islet transplantation. In the B6-to-B6 group, only 20% of the mice had normoglycemia until day 13 posttransplantation. However, in the *lpr/lpr*-to-*gld/gld* group, 33% of the mice achieved normoglycemia on day 5 (with some fluctuation between day 5 and day 10), and 44% of the mice achieved that on day 13. The difference between the two groups was highly significant ( $P < 0.001$ , two-way repeated measurement of ANOVA). These data indicate that with nonfunctional FasL and Fas in the donors and recipients, respectively, the efficiency of islet transplantation was improved. This suggests that Fas-mediated apoptosis contributes to PNF.

## DISCUSSION

In this study, we reported that a soluble death decoy DcR3/TR6 could prevent islet PNF, and this protective effect was, at least in part, due to interference of TR6 with Fas-mediated islet apoptosis. The mechanisms of PNF have not been clearly delineated, but multiple factors, such as inflammatory cytokines, free radicals, macrophages, and withdrawal of trophic support from tissues surrounding the islets, are likely involved. The effective prevention of PNF by TR6 suggested that Fas-mediated islet apoptosis was an essential mechanism for PNF, because TR6 is known to bind to FasL and prevent FasL-triggered apoptosis. Indeed, Fas is reported being expressed on murine islets (18), which undergo apoptosis when infected ex vivo with FasL-expression adenovirus vector, resulting in PNF when transplanted into isogeneic or allogeneic diabetic recipients (19). Using *lpr/lpr* mice as



**FIG. 4.** TR6 protects islets from IFN- $\gamma$  plus FasL-induced death. Mouse islets were cultured in complete RPMI medium containing 10% FCS for 24 h after isolation. IFN- $\gamma$  (1,000 units/ml), FasL (60 ng/ml), and TR6-Fc (10  $\mu$ g/ml) were then added alone or in combination, as indicated. After an additional 48 h, the islets were stained with EB/AO, and then examined under a fluorescent microscope. Dead cells in the islets were stained yellow (A). The islet viability was also semiquantified. Islets with <50% of its mass stained yellow was defined as viable. The samples were in triplicate, and means  $\pm$  SD were plotted (B). The viability of control versus FasL/IFN- $\gamma$ -treated sample, and FasL/IFN- $\gamma$ -treated versus FasL/IFN- $\gamma$ /TR6-Fc-treated sample was significantly different ( $P < 0.01$ , marked with asterisks, Student's  $t$  test). The experiments were repeated twice, and similar results were obtained.



**FIG. 5.** TR6 protects IFN- $\gamma$  plus FasL-induced apoptosis of mouse islets. Mouse islets were cultured under conditions as described in Fig. 4 and were analyzed by TUNEL assay. TUNEL-positive apoptotic cells in the islets were stained brown. Islet apoptosis according to TUNEL was also assessed semiquantitatively by one-way blinded examination of triplicate samples, with the sample identities undisclosed to the examiner. Islets containing >50% TUNEL-positive cells were considered apoptotic. The experiment was performed twice, and means  $\pm$  SD of a representative experiment are shown in the bar graph. The difference between control versus IFN-g/FasL-treated and IFN-g/FasL versus IFN-g/FasL/TR6-Fc-treated samples was significantly different ( $P < 0.01$  in both cases, Student's  $t$  test).

donors and syngeneic *gld/gld* mice as recipients, we demonstrated that without effective FasL and Fas interaction, the efficacy of islet transplantation was significantly improved. This convincingly confirms that after transplantation, in the absence of allograft rejection, there is significant loss of islet function (i.e., PNF) as a result of Fas-mediated apoptosis.

Soluble FasL alone at the concentration used (60 ng/ml) or at a higher concentration (1  $\mu$ g/ml, data not shown) did not cause significant islet death in our in vitro experiments, even though the FasL used was in a form capable of inducing apoptosis of many cell lines at a concentration of 10–100 ng/ml in solution. In vivo, leukocytes of donor origin or leukocytes attracted to islets by inflammatory cytokines might be a source of membrane-bound FasL, which might give a more potent signal through Fas. Alternatively, soluble FasL needed cofactors to effectively induce islet apoptosis.

In many in vitro apoptosis models, cells need to be sensitized or conditioned by measures such as protein synthesis inhibition, using cycloheximide, in addition to FasL, for apoptosis induction (20,21). In our model, when FasL was applied in combination with IFN- $\gamma$ , significant islet damage was observed, and the TUNEL assay revealed that it was due to apoptosis. What is the physiologic relevance of IFN- $\gamma$  in PNF, which is defined as a reaction unrelated to classical allograft rejection? Although a definitive cell source of IFN- $\gamma$  production during PNF remains to be elucidated, IFN- $\gamma$  mRNA can indeed be detected in freshly isolated islets (22). Also, it is known that memory T-cells under the influence of inflammatory cytokines can nonspecifically produce IFN- $\gamma$  without T-cell receptor ligation (23). It is possible that some non-specific memory T-cells of donor or recipient origin in the islets produce some IFN- $\gamma$ , which sensitizes the islets. Another potential source of IFN- $\gamma$  is NK cells. In the

clinical islet transplantation, IFN- $\gamma$  can also be produced by alloantigen-specific host T-cells within hours of alloantigen stimulation. Therefore, IFN- $\gamma$  might be available in vivo locally during islet transplantation and serves as a conditioner for Fas-mediated islet apoptosis. We have tested only the combination of IFN- $\gamma$  and FasL in our in vitro experiments. In vivo, other cytokines, in addition to IFN- $\gamma$ , might also participate in the conditioning process for Fas-mediated apoptosis.

In addition to FasL, TR6 could bind to LIGHT, which is a ligand of LT $\beta$ R and is sufficient to trigger apoptosis via LT $\beta$ R in some tumor cells (16). This has raised a possibility that TR6 might also inhibit islet apoptosis mediated by LT $\beta$ R in vivo. Two recent articles showed that the administration of soluble LT $\beta$ R-Ig or transgenic overexpression of LT $\beta$ R-Ig effectively prevents or reverses insulinitis and diabetes in NOD mice (24,25); prevention of LT $\beta$ R-mediated islet apoptosis by soluble LT $\beta$ R-Ig might be implicated in these models. Therefore, the protective action of TR6 might involve multiple mechanisms.

It is worth pointing out that for the purpose of quantifying, we arbitrarily defined the PNF as failure of glucose decrease <18 mmol/l with 48 h after islet transplantation. Although TR6 treatment rescued all PNF under such a definition,  $\sim$ 50% of the mice that received TR6 still had blood glucose >12 mmol/l at 48 h (data not shown). Thus, the result of this study should not be interpreted in such a way that the TR6 treatment is a cure for PNF. We believe that there are still islets not being able to function properly in vivo despite the treatment, because 1) TR6-Fc in its present form has only a very short half-life of 20 min (data not shown), and there is certainly room to improve its effect in this regard; 2) the 400 islets transplanted to a recipient are empirically determined to achieve euglycemia in  $\sim$ 80% of the mice, and probably this number has already factored in nonfunctional islets; and 3) PNF is

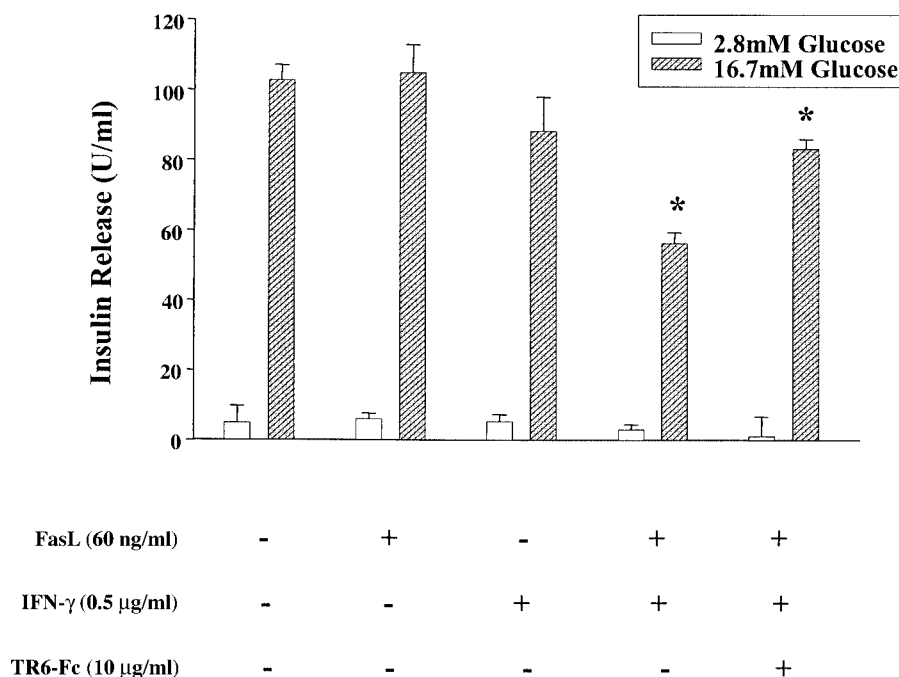


FIG. 6. TR6 protects  $\beta$ -cell function. Islets were cultured for 48 h with various reagents as indicated and then challenged with 2.8 or 16.7 mmol/l glucose. Insulin released to the supernatants was assayed by enzyme-linked immunosorbent assay. The samples were in triplicate. The experiments were repeated more than twice with similar results. Data from a representative experiment are shown. The difference between control versus IFN- $\gamma$ /FasL-treated and IFN- $\gamma$ /FasL/TR6-Fc-treated samples was highly significant ( $P < 0.01$  in both cases, marked with asterisks, Student's  $t$  test).

probably caused by multiple factors, not all of which could be corrected by TR6; indeed, TR6 was not able to protect islet function damaged by a combination of TNF- $\alpha$  and interleukin-1 $\alpha$  (data not shown).

The Edmonton protocol has proved that islet transplantation is a successful treatment for diabetes. Rough calculations show that  $>60\%$  of transplanted islets are nonfunctional. PNF and rejection are the chief reasons for

such a low efficiency. Considering the organ donor shortage, to reduce the number of islets needed for successful transplantation becomes an essential task to significantly enhance our capability for treating more patients with diabetes. Many preclinical and clinical studies are under way to optimize immunosuppressant regimens in islet transplantation, but the problem of PNF remains to be addressed. In vivo, tumor cells may use TR6 to protect

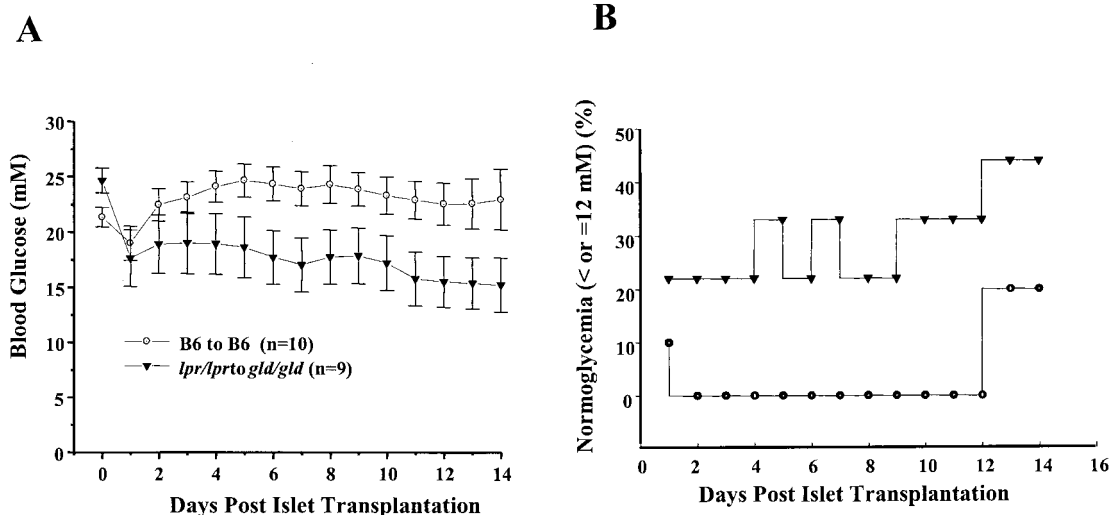


FIG. 7. Islet transplantation from *lpr/lpr* ( $H-2^b$ ) donors to *gld/gld* ( $H-2^b$ ) isogenic recipients had significantly higher efficacy compared with that between C57BL/6 mice (B6). The *lpr/lpr* mice (in  $H-2^b$  background) were used as donors, and streptozotocin-induced diabetic *gld/gld* mice (also in  $H-2^b$  background) were used as recipients. A marginal dose of 250–270 islets was transplanted intraperitoneally to each recipient. The recipient mice had blood glucose levels between 20 and 30 mmol/l before islet transplantation (day 0). After islet transplantation, blood glucose was monitored daily until day 14 and was plotted (A). The two groups had a significant difference in blood glucose levels starting from day 6 posttransplantation (ANOVA,  $P < 0.05$  at all points from day 6 and on). B: Percentage of mice that returned to normoglycemia (defined as  $\leq 12$  mmol/l) after the transplantation. The difference between the two groups was also highly significant ( $P < 0.001$ , two-way repeated ANOVA).

themselves from immune surveillance and gain survival advantages (9). By the same token, islets can benefit from the protective role of TR6. Our animal study proved that this is indeed the case. We envision that such an approach could be applied for clinical islet transplantation. Soluble TR6 can be added to buffers used for islet isolation, transportation, and injection. Alternatively, islets can be transfected with TR6 expression adenovirus vectors for long-lasting secretion of TR6 in situ. As discussed above, such treatments with TR6 might not totally prevent PNF, but they could probably reduce PNF significantly and bring down the number of islets needed for insulin independence after islet transplantation.

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#### REFERENCES

- Kenmochi T, Miyamoto M, Mullen Y: Protection of mouse islet isografts from nonspecific inflammatory damage by recipient treatment with nicotinamide and 15-deoxyspergualin. *Cell Transplant* 5:41–47, 1996
- Wahoff DC, Papalouis BE, Najarian JS, Kendall DM, Farney AC, Leone JP, Jessurun J, Dunn DL, Robertson RP, Sutherland D: Autologous islet transplantation to prevent diabetes after pancreatic resection. *Ann Surg* 222:562–575, 1995
- Shapiro AMJ, Lakey R, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
- Rosenberg L: Clinical islet cell transplantation. Are we there yet? *Int J Pancreatol* 24:145–168, 1998
- Rosenberg L, Wang R, Paraskevas S, Maysinger D: Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery* 126:393–398, 1999
- Thomas FT, Contreras JL, Bilbao G, Ricordi C, Curiel D, Thomas JM: Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. *Surgery* 126:299–304, 1999
- Soon-Shiong P, Feldman E, Nelson R, Komtebedde J, Smidsrod O, Skjak-Braek G, Espevik T, Heintz R, Lee M: Successful reversal of spontaneous diabetes in dogs by intraperitoneal microencapsulated islets. *Transplantation* 54:769–774, 1992
- Lanza RP, Sullivan SJ, Chick WL: Perspectives in diabetes. Islet transplantation with immunoisolation. *Diabetes* 41:1503–1510, 1992
- Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, Huang A, Donahue CJ, Sherwood SW, Baldwin DT, Godowski PJ, Wood WI, Gurney AL, Hillan KJ, Cohen RL, Goddard AD, Botstein D, Ashkenazi A: Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396:699–703, 1998
- Zhang J, Salcedo TW, Wan X, Ullrich S, Hu B, Gregorio T, Feng P, Qi S, Chen H, Cho YH, Li Y, Moore PA, Wu J: Modulation of T-cell responses to alloantigens by TR6/DcR3. *J Clin Invest* 107:1459–1468, 2001
- Yu KY, Kwon B, Ni J, Zhai Y, Ebner R, Kwon BS: A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J Biol Chem* 274:13733–13736, 1999
- Bai C, Connolly B, Metzke ML, Hilliard CA, Liu X, Sandig V, Soderman A, Galloway SM, Liu Q, Austin CP, Caskey CT: Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc Natl Acad Sci U S A* 97:1230–1235, 2000
- Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, Ruben S, Murphy M, Eisenberg RJ, Cohen GH, Spear PG, Ware CF: LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* 8:21–30, 1998
- Tamada K, Shimozaki K, Chapoval AI, Zhai Y, Su J, Chen SF, Hsieh SL, Nagata S, Ni J, Chen L: LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. *J Immunol* 164:4105–4110, 2000
- Zhai Y, Guo R, Hsu TL, Yu GL, Ni J, Kwon BS, Jiang GW, Lu J, Tan J, Ugustus M, Carter K, Rojas L, Zhu F, Lincoln C, Endress G, Xing L, Wang S, Oh KO, Gentz R, Ruben S, Lippman ME, Hsieh SL, Yang D: LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J Clin Invest* 102:1142–1151, 1998
- Rooney IA, Butrovich KD, Glass AA, Borboroglu S, Benedict CA, Whitbeck JC, Cohen GH, Eisenberg RJ, Ware CF: The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J Biol Chem* 275:14307–14315, 2000
- Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen SF, Zhou JX, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gentz R, Feng P, Moore PA, Ruben SM, Wei P: TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* 16:479–492, 2002
- Lee MS, Kim S, Chung JH, Lee MK, Kim KW: Fas is expressed in murine pancreatic islet cells and an insulinoma cell line but does not mediate their apoptosis in vitro. *Autoimmunity* 29:189–199, 1999
- Muruve DA, Nicolson AG, Manfro RC, Strom TB, Sukhatme VP, Libermann TA: Adenovirus-mediated expression of Fas ligand induces hepatic apoptosis after systemic administration and apoptosis of ex vivo-infected pancreatic islet allografts and isografts. *Hum Gene Ther* 8:955–963, 1997
- Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J: NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol* 21:5299–5305, 2001
- Mullauer L, Mosberger I, Grusch M, Rudas M, Chott A: Fas ligand is expressed in normal breast epithelial cells and is frequently up-regulated in breast cancer. *J Pathol* 190:20–30, 2000
- Ozasa T, Newton MR, Dallman MJ, Shimizu S, Gray DW, Morris PJ: Cytokine gene expression in pancreatic islet grafts in the rat. *Transplantation* 64:1152–1159, 1997
- Unutmaz D, Pileri P, Abrignani S: Antigen-independent activation of naive and memory resting T cells by a cytokine combination. *J Exp Med* 180:1159–1164, 1994
- Ettinger R, Munson SH, Chao CC, Vadeboncoeur M, Toma J, McDevitt HO: A critical role for lymphotoxin-beta receptor in the development of diabetes in nonobese diabetic mice. *J Exp Med* 193:1333–1340, 2001
- Wu Q, Salomon B, Chen M, Wang Y, Hoffman LM, Bluestone JA, Fu YX: Reversal of spontaneous autoimmune insulinitis in nonobese diabetic mice by soluble lymphotoxin receptor. *J Exp Med* 193:1327, 2001