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ORIGINAL ARTICLE

Immunomodulation by blockade of the TRANCE co-stimulatory pathway in murine allogeneic islet transplantation

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Summary

We explore herein the effect of TNF-related activation-induced cytokine (TRANCE) co-stimulatory pathway blockade on islet survival after allograft transplantation. Expression of TRANCE on murine C57Bl/6 (B6) CD4+ T cells after allogeneic activation was analyzed by fluorescence-activated cell sorter (FACS). The effect of a TRANCE receptor fusion protein (TR-Fc) and anti-CD154 antibody (MR1) on B6 spleen cell proliferation after allogeneic activation was assessed by mixed lymphocyte reaction (MLR). Three groups of B6 mice were transplanted with allogeneic islets (DBA2): Control; short-term TR-Fc-treatment (days 0–4); and prolonged TR-Fc-treatment (days −1 to 13). Donor-specific transfusion (DST) was performed at the time of islet transplantation in one independent experiment. Transplantectomy samples were analyzed by immunohistochemistry. TRANCE expression was upregulated in stimulated CD4+ T cells *in vitro*. In MLR experiments, TR-Fc and MR1 both reduced spleen cell proliferation, but less than the combination of both molecules. Short-course TR-Fc treatment did not prolong islet graft survival when compared with controls (10.6 ± 1.9 vs. 10.7 ± 1.5 days) in contrast to prolonged treatment (20.7 ± 3.2 days; $P < 0.05$). After DST, primary non function (PNF) was observed in half of control mice, but never in TR-Fc-treated mice. Immunofluorescence staining for Mac-1 showed a clear decrease in macrophage recruitment in the treated groups. TRANCE-targeting may be an effective strategy for the prolongation of allogeneic islet graft survival, thanks to its inhibitory effects on co-stimulatory signals and macrophage recruitment.

Introduction

Pancreatic islet transplantation offers a great potential for the treatment of type 1 diabetes mellitus. The protocol introduced by the group at the University of Alberta has helped improve the results of islet transplantation to a great extent, achieving 80% insulin-independence at 1-year follow-up [1,2]. Among the issues that have surfaced are the rapid loss of islet graft function over time and the high incidence of side-effects of the immunosuppressive (IS) regimen. [3,4] For this reason, alternative, less toxic IS regimens are desirable. The use of agents blocking co-stimulatory signals of T-cell activation at the time of cognate interaction between T-cell receptor and the foreign antigen is a promising approach. Blockade of the CD28-B7-1 or 2 axis with the use of belatacept has recently been successfully used in a phase II trial of kidney transplantation, with a remarkably low incidence of side-effects [5]. CD154, another key molecule for T-cell activation, has been shown critical in the delivery of co-stimulation signals during allograft rejection [6]. Anti-CD154 monoclonal antibody (MR1) used as a blocker of

this co-stimulation pathway, induces operational tolerance in allogeneic [7,8] or xenogenic [9] rodent models of islet transplantation. Specific blockade of this pathway also prevents acute allograft rejection in primate models of islet [10] and kidney [11] transplantation. Unfortunately, these promising preliminary results could not reach the stage of clinical application because anti-CD154 antibody therapy was reported to cause unexpected thrombo-embolic complications in both primates and humans [10,12,13].

The role of TNF-related activation-induced cytokine (TRANCE) in the immune system has recently emerged [14–16]. TRANCE provides signals for the morphogenesis of lymph nodes, participates in B and T-cell development [17] and belongs to another T-cell co-stimulatory pathway. TRANCE mediates its effects via the TRANCE receptor (TRANCE-R), also known as receptor-activating NF-κB (RANK), expressed on antigen-presenting cells (APCs). While CD154 is only expressed on activated CD4+ T cells, TRANCE is expressed on both activated CD4+ and CD8+ T cells [18], suggesting an independent role of TRANCE on APCs through CD8+ activation. In the absence of the CD40/CD154 pathway, TRANCE can independently activate CD4+T cells, following lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus or Leshmania infection in mice [19,20]. An involvement of TRANCE in the allogeneic immune response has recently been suggested in a rodent model of heart allograft [21].

We hypothesized that blockade of the TRANCE co-stimulatory pathway might prolong the survival of allogeneic pancreatic islets. Therefore, we have first demonstrated the independent role of TRANCE in the T-cell response in an *in vitro* allogeneic model. Second, we have studied the effects of TRANCE blockade on graft rejection in an *in vivo* model of allogeneic islet transplantation.

Materials and methods

Animals

Adult male C57Bl/6 (B6) and DBA2 mice were purchased from Janvier Laboratories (Le Genest Saint Isle, France). Eight- to 10-week-old mice were used for *in vitro* experiments and 6–9-week-old mice were used for *in vivo* experiments. Animals were maintained in our own housing facilities with free access to food and water. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee and by the State of Geneva Veterinary Authorities.

Spleen cells

After skin disinfection, spleens from B6 or DBA2 mice were removed though a large midline laparotomy. Cells were obtained by gentle mechanical disruption of the spleen followed by filtration through a 100-μm mesh. Erythrocytes were lysed using a commercial kit (Mouse Erythrocyte Lysing kit; R&D Systems, Minneapolis, MN, USA), and mononuclear cells were purified by Ficoll-hypaque density gradient centrifugation (Histopaque-1077; Sigma, St Louis, MO, USA).

In vitro T-cell activation

Supra-physiologic activation of murine CD4+ T cells was induced by incubation with anti-CD3 and anti-CD28 mAb as described previously [19]. Briefly, 400×10^5 mononuclear cells isolated from the spleens of B6 mice were transferred into 96-well round bottom plates (Nunclon Surface, DK 4000, Roskilde, Denmark) previously coated with saturating amounts of rat anti-mouse CD3 IgG (Serotec, Oxford, UK) in 200 μl Iscove modified Dulbecco's culture medium (Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (Gibco), 1 mM sodium pyruvate (Gibco), MEM Non Essential Amino Acids (Gibco) diluted 100×, 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma, St Louis, MO, USA), 0.292 mg/ml L-Glutamine (Sigma), and 50 μM 2-Mercapto-ethanol (Sigma). 2.5 μg/ml of a hamster anti-mouse CD28 IgG (Becton Dickinson, Basel, Switzerland) antibody was added to each well. As control, equal numbers of cells were transferred into 96-wells plates untreated with anti-CD3 and anti-CD28 antibodies. After 2 days of incubation, the cells were harvested, stained for CD4 and TRANCE, and analyzed by flow cytometry as described below. Experiments were run in triplicate and repeated five times.

Flow cytometry

Cells were stained using a monoclonal phycoerythrin (PE)-conjugated rat anti-mouse CD4 IgG (1/400; Becton Dickinson, Basel, Switzerland, réf. 553048) and a monoclonal goat anti-mouse TRANCE IgG (dilution 1/1; R&D Systems, ref. AF 462) followed by fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat $F(ab')_2$ IgG (1/400; AffiniPure, Jackson Laboratories, Basel, Switzerland). As negative isotype control, cells were incubated with an irrelevant goat IgG (1/1, AB108c; R&D Systems).

All antibodies were incubated with cells for 1 h. Fluorescence of stained cells was measured on a FACSTrack fluorocytometer (Becton Dickinson) and analyzed with the WinMDI software (Scripps Research Institute, La Jolla, CA, USA).

Mixed lymphocyte reactions

To assess TRANCE expression in response to allogeneic stimulation, 3×10^5 DBA2 spleen cells and 3×10^5 B6

spleen cells were co-cultured in 96-well round-bottom plates (Nunc, Wiesbaden, Germany). As controls (non activated cells), either 6×10^5 DBA2 spleen cells or 6×10^5 C57Bl/6 spleen cells were cultured independently. Spleen cells were cultured in 200 μl Iscove modified Dulbecco's culture medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, MEM Non Essential Amino Acids diluted 100×, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 mg/ml L-Glutamine, 50 μM 2-Mercapto-ethanol and 2.5 μg/ml of anti-CD28 IgG. For the experimental and control groups, cell harvesting and culture were performed in parallel, in quadruplicates. Plates were incubated at 37 °C, in a 5% CO_2 atmosphere. After 5 days, cells were harvested and washed three times with PBS (Sigma) supplemented with BSA (Sigma) 0.1% and Azide 0.1%. Experiment was repeated five times.

To study the effect of co-stimulatory blockade on T-cell proliferation, spleen cells obtained from C57Bl/6 mice were used as responder cells, and spleen cells from DBA2 as stimulator cells. Stimulator cells were irradiated with a total dose of 35 Gy using a Cesium source. Cells were cultured in a 2:1 stimulator/responder ratio, i.e. 4×10^5 DBA2 cells were cultured with 2×10^5 C57Bl/6 cells. Culture was performed in quadruplicate for each condition, in 96-well round bottom plates containing 200 μl fresh Iscove modified Dulbecco's medium supplemented as described above. Each experiment was repeated three times. Cells were incubated for 5 days at 37 °C in 5% CO_2. Inhibition of TRANCE was obtained by adding a recombinant protein of the extracellular domain of TRANCE-R fused to the constant region of human IgG1 (TR-Fc; Dr. Y. Choi, University of Pennsylvania, Philadelphia). Production of TR-Fc has been described previously [19]. Inhibition of CD154 was performed using a hamster anti-mouse CD40L antibody, MR-1 (Bio Express Inc, West Lebanon, NH, USA).

After 4 days incubation, 1 mCi ^{3}H-thymidine (Amersham, Otelfingen, Switzerland) was added and cells were further incubated for6 h. Plates were harvested and cell proliferation was assessed by measuring ^{3}H incorporation using a beta counter (Wallac, Perkin-Elmer; Geneva, Switzerland).

Induction of diabetes

C57Bl/6 mice were rendered diabetic by a single intraperitoneal injection of 200 mg/kg streptozotocin (Sigma, Buchs, Switzerland). Blood glucose levels were monitored on whole-blood samples collected from the tail vein using a strip glucometer (Precision Q.I.D; Abbott MediSense, Wiesbaden, Germany). Mice with blood glucose >20 mmol/l for more than 3 consecutive days were used as transplant recipients.

Islet isolation

Murine islets were isolated using a technique routinely used in our laboratory. Briefly, a collagenase (type XI, Sigma) solution was prepared at a final concentration of 2 mg/ml in Hank's balanced saline solution (HBSS). After anesthesia by intraperitoneal ketamine injection (12 mg/100 g of animal body weight), mice were euthanized, the abdomen was opened, and the pancreas was exposed. After clamping at the porta hepatis, the main bile duct was cannulated with a 10×0.4 mm needle (Venisystem, butterfly-27G) and injected in retrograde fashion with 2 ml of collagenase solution in order to distend the pancreas. The pancreas was then removed and placed in 7.5 ml ice-cold HBSS in 50-ml conical tubes (2 pancreata per tube). Digestion was started by raising the temperature to 37 °C. After 17 min, tubes containing the pancreata were put on ice and cold HBSS was added up to 50 ml in order to stop the digestion. Mechanical disruption of the digested pancreatic tissue was achieved by vigorous tube shaking. Islets were then purified on a discontinuous Ficoll gradient, washed twice with HBSS solution and kept on ice until transplantation.

Islet transplantation

Allogeneic murine islet transplantation was performed in the fully mismatched DBA2 (H2d) to B6 (H2b) combination. Prior to transplantation, islets were divided in aliquots of 750 islets. Islet viability, assessed by fluoresceine diacetate/propidium iodide staining was always >90%. Under general anesthesia by isoflurane (Forene®, Abbott AG, 6340 Baar) inhalation using a custom-made breathing apparatus, the left kidney was exposed. Islets were slowly injected under the kidney capsule, as described previously [22]. Islet transplantation was considered successful when blood glucose levels <11 mmol/l were measured on at least 2 consecutive days, and rejection was defined as sustained hyperglycemia >15 mmol/l on at least 3 consecutive days. Primary non function was defined as sustained hyperglycemia after transplantation in the absence of technical problem.

Animals were kept up to 100 days if no islet rejection occurred, or 3 to 10 days after recurrence of diabetes. Animals were sacrificed and graft-bearing kidneys were retrieved. Kidneys were embedded in Tissue-Tek (Miles, Elkhart, IN, USA), snap-frozen in liquid methylbutane pre-equilibrated with liquid nitrogen and stored at −80 °C.

Experimental groups

In a first set of experiments, the treated group ($n = 8$) received 0.2 mg TR-Fc intraperitoneally on days 0, 2 and 4, day 0 being the day of islet transplantation (Short-term treatment). Controls ($n = 7$) were injected intraperitoneally with vehicle. In a second set of experiments, the treated group ($n = 4$) received 0.2 mg of TR-Fc intraperitoneally on days −1, 1, 3, 5, 7, 9, 11, 13 (Long-term treatment). In a third set of experiments, 5×10^6 donor-specific spleen cells were injected intravenously into the tail vein 1 day before transplantation and TR-Fc injections (treated group, $n = 5$) or vehicle (control group, $n = 9$) were administered at the same dose and on the same days as in the short-term treatment group.

Histology and immunohistochemistry

Serial frozen sections were cut at 5 μm intervals using a cryostat (Leica, Glattbrugg, Switzerland). Sections were stained with hematoxylin and eosin (HE). For immunofluorescence staining, tissue sections were fixed with absolute ethanol for 1 min and then incubated with phosphate-buffered saline supplemented with 0.5% bovine serum albumin (Sigma, St. Louis, MO, USA) for 15 min to block nonspecific binding. Insulin-staining was performed using a guinea pig anti-insulin primary antibody (Ab) (1/100; DAKO, Glostrup, Denmark) and a rhodamine-conjugated goat anti-guinea pig secondary Ab (1/400; Jackson Laboratories, Basel, Switzerland). T-lymphocyte staining was performed using a rat anti-mouse CD4 Ab (1/20; Becton Dickinson, Basel, Switzerland) and rat anti-mouse CD8 Ab (1/100; Serotec, Kidlington, UK). Macrophage staining was performed with a rat anti-mouse Mac-1 Ab (1/10, ImmunoKontact, Switzerland). For all T-cell and macrophage stainings, FITC-conjugated donkey anti-rat was used as secondary Ab. Slides were examined under a fluorescence microscope (Zeiss Axiophot, Göttingen, Germany). Sections were analyzed by two independent observers (AW, MA), and intensity of staining was classified semi-quantitatively as absent (0), borderline (+/−), mild (+), moderate (++), or strong (+++).

Statistical analysis

The Statistica 6.0 software package (Statsoft, Tulsa, OK, USA) was used for statistical analysis. Data were expressed as median and range or mean ± SD, wherever appropriate. Comparison of continuous variables was done using bilateral Student's *t*-test. Kaplan–Meier analysis was performed for diabetes-free survival determination, and differences were assessed with the Mantel-Cox log-rank test. Values of $P < 0.05$ were considered as significant.

Results

Expression of TRANCE by T cells

To test whether TRANCE/TRANCE-R interaction plays a role in T-cell activation, surface expression of TRANCE was analyzed on activated CD4+ T cells after stimulation with both anti-CD3 and anti-CD28 monoclonal antibodies (Fig. 1a–c). After CD4/TRANCE double staining of murine B6 spleen cells, CD4+ T cells showed a clear increase in TRANCE expression when compared with control conditions in which spleen cells were incubated in with culture medium only. No increase in TRANCE expression was detected after stimulation by anti-CD3 or anti-CD28 alone.

Five-day mixed lymphocyte culture of spleen cells isolated from B6 and DBA2 mice led to an increased expression of TRANCE by B6 CD4+ T cells, as detected by a slight but sizeable shift to the right of the fluorescence peak on FACS analysis (Fig. 1a and f). TRANCE expression by CD4+ T cells was not increased in control B6 or DBA/2 spleen cells cultured independently (Fig. 1, panels D and E).

Inhibition of T-cell activation by TRANCE-blockade

In mixed lymphocyte culture experiments of spleen cells isolated from B6 and DBA/2 mice, cell proliferation was largely increased in control conditions as a result of allogeneic stimulation by DBA2 irradiated cells on proliferation of B6 stimulator cells. This response was inhibited individually by blockade of the TRANCE, with a trend toward significance ($P = 0.19$) and CD154 pathways ($P < 0.02$), using TR-Fc and anti-CD154 mAb (MR-1) respectively. A cumulative effect on the inhibition of cell proliferation was observed with simultaneous blockade of both pathways ($P < 0.01$; Fig. 2).

Effect of TRANCE blockade on allogeneic islet graft survival

Based on these results, the effects of systemic TR-Fc treatment in an *in vivo* allogeneic model of islet allograft transplantation were tested. A full-mass islet graft (750 IEQ) restored normoglycemia in all recipient mice within 2 days. In the short-term treatment group, mice did not exhibit a significant prolongation of graft survival ($n = 8$; 10.6 ± 1.9 days) compared with controls ($n = 6$; 10.7 ± 1.5 days, $P > 0.1$; Fig. 3). A significant prolongation of graft survival was observed in the long-term treatment group ($n = 4$; 20.7 ± 3.2 days, $P < 0.05$; Fig. 3).

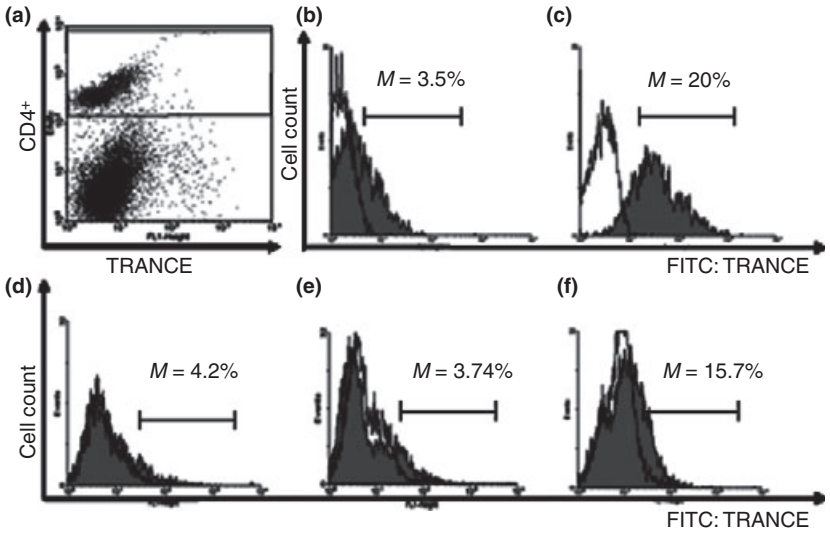
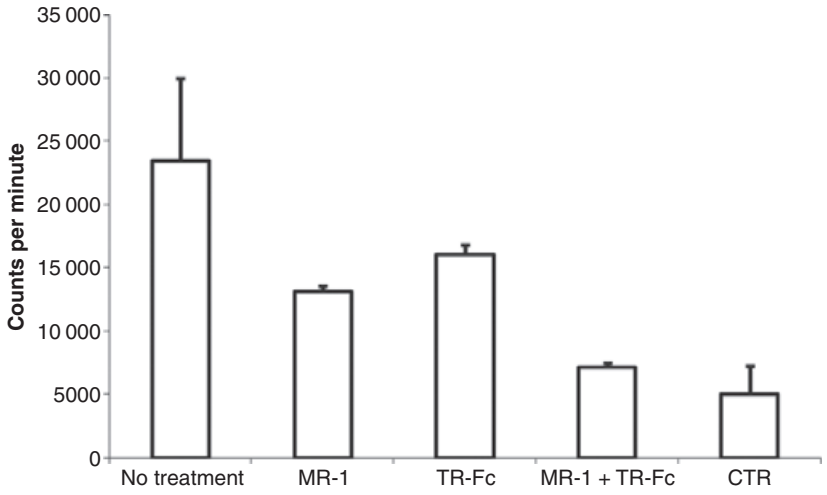


Figure 1 FACS analysis of TRANCE expression by stimulated CD4+ T cells. Panel a: CD4-PE and TRANCE-FITC double staining of C57Bl/6 spleen cells. All further analyses were performed on the same CD4+-gated cell population shown here. Panels b, c: TRANCE expression (CD4+-gated) of C57Bl/6 spleen cells cultured in medium alone (b), or incubated with anti-CD3 and anti-CD28 mAb (c). A shift from 3.5% to 20% FITC-positive cells is observed, indicating an increase in TRANCE expression secondary to nonspecific T-cell stimulation. Panels d, e, f: TRANCE expression (CD4+-gated) in 600×10^5 C57Bl/6 spleen cells cultured alone (d), 600×10^5 DBA2 spleen cells cultured alone (e) and co-culture of 300×10^5 C57Bl/6with 300×10^5 DBA2 spleen cells (f). A shift from 4.2% or 3.7% (basal condition) to 15.7% FITC-positive cells is observed in the mixed lymphocyte culture (f) indicating increased TRANCE expression secondary to allogeneic stimulation. In panels b–f, empty curves correspond to control isotype staining, and full curves correspond to staining with anti-TRANCE antibody.



p	No TTT	MR-1	TR-Fc	MR-1 + TR-Fc
No treatment				
MR-1	<0.02			
TR-Fc	0.19	<0.01		
MR-1 + TR-Fc	0.02	<0.0001	<0.01	
CTR (control)	<0.01	<0.001	<0.01	0.3

Figure 2 Effect of TRANCE blockade on T-cell proliferation in mixed lymphocyte cultures. In this experiment, 2×10^5 C57Bl/6 spleen cells (responders) were co-cultured for 5 days with 4×10^5 DBA2 irradiated spleen cells (stimulators). T-cell proliferation was measured by ^{3}H incorporation with a beta counter and expressed as counts per minute (c.p.m.) +/– SD. T-cell proliferation was partially inhibited by individual blockade of the TRANCE and CD154 pathways, an inhibition that became complete after combined blockade of both pathways. *P* values of the differences observed between experiments are shown. In control experiments (CTR), C57Bl/6 spleen cells were used both as responders and stimulators at the same ratio. The figure is representative of three separate experiments.

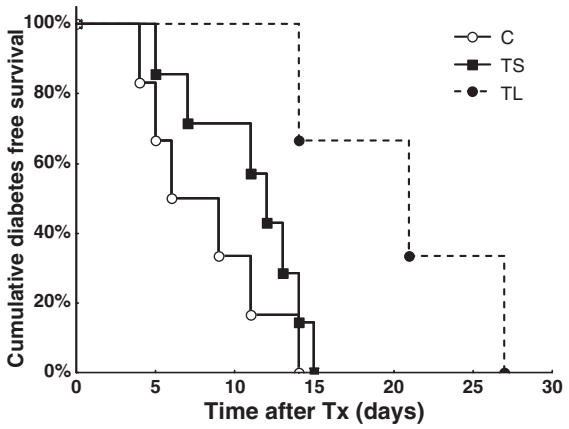


Figure 3 Effect of *in vivo* TRANCE blockade on islet graft survival. Chemically diabetic C57Bl/6 mice were transplanted under the kidney capsule with 750 IEQ isolated from DBA/2 mice. Graft survival in animals treated with 3 injections of 200 μg TR-Fc on days 0, 2 and 4 (short-term treatment; TS: $n = 8$; full line, full squares) was identical to that of untreated control animals (C: $n = 6$; full line, empty circles). A significantly prolonged islet graft survival was observed in animals treated with 8 injections of 200 μg TR-Fc from day-1 to Day 13 post transplantation (long-term treatment; TL $n = 4$, dashed line, full circles; $P < 0.05$).

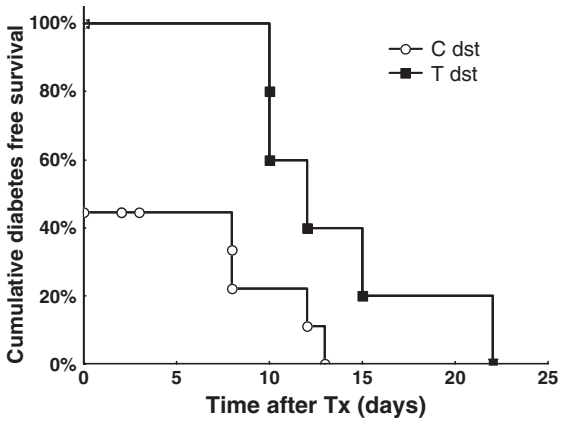


Figure 4 Effect of *in vivo* TRANCE blockade combined with donor-specific transfusion (DST) on islet graft survival. Chemically diabetic C57Bl/6 mice were transplanted under the kidney capsule with 750 IEQ isolated from DBA/2 mice. Treated animals also received three injections of 200 μg TR-Fc on days 0, 2 and 4. All mice from the treated (T DST: $n = 5$) and control (C DST: $n = 9$) groups received an intravenous injection of 5×10^6 DBA/2 spleen cells 1 day prior to islet transplantation. Graft survival in treated animals was significantly prolonged when compared with controls because of the high frequency of primary non function observed in the control group ($P < 0.05$).

Primary non function did not occur in the control or treated groups.

One mouse in each group did not reject its allograft (graft survival >100 days). In these mice, blood sugar levels increased after nephrectomy showing that the observed normoglycemia was attributable only to a functional islet graft.

Effect of TRANCE blockade and donor-specific transfusion (DST) on allogeneic islet graft survival

As it had been shown that TRANCE blockade could prolong murine heart graft survival only when combined with donor-specific spleen cell transfusion [21], we then used the same strategy in our model of islet transplantation. A marked increase in the rate of PNF occurred in the control group ($n = 5$, 56% of transplanted mice) compared with the treated group ($n = 0$). Median islet graft survival was 13.8 ± 5.0 days in the treated group ($n = 5$) vs. 10.3 ± 2.6 days in the control group, when excluding grafts that underwent PNF from analysis ($n = 4$; $P = 0.31$). When islet grafts that underwent PNF were considered in the control group, survival was 5.9 ± 4.5 days ($n = 9$, $P = 0.026$; Fig. 4). No long-term survival was observed.

Histology

Histological examination of islet allografts was performed 3 to 10 days after rejection in all groups of recipients. Islet grafts were identified by HE and insulin staining. Inflammatory cells were stained for Mac-1, CD4 and CD8.

In controls (Fig. 5a, e, i and m), classic signs of rejection were evident, with infiltration by T cells, identified by strong (+++) CD4 or CD8 staining, and macrophages, identified by strong (+++) Mac-1 staining. Disruption of islet architecture was observed, as well as a decrease of insulin staining (mild, +).

In the group of mice that received DST but no TR-Fc treatment (Fig. 5b, f, j and n), intense inflammation and near-complete islet destruction were seen. Only rare islets stained positive for insulin (+/−) and a marked macrophage infiltration (Mac-1; +++) with mild (+) CD4+ and CD8+ T-cell infiltration was observed. Only small remnants of insulin-containing tissue permitted islet identification.

In TR-Fc-treated mice with DST (Fig. 5c, g, k and o), inflammatory cell infiltration was observed on HE staining and, despite rejection, macrophage infiltration was borderline (+/−). CD4+ T-cell infiltration was borderline (+/−) and CD8+ T-cell infiltration was moderate (++). Insulin staining was moderate (++), with preservation of islet structure.

In long-term TR-Fc-treated mice (no DST; Fig. 5d, h, l and p), macrophage infiltration was borderline (+/−). CD4+ T-cell infiltration was absent (0) and CD8+ T-cell infiltration was moderate (++). Insulin staining was strong (+++) with preserved islet architecture.

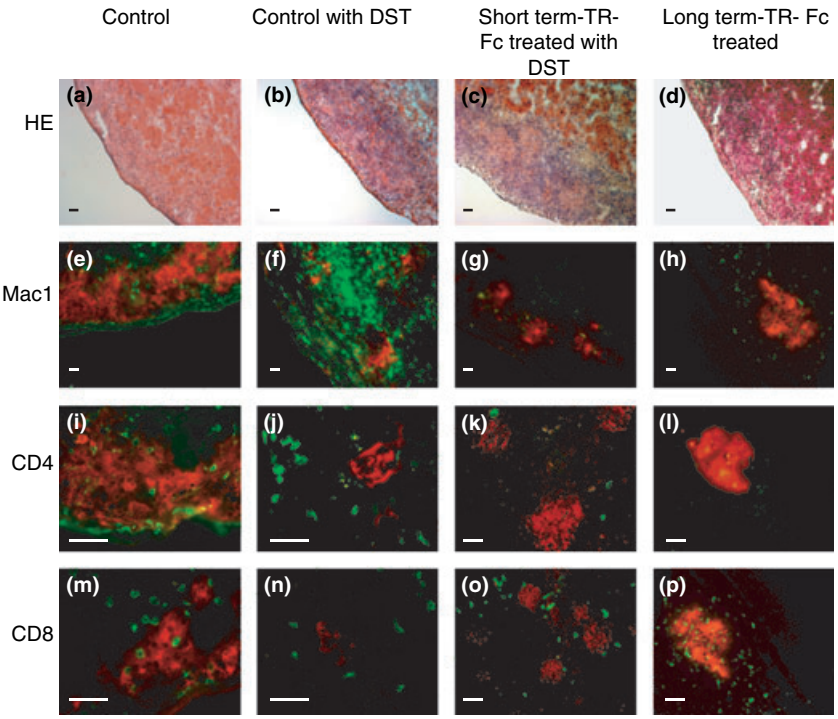


Figure 5 Immunohistochemistry of islet graft sections. After islet rejection, kidneys were removed, sectioned and stained with HE (a–d). Insulin staining with a rhodamine-conjugated secondary Ab was performed to identify beta-cells within the transplanted islets (e–p). Mac-1 (e–h), CD4 (i–l) and CD8 (m–p) staining with a FITC-conjugated secondary Ab was performed to label immune cellular infiltration within the islet grafts. Scale bar: 50 μm.

Discussion

In this study, we have shown in an allogeneic model of islet transplantation that blockade of the TRANCE pathway can prevent islet-graft primary nonfunction and delay graft rejection. We first showed that TRANCE expression is upregulated during T-cell activation, and that TRANCE blockade impairs T-cell proliferation. Second, we demonstrated *in vivo* that TRANCE blockade delays acute islet allograft rejection in a murine model, while decreasing macrophage infiltration in the graft.

The role of TRANCE/TRANCE-R interaction as a co-stimulatory pathway of T-cell activation was first suggested by Josien *et al.* [18]. We showed here that the expression of TRANCE at the membrane surface of T cells was highly upregulated upon antibody stimulation of the CD3 and CD28 T-cell surface molecules. Moreover, we have pointed out the role of allogeneic stimulation in the increase of TRANCE expression on CD4+ T cells in an MLR experiment using DBA2 and B6 spleen-cells. In the same MLR combination, TRANCE blockade by a recombinant protein of the extracellular domain of TRANCE-receptor fused to the constant region of human IgG1 (TR-Fc) led to a decrease in T-cell proliferation. Blockade of the CD154 pathway led to a decrease of T-cell proliferation to a similar extent, and blockade of both pathways further decreased T-cell proliferation to levels close to background. This suggests that TRANCE and CD154 engagement have at least an independent additive effect on T-cell activation secondary to an allogeneic stimulus. Taken together, these results suggest that TRANCE and CD154 may share similar *in vivo* co-stimulatory functions during T-cell activation in response to an allogeneic stimulus. TRANCE may also be responsible for CD154-independent CD4+ T-cell alloimmune responses, as observed in murine models of viral infections [19,20].

To determine whether the role of TRANCE as a co-stimulatory role is significant enough to prevent or delay graft rejection, we blocked the TRANCE pathway by *in vivo* treatment with TR-Fc in a murine model of allogeneic islet transplantation. Short-term TR-Fc treatment had no effect on the timing and occurrence of rejection. By contrast, long-term TR-Fc treatment delayed graft rejection and doubled graft survival time. Long-term graft survival was not achieved. Interestingly, graft rejection occurred in both treatment groups despite low graft infiltration by T cells, as shown by weak CD4 staining. These experiments show that, in contrast to CD154 blockade, TRANCE blockade alone is not sufficient for long-term prevention of rejection. We did not attempt to

combine TRANCE and CD154 blockade *in vivo*, because in our hands, as in others', CD154 blockade alone is able to yield indefinite graft survival in the murine islet allogeneic graft model [7,22,23].

A previous report by Guillonneau *et al.* [21] showed that short-term TRANCE blockade was only effective in prolonging heart transplant survival when combined with donor-specific transfusion administered on the day of transplantation. Although no clear explanation was offered for this observation, we used the same approach in our model. Surprisingly, DST led to a high incidence of PNF in the control group. At variance with Guillonneau's study [21], TRANCE blockade did not delay graft rejection in DST-treated animals. However, it completely prevented the occurrence of PNF. Differences in graft survival can partly be explained by the different nature of heart and islet transplantation, i.e. between a vascularized and a non vascularized graft. TRANCE has been described as a potent angiogenetic factor [24] and its blockade may have affected neo-vessel formation around transplanted islets, which is critical for their engraftment and survival. It should also be mentioned that in the Guillonneau study [21] DST was given on the same day as heart transplantation rather than 1 day before, and in a different mouse strain combination, both elements that could account for the differences observed.

The role of DST *per se* in islet transplantation must also be discussed. In our hands, DST administered on the day of transplant caused a high rate of islet PNF. A similar phenomenon was reported in the Balb/c to NOD combination, even when DST administered 5–8 days before islet transplantation was combined with CD154 co-stimulatory blockade [25]. In contrast, the same group reported a beneficial effect of DST, which was able to indefinitely prolong islet graft survival in the DBA/2 to B6 combination, when combined with CD154 co-stimulatory blockade [26]. Of note, DST was administered 28 days prior to islet transplantation in the latter study. These somewhat conflicting sets of data make it difficult to interpret the role of DST in allogeneic murine islet transplantation. However, it seems likely that there exists in mice a delicate balance between the tolerizing and sensitizing effects of DST that is dependent on timing of administration and genetic backgrounds of donor and recipient.

It is well known that macrophage activation is one of the key determinants of islet of Langerhans PNF [27]. In our study, DST caused a massive macrophage infiltration in the islet grafts of control mice, whereas a weak infiltration was observed in TR-Fc-treated mice. TRANCE-R (RANK) is expressed on the membrane surface of antigen-presenting cells, notably dendritic cells (DC), which belong to the same cell lineage as macrophages. TRANCE-RANK engagement not only results in co-stimulatory signals to T cells, but also in survival, maturation and secretory signals to DCs [28], notably leading to the secretion of pro-inflammatory cytokines such as IL-1 and IL-6 [18]. It is therefore likely that macrophage maturation and recruitment are inhibited by TRANCE blockade, as is the case of dendritic cells. This lack of recruitment could well explain the weak macrophage infiltration observed in islet grafts in TR-Fc-treated mice, which is even more obvious in DST-treated animals, and thus the protection against PNF conferred by TRANCE-RANK engagement blockade.

In accordance with the increasing evidence about the role of innate immunity in allograft rejection, the effect of TRANCE blockade on macrophage function at the site of engraftment could also be involved in the delay of rejection observed in long-term TR-Fc treated mice.

Taken together, our data indicate that TRANCE expression is upregulated on T cells after allogeneic stimulation and that TRANCE blockade by TR-Fc *in vitro* can depress T-cell proliferation in a CD154-independent pathway. This effect is not sufficient to confer operational tolerance to islet grafts *in vivo*, but can prolong islet graft survival if TR-Fc is administered over a prolonged period of time. This important difference with the effects of CD154 blockade can be explained by TRANCE being less critical for T-cell priming than CD154, but also by the blockade of the angiogeneic properties of TRANCE in the context of islet grafts that must revascularize in order to ensure engraftment. On the other hand, the tampering of macrophage recruitment and activity by TRANCE blockade may be of high interest in the promotion of islet engraftment.

Authorship

AW and AA contributed equally to the manuscript. TB, PM, and DB: designed study. AW, AA, SC, MA, and CT: performed research. AW, AA, SC and MA: collected data. AW, AA and DB: analyzed data. YC: contributed major reagents. AW, AA, and TB: wrote the paper. CT, YC, DB and PM: reviewed the paper.

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References

1. Shapiro AM, Lakey JR, Ryan EA, *et al.* Islet transplantation in seven patients with type 1 diabetes mellitus using a

glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 2000; **343**: 230.
2. Ryan EA, Lakey JR, Paty BW, *et al.* Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes* 2002; **51**: 2148.
3. Ryan EA, Paty BW, Senior PA, *et al.* Five-year follow-up after clinical islet transplantation. *Diabetes* 2005; **54**: 2060.
4. Hafiz MM, Faradji RN, Froud T, *et al.* Immunosuppression and procedure-related complications in 26 patients with type 1 diabetes mellitus receiving allogeneic islet cell transplantation. *Transplantation* 2005; **80**: 1718.
5. Vincenti F, Larsen C, Durrbach A, *et al.* Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* 2005; **353**: 770.
6. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* 1998; **16**: 111.
7. Molano RD, Berney T, Li H, *et al.* Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* 2001; **50**: 270.
8. Phillips NE, Markees TG, Mordes JP, Greiner DL, Rossini AA. Blockade of CD40-mediated signaling is sufficient for inducing islet but not skin transplantation tolerance. *J Immunol* 2003; **170**: 3015.
9. Bucher P, Gang M, Morel P, *et al.* Transplantation of discordant xenogeneic islets using repeated therapy with anti-CD154. *Transplantation* 2005; **79**: 1545.
10. Kenyon NS, Chatzipetrou M, Masetti M, *et al.* Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A* 1999; **96**: 8132.
11. Kirk AD, Burkly LC, Batty DS, *et al.* Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med* 1999; **5**: 686.
12. Kenyon NS, Fernandez LA, Lehmann R, *et al.* Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. *Diabetes* 1999; **48**: 1473.
13. Kawai T, Abrahamian G, Sogawa H, *et al.* Costimulatory blockade for induction of mixed chimerism and renal allograft tolerance in nonhuman primates. *Transplant Proc* 2001; **33**: 221.
14. Wong BR, Josien R, Choi Y. TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. *J Leukoc Biol* 1999; **65**: 715.
15. Kong YY, Feige U, Sarosi I, *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999; **402**: 304.
16. Kong YY, Yoshida H, Sarosi I, *et al.* OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999; **397**: 315.
17. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001; **104**: 487.
18. Josien R, Wong BR, Li HL, Steinman RM, Choi Y. TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol* 1999; **162**: 2562.
19. Bachmann MF, Wong BR, Josien R, Steinman RM, Oxenius A, Choi Y. TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. *J Exp Med* 1999; **189**: 1025.
20. Padigel UM, Kim N, Choi Y, Farrell JP. TRANCE-RANK costimulation is required for IL-12 production and the initiation of a Th1-type response to Leishmania major infection in CD40L-deficient mice. *J Immunol* 2003; **171**: 5437.
21. Guillonneau C, Louvet C, Renaudin K, *et al.* The role of TNF-related activation-induced cytokine-receptor activating NF-kappa B interaction in acute allograft rejection and CD40L-independent chronic allograft rejection. *J Immunol* 2004; **172**: 1619.
22. Mai G, Bucher P, Morel P, *et al.* Anti-CD154 mAb treatment but not recipient CD154 deficiency leads to long-term survival of xenogeneic islet grafts. *Am J Transplant* 2005; **5**: 1021.
23. Nicolls MR, Coulombe M, Beilke J, Gelhaus HC, Gill RG. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J Immunol* 2002; **169**: 4831.
24. Kim YM, Kim YM, Lee YM, *et al.* TNF-related activation-induced cytokine (TRANCE) induces angiogenesis through the activation of Src and phospholipase C (PLC) in human endothelial cells. *J Biol Chem* 2002; **277**: 6799.
25. Markees TG, Serreze DV, Phillips NE, *et al.* NOD mice have a generalized defect in their response to transplantation tolerance induction. *Diabetes* 1999; **48**: 967.
26. Zheng XX, Markees TG, Hancock WW, *et al.* CTLA4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *J Immunol* 1999; **162**: 4983.
27. Bottino R, Fernandez LA, Ricordi C, *et al.* Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation. *Diabetes* 1998; **47**: 316.
28. Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, *et al.* TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T-cells, is a dendritic cell-specific survival factor. *J Exp Med* 1997; **186**: 2075.

