

CD40–CD40 Ligand Interaction Activates Proinflammatory Pathways in Pancreatic Islets

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Pancreatic islet transplantation is becoming an alternative to insulin therapy in patients suffering from brittle type 1 diabetes. A major obstacle to the procedure is the early graft loss caused by nonspecific inflammation at the site of implantation. We recently discovered that CD40, a member of tumor necrosis factor (TNF) receptor family, is expressed in pancreatic β -cells. CD40 expression in nonhematopoietic cells is generally associated with inflammation. Therefore, we investigated the potential proinflammatory role of CD40 in human and nonhuman primate islets. Islet β -cells responded to CD40L interaction by secreting interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1, and macrophage inflammatory protein (MIP)-1 β , the latter a chemokine first reported to be produced by islets. Induction of IL-8 and MIP-1 β was confirmed at the transcriptional level by quantitative RT-PCR. MIP-1 β expression in β -cells was verified by double-immunofluorescence staining. CD40-CD40L interaction activates extracellular signal-regulated kinase 1/2 and nuclear factor- κ B pathways in insulinoma NIT-1 cells, and inhibitors of either pathway suppress cytokine/chemokine production in islets. Moreover, ligation of CD40 receptor upregulates intercellular adhesion molecule-1, associated with inflammation, at both transcriptional and translational levels. Our results in vitro indicate that the CD40 receptor expressed by β -cells could be activated in vivo, inducing proinflammatory responses contributing to early islet graft loss after transplantation. *Diabetes* 55:2437–2445, 2006

CD40 is a member of tumor necrosis factor (TNF) receptor family expressed in various cell types. In B-lymphocytes, macrophages, and antigen-presenting cells (APCs), the engagement of CD40 by CD40L (CD154) has a principal role in the immune system, inducing differentiation, cell survival, and

proliferation. Additionally, in B-cells, CD40-CD40L interaction results in isotype class switching (1). The critical role of CD40 in T-cell activation is well recognized in allograft rejection (2,3) and autoimmunity (4) as confirmed by studies in transgenic and knockout mice (1). In rodents (5) and nonhuman primates (NHPs) (2), blockade of CD40L prolongs allogeneic islet graft survival without immunosuppressive drugs.

CD40 expression has been reported in nonhematopoietic cells, such as thymic epithelial and endothelial cells, normal and neoplastic thyroid tissue, neurons, and pancreatic duct cells (6–10). Activation of CD40 receptor in nonhematopoietic cells is mostly associated with nonspecific inflammatory responses. These events engage costimulatory molecules, inducing cytokine/chemokine synthesis and secretion, which results in recruitment and activation of immune cells. For instance, CD40 stimulation plays a role in psoriasis, atherosclerosis, and cystic fibrosis (11–13).

At the time of engraftment, transplanted islets are susceptible to rapid destruction caused by the local secretion of proinflammatory cytokines, including interleukin (IL)-1, γ -interferon (IFN- γ), and TNF- α (14,15). This autocrine production of cytokines may induce the pancreatic β -cells to express proinflammatory molecules (16) and chemokines, such as monocyte chemoattractant protein (MCP)-1 (17,18), which develop an inflammatory milieu resulting in impaired function and viability of islets, leading to graft damage. In vitro studies indicate that CD40 activation in duct cells induces secretion of IL-1 β and TNF- α (10), which in turn activates dendritic cells and triggers apoptosis in neighboring β -cells (19). Moreover, it has been reported that CD40 signaling activates mitogen-activated protein kinase (MAPK), pathways frequently involved in inflammatory responses in other cell types such as synovial cells (20) and human proximal tubule cells (21).

We previously demonstrated that CD40 is expressed in mouse and human pancreatic β -cells, and its expression is upregulated by incubation with a cocktail of proinflammatory cytokines (IL-1 β , IFN- γ , and TNF- α). Initial studies indicate that CD40 signaling activates the proinflammatory transcription factor nuclear factor (NF)- κ B (22). This suggests that CD40-CD40L interaction in islets could activate MAPK pathways as well and induce a proinflammatory response. Here, we substantiate this hypothesis by showing that stimulation of CD40 in β -cells activates secretion of the proinflammatory cytokines/chemokines IL-6, IL-8, MCP-1, and macrophage inflammatory protein 1 β (MIP-1 β) through Raf/mitogen extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK) and NF- κ B pathways. Additionally, activated CD40 receptor

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APC, antigen-presenting cell; 7-AAD, 7-aminoactinomycin D; ERK, extracellular signal-regulated kinase; ICAM, intercellular adhesion molecule; I κ B, inhibitor of κ B; IL, interleukin; IFN- γ , γ -interferon; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; MEK, mitogen extracellular kinase; MIP, macrophage inflammatory protein; NF, nuclear factor; NHP, nonhuman primate; Th1, T-helper 1; TNF, tumor necrosis factor.

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TABLE 1

Cytokine/chemokine secretion is associated with the Raf/MEK/ERK and NF- κ B pathways in human and NHP islet cells

	Control	CD40L	PD98059 + CD40L	Bay 117082 + CD40L
NHP/1 (pg/ml)				
IL-6	4.12 \pm 2.61	10.18 \pm 1.09*†‡	5.10 \pm 0.32	2.69 \pm 0.37
IL-8	635 \pm 248	1,701 \pm 396*†‡	463 \pm 7.69	55 \pm 1.88
MCP-1	181 \pm 43	361 \pm 57*†‡	159 \pm 8.30	17.39 \pm 0.10
MIP-1 β	2.55 \pm 0.55	31.60 \pm 2.59*†‡	10.62 \pm 0.24	1.20 \pm 0.09
Human islets (pg/ml)				
IL-6	6.54 \pm 0.30	8.90 \pm 0.84*†‡	6.79 \pm 0.53	1.98 \pm 0.19
IL-8	847 \pm 32.45	16,683 \pm 4,474*†‡	1,791 \pm 145	325 \pm 3.51
MCP-1	0.10 \pm 0.01	2.33 \pm 0.01*†‡	0.28 \pm 0.26	0.10 \pm 0.01
MIP-1 β	4.50 \pm 0.12	12.66 \pm 0.58*†‡	4.26 \pm 0.21	0.775 \pm 0.08
NHP/2 (pg/ml)				
IL-6	62.21 \pm 3.75	216 \pm 45*‡	ND	57.36 \pm 9.40
IL-8	2.01 \pm 0.01	6,841 \pm 764*‡	ND	1,842 \pm 272
MCP-1	1,325 \pm 56	2,897 \pm 448*‡	ND	616 \pm 145
MIP-1 β	19 \pm 0.90	84 \pm 11.55*‡	ND	8.90 \pm 1.27
NHP/3 (pg/ml)				
IL-6	0.10 \pm 0.01	1.29 \pm 0.21*	1.29 \pm 1.29	ND
IL-8	162 \pm 3.67	567 \pm 51.9*†	246 \pm 82.39	ND
MCP-1	103 \pm 6.09	356 \pm 3.13*†	161 \pm 43.55	ND
MIP-1 β	4.04 \pm 0.11	17 \pm 0.81*†	6.39 \pm 2.05	ND

Data are means \pm SD. NHP/1, 2, and 3 represent three different islet preparations, respectively. * $P \leq 0.05$ vs. control, † $P \leq 0.05$ vs. PD + CD40L, ‡ $P \leq 0.05$ vs. Bay + CD40L. ND, not determined.

enhances expression of intercellular adhesion molecule (ICAM)-1 (CD54), known for its critical role in the pathophysiology of inflammation. Our data in vitro show that CD40-CD40L interaction in islet cells induces secretion of proinflammatory molecules, which are likely to be produced in the transplantation setting and might contribute to β -cell death and early graft loss.

RESEARCH DESIGN AND METHODS

Human pancreata were obtained from brain-dead multiorgan donors ($n = 12$, age range 23–61 years). Pancreatic islets were obtained from the Diabetes Research Institute's human islet cell processing facility and through the National Institutes of Health islet cell resources grants, using the automated method developed by Ricordi et al. (23). All animal studies were performed under protocols approved by the University of Miami animal care and use committee. NHP *Macaca fascicularis* islets (≥ 4 years of age) were isolated at the JDRF (Juvenile Diabetes Research Foundation International) Preclinical Cell Processing Core at the Diabetes Research Institute, using a modification of the automated method, as described previously (2). We chose to perform experiments with NHP islets as well because the cytoarchitectural structure, cell composition, and physiological parameters such as cytoplasmic free Ca^{2+} are similar to those in humans and are very different from the mouse islets (24,25). We used aliquots of those islets that were not used for transplantation protocols and thus did not require killing additional animals.

Islet cell dissociation. Islets were dissociated for 10–12 min at 37°C into single-cell suspensions, using a cocktail of digestive enzymes (Accutase; Innovative Cell Technologies, San Diego, CA) (26). Accutase was deactivated with fetal bovine serum.

Magnetic bead depletion. Dissociated human islet cells were depleted of carbohydrate antigen CA19-9–positive duct cells, using a pancreatic membrane antibody (Novocastra, Newcastle, U.K.) (27) in an indirect labeling protocol utilizing MACS microbeads (Miltenyi Biotec, Auburn, CA). Cells were incubated for 15 min with mouse anti-human CA19-9 monoclonal antibodies (mAbs; 1:100 dilution) and, after washing, for 15–20 min with MACS goat anti-mouse IgG microbeads (Miltenyi Biotec). The cell suspension was passed twice through a magnetic column. The efficiency of depletion was confirmed by flow cytometry (FacsCalibur; Becton Dickinson, Mountain View, CA) with anti-mouse Alexa fluor 488–conjugated secondary antibody (Invitrogen, La Jolla, CA). The purity of CA19-9 negative fraction was $>99\%$. Viability of depleted islet cells was $>70\%$, depending on the preparation. The assessment was performed with 7-aminoactinomycin D (7-AAD; Invitrogen). Depleted islet cell population was screened for other CD40-expressing cells, such as B-cells and dendritic cells (CD19 and CD11c, respectively; BD-Pharmingen, La

Jolla, CA) and monocytes (CD14; R&D Systems, Minneapolis, MN). NHP islets contain consistently a negligible amount of duct cells, as assessed by flow cytometry, using CK7, CK19 (M0888; Dako Cytomation), and CA19-9 antibodies.

Fluorescence activated cell sorting. Dispersed human and NHP islets were stained 90 min at 37°C in PBS with 1 μ M Newport Green (Invitrogen), which selectively binds to insulin in β -cells. Negative 7-AAD cells were sorted according to their Newport Green staining into bright Newport Green (β -cells) and dim/negative Newport Green (non- β -cells) subsets, using a FACSvantage cytometer (Becton Dickinson) (26). The purity of sorted β -cells was $>90\%$, as assessed by staining with anti-insulin antibody (Invitrogen/Zymed).

Cytokine induction and detection. Depleted islet cells, sorted human β -cells, or NHP dissociated islet cells were cultured in suspension in ultralow attachment plates at a density of 900,000 cells per ml of CMRL-based media supplemented with human serum albumin (0.5%). The cells were stimulated for 24 h at 37°C with rhCD40L (recombinant human soluble CD40 ligand; 1 μ g/ml; Alexis Biochemicals, San Diego, CA). After culture, ~ 30 – 50% of the islet cells remained alive, as assessed by dye exclusion. To identify intracellular pathways involved in CD40 signaling, the MEK1/2 phosphorylation inhibitor PD98059 (50 μ M; Cell Signaling Technology, Beverly, MA) and inhibitor of κ B (I κ B) phosphorylation inhibitor Bay117082 (5 μ M; Biomol, Plymouth Meeting, PA) were added to the media 1 h before the stimulation and maintained for 24 h. Concentrations of IL-1 β , IL-6, IL-8, IL-10, IFN- γ , MCP-1, MIP-1 β , and TNF- α in supernatants from controls and treated cells were determined, using Multi-Plex cytokine kits following the manufacturer's protocol (Bio-Plex platform technology; Bio-Rad Laboratories).

RT-PCR. Total RNA was isolated with an RNeasy kit (Qiagen, Valencia, CA), treated with DNase I, and used for cDNA synthesis. Human IL-6 forward and reverse primers were: 5'-ACATCTCGACGGCATCTCAG-3' and 5'-TGGCTT GTTCTCCTACTCT-3', respectively. Primers for human IL-8 and MIP-1 β were obtained from Biosource International (Camarillo, CA). The β -actin served as an internal control. PCR products were separated on 1% agarose gel.

Relative quantification real-time PCR. The assay was performed using the 7500 Fast Real-Time PCR system utilizing TaqMan Universal PCR mix (Applied Biosystems, Foster City, CA). Relative quantification determines the change in expression of target transcripts in a test sample (CD40L-treated cells) relative to a calibrator sample (untreated control). Relative quantification was calculated using Applied Biosystems SDS software based on the equation $RQ = 2^{-\Delta\Delta Ct}$, where RQ is relative quantification and Ct is the threshold cycle to detect fluorescence. Threshold cycle data were normalized to the internal standard, β -actin, using the formula: $\Delta Ct = \text{target Ct} - \beta\text{-actin Ct}$. Also, $\Delta\Delta Ct$ was calculated as $\Delta\Delta Ct = \Delta Ct (\text{CD40L treated}) - \Delta Ct (\text{control/calibrator})$. The results are expressed as relative quantification values ($2^{-\Delta\Delta Ct}$) with the range of minimum relative quantification ($2^{-\Delta\Delta Ct - 5}$) and

maximum relative quantification ($2^{-\Delta\Delta Ct + s}$), where s is the SD of the $\Delta\Delta Ct$ value. The primers and TaqMan probes for detection of ICAM-1, IL-6, IL-8, MIP-1 β , MCP-1, and β -actin transcripts were from Applied Biosystems.

Immunofluorescent staining of islet cells. Dissociated cells on slides were air-dried at room temperature, fixed for 15 min in 2.5% paraformaldehyde washed with Optimax (Bio-Genex, San Ramon, CA), and treated with universal blocker (Bio-Genex). The slides were incubated overnight at 4°C with goat anti-human MIP-1 β (5 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently for 20 min with secondary antibody Alexa Fluor 488 chicken anti-goat 1:200 (Invitrogen). Insulin staining was performed with anti-insulin antibody and Alexa Fluor 568 secondary antibody (1:200). The slides were mounted in Prolong antifade mounting solution and evaluated by confocal microscopy (LSM-510; Zeiss). The percentage of β -cells expressing MIP-1 β was calculated using laser scanning cytometry (CompuCyte, Cambridge, MA) or Metamorph imaging.

Western blot: CD40 expression. NHP islets were lysed in SDS and Tris-HCl buffer (pH 6.8), and aliquots corresponding to 50 μ g of protein were subjected to Western blot analysis as previously described (22). Membranes were incubated with 1:200 rabbit anti-human CD40 antibody (C20; Santa Cruz Biotechnology) followed by secondary anti-rabbit horseradish peroxidase-conjugated antibody (1:25,000) and chemiluminescent detection (Amersham Biosciences, San Francisco, CA). The same membranes were stripped and reprobed with an anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO).

Western blot identification of stress signaling pathways induced by CD40 agonistic antibody in NIT-1 insulinoma cell line. NIT-1 cells (CRL 2055; American Type Culture Collection, Manassas, VA) were plated and cultured in F12-K medium supplemented with 10% dialyzed fetal bovine serum. Cells were activated with CD40 agonist mouse mAb HM40-3 (10 μ g/ml; B&D Biosciences). We found that in NIT-1 cells the agonistic anti-CD40 antibodies were more efficient in eliciting a CD40 response than CD40L. Samples treated with PD98050 (50 μ mol/l) and Bay117082 (5 μ mol/l) were included.

Membranes were probed with antibodies specific to total and deactivated I κ B α (phosphorylated at Ser32/Ser36), total and activated ERK1/2 (phosphorylated at Thr202/Tyr204), total and activated MEK1/2 (phosphorylated at Ser217/221), total and activated p38 (phosphorylated at Thr180/Tyr182), and activated stress-activated protein kinase/c-Jun-NH $_2$ -terminal kinase (phosphorylated at Thr183/Tyr185), all at 1:1,000 dilution (Cell Signaling Technology). Enzymatic activity of ERK1/2 was measured using the protein Elk-1 as a substrate, using a kit from Cell Signaling.

Flow cytometry studies. A total of 1,000 human or NHP islet equivalents were dissociated with Accutase, stained with Newport Green, washed, and incubated 30 min on ice with either APC/anti-human CD40 mAbs (Caltag, Burlingame, CA) or anti-human ICAM-1 (CD54; BD Pharmingen). 7-AAD was added to identify dead cells. Flow cytometry was performed using CellQuest software gating in the bright Newport Green population (β -cells) (26). To evaluate ICAM-1 induction, islet cells were stimulated for 24 h with CD40L.

Statistical analysis. Results in Figs. 3, 4B, and 7B were analyzed with Wilcoxon's signed-rank test for paired nonparametric samples, with 95% CIs, using Prism software. This test is specifically suitable for a small number of samples with unknown distribution. Two-tailed P values <0.05 were considered statistically significant. Group comparisons (Table 1 and Figs. 5 and 6,

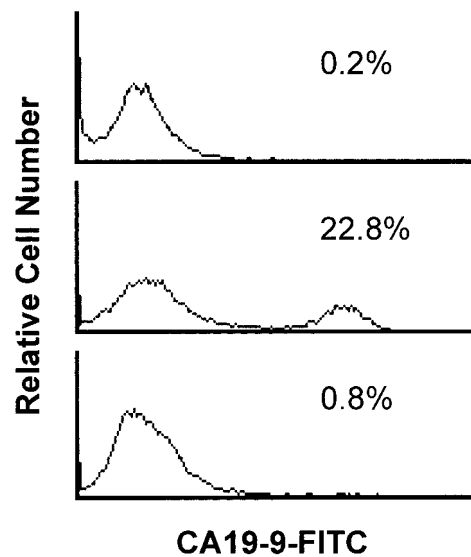


FIG. 1. Islet depletion of duct cells. Panels show FACS analysis of depletion ($n = 6$ islet preparations). *Top*, isotype; *middle*, before depletion; *bottom*, depleted.

Western blots) were performed using one-way ANOVA and Bonferroni's test for post hoc analysis, using SigmaStat 2 software.

RESULTS

CD40/CD40L stimulation results in secretion of proinflammatory cytokines/chemokines in both human and NHP islet cells. We investigated whether CD40 stimulation in human and NHP islets is associated with secretion of proinflammatory mediators. Human islets were depleted of contaminating duct cells. The proportion of duct cells in the seven preparations studied was $34.6 \pm 15.2\%$. Standard depletion resulted in islet cells containing $<0.5\%$ duct cells (Fig. 1). Depleted populations contained negligible amounts of other CD40-expressing cells, such as B-cells ($0.75 \pm 0.01\%$), dendritic cells ($0.72 \pm 0.48\%$), and monocytes ($0.52 \pm 0.40\%$), as assessed by flow cytometry. NHP islets have a higher percentage of β -cells than human islets and a lower percentage of duct cells: on average, $1.78 \pm 1.3\%$ as determined by flow cytometry ($n = 4$ donors). Therefore, the islet cells may be activated without depletion.

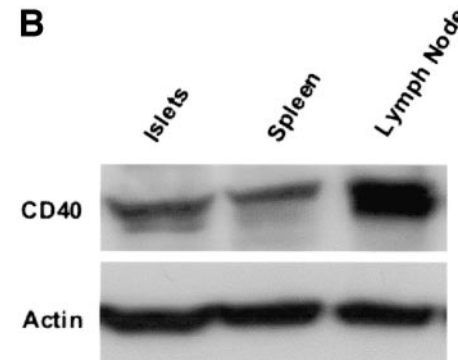
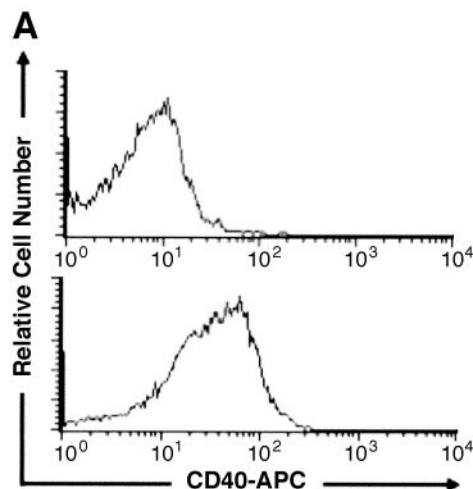


FIG. 2. CD40 expression in NHP β -cells. *A*: FACS analysis for isotype (*top panel*) and CD40 (*bottom panel*). *B*: Western blot, spleen and lymph nodes; positive controls ($n = 3$).

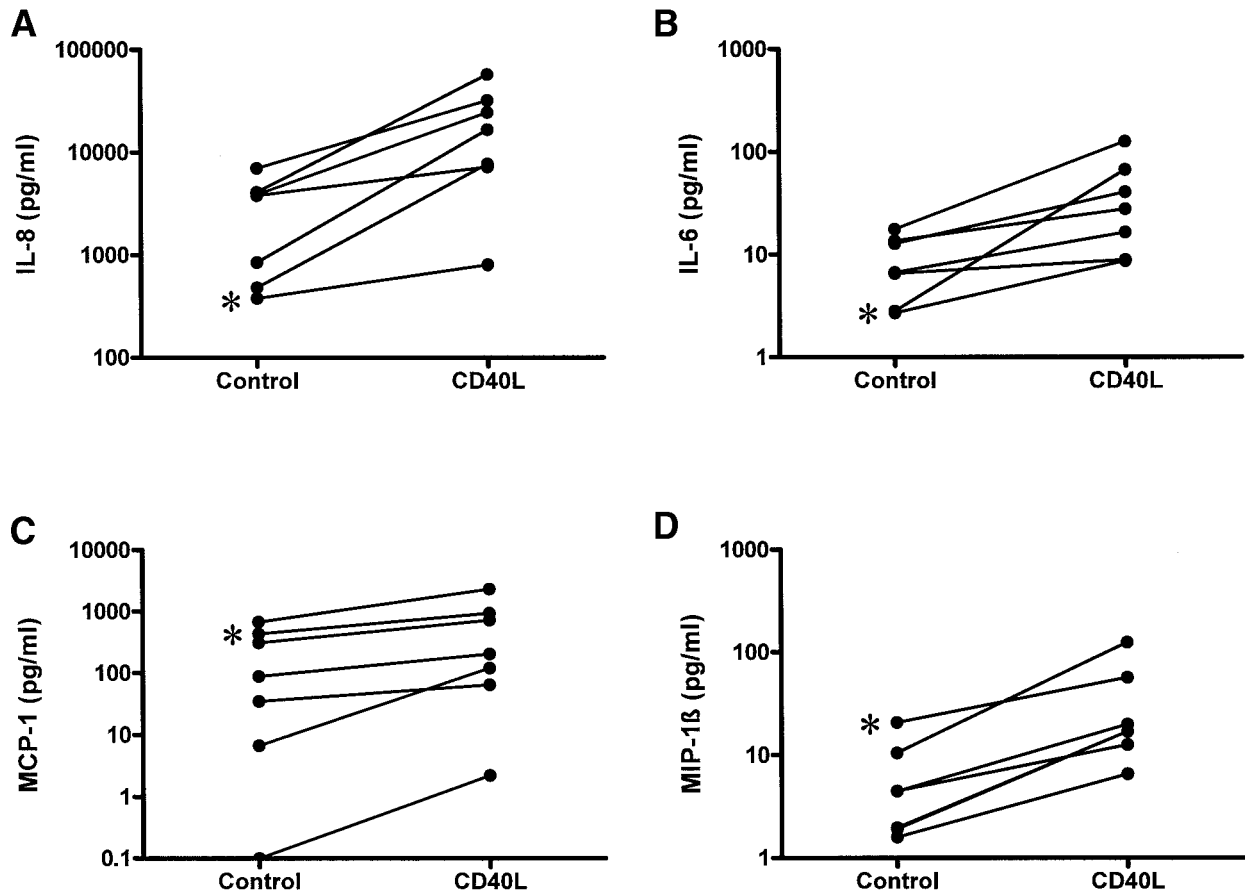


FIG. 3. Cytokine/chemokine secretion in CD40L-treated islets. Islet cells were treated with CD40L, and cytokines/chemokines were determined in the supernatant ($n = 7$), with six human preparations and one NHP preparation. Data are the means \pm SE (pg/ml) for control and CD40L, respectively, for IL-8 ($3,363.3 \pm 982$ and $24,023 \pm 7,179$) (A), IL-6 (18.6 ± 10.7 and 48.1 ± 17.6) (B), MCP-1 (186.9 ± 109.1 and 571.4 ± 364) (C), and MIP-1 β (182.8 ± 110.3 and 565 ± 365.6) (D). $P < 0.05$ was considered statistically significant. $P = 0.031$ for all cytokines/chemokines. *NHP preparation.

Because CD40 expression has not been previously reported in NHP β -cells, we confirmed it first with APC-conjugated human anti-CD40 mAb and Newport Green staining. Flow cytometry analysis confirmed the expression of CD40 on NHP β -cells by colocalization of bright Newport Green staining and CD40 staining (Fig. 2A). Western blot analysis further confirmed the presence of CD40 protein in NHP islets (Fig. 2B).

Supernatants from depleted human islet cells and NHP dissociated islets were assayed for the presence of cytokines/chemokines. Figure 3 shows that CD40 engagement consistently elevates secretion of IL-6, IL-8, MCP-1, and MIP-1 β in both human and NHP islet cells. Interestingly, undetectable or very low levels of the other cytokines tested (namely IL-1 β , IFN- γ , IL-10, and TNF- α) were found. **RT-PCR and immunostaining studies.** CD40L-mediated activation was confirmed by RT-PCR (Fig. 4A) in RNA isolated from sorted β -cells (MCP-1 not nested). Human depleted islet cells were used for quantitative PCR (Fig. 4B). The results are shown as the relative quantification fold increase of mRNA in CD40L-treated cells versus control cells for each cytokine/chemokine. Analysis of the raw data, using Wilcoxon's paired signed-rank test, showed statistical significance ($P < 0.05$) for IL-8 ($P < 0.01562$, $n = 7$) and MIP-1 β ($P < 0.01562$, $n = 7$) but not for IL-6 ($P < 0.578$, $n = 7$) and MCP-1 ($P < 0.156$, $n = 7$). Figure 4C shows MIP-1 β expression in sorted β -cells by double-immunofluorescent staining and confocal micros-

copy. Staining with the MIP-1 β antibody clearly colocalizes with insulin staining. The percentage of β -cells expressing MIP-1 β after CD40L treatment was $66 \pm 5\%$ ($n = 4$ preparations) as assessed by double immunostaining and evaluated by laser scanning cytometry and MetaMorph imaging software. Of note, we did not detect MIP-1 β in pancreatic tissue sections (data not shown), suggesting that this chemokine may not be constitutively expressed but is induced by CD40/CD40L interaction.

Proinflammatory cytokine/chemokine synthesis and secretion require the activation of MEK/ERK and NF- κ B pathways. The importance of CD40 signaling through MEK/ERK MAPK family members in inflammatory responses of monocytes and macrophages (28), synovial cells (20), and human proximal tubule cells (21) is well recognized. To study CD40-mediated activation of this pathway in β -cells, we began our experiments using NIT-1 cells (29) because these are an acceptable surrogate of primary β -cells, given the limited availability and cost of human or NHP islets for research. We first tested the activation of MEK1/2, a kinase immediately upstream of ERK1/2. Phosphorylation of MEK1/2 at Ser217/221 was already detected after 5 min of CD40 engagement and persisted over 30 min (Fig. 5A). Analysis of ERK1/2 activation showed that CD40 ligation increases ERK1/2 phosphorylation 5 min posttreatment and declines over time (Fig. 5B). Concurrently, assessment of ERK1/2 enzymatic activity peaked 5 min after stimulation (Fig. 5C).

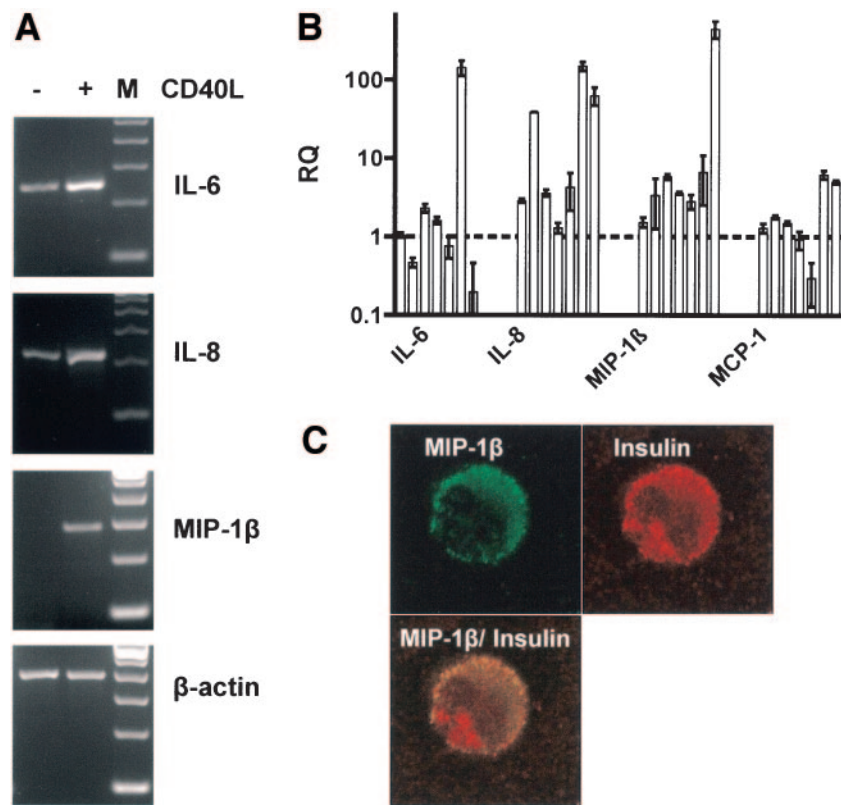


FIG. 4. RT-PCR of cytokine/chemokine transcripts and MIP-1 β immunofluorescence. **A:** RT-PCR of sorted β -cells (two donors). Panels show agarose gel electrophoresis of amplicons. -, unstimulated; +, stimulated; M, marker. **B:** Relative quantification (RQ) values for quantitative RT-PCR of depleted islet cells ($n = 7$ human islet preparations). The dotted line represents the threshold of the activation. **C:** Confocal microscopy of sorted β -cells double stained for MIP-1 β and insulin (magnification $\times 630$).

CD40-mediated ERK1/2 phosphorylation and enzymatic activity were abrogated by preincubation with the MEK1/2 inhibitor PD98059 (Fig. 5C and D).

Using a luciferase reporter gene, we previously showed that CD40 signaling in NIT-1 cells activated the NF- κ B pathway (22). NF- κ B involvement in the CD40 signal transduction pathway was further investigated by analyzing I κ B α phosphorylation and degradation. I κ B α is one of the inhibitory I κ B proteins that complex NF- κ B in the cytosol. CD40 ligation in NIT-1 cells caused an increase of the deactivated form of I κ B α (phosphorylated I κ B α) in a time-dependent manner (0, 5, 10, and 15 min), reaching maximum phosphorylation at 10 min. In parallel, I κ B α degradation was already apparent 5 min after the stimulus (Fig. 6A). As expected, I κ B α phosphorylation was inhibited in a concentration-dependent manner by preincubation with the inhibitor of I κ B phosphorylation Bay 117082 (Fig. 6B). We assessed the possible induction of p38 and stress-activated protein kinase/c-Jun-NH $_2$ -terminal kinase MAP kinases by CD40 stimulation in NIT-1 cells. We could not find any activation of these two kinases in our experiments (not shown).

Based on the above findings, we expanded our signal transduction studies to human and NHP islets. We observed that Raf/MEK/ERK MAPK and NF- κ B pathways are activated in both human and NHP islets. Moreover, the addition of selective inhibitors of these pathways resulted in a sizable reduction of IL-6, IL-8, MCP-1, and MIP-1 β secretion caused by blocking their CD40 signaling-dependent production (Table 1). We calculated means and SDs and compared changes associated with the different culture conditions. Results with the one-way ANOVA and the

Bonferroni's test for post hoc analysis demonstrate significant differences in the various conditions tested in each sample. These results also show concordant patterns across species. Overall, our results provide evidence linking CD40 signaling in both human and NHP islets with the Raf/MEK/ERK and NF- κ B pathways.

CD40-CD40L interaction-induced expression of ICAM. ICAM-1 is an ICAM regulated by the NF- κ B signaling pathway (30). ICAM-1 expression is associated with inflammation. Reports indicate that ICAM-1 expression is enhanced in human renal proximal tubule cells by CD40-CD40L interaction (21). Therefore, we investigated whether ligation of the CD40 receptor affects the expression of ICAM-1 in human islets. Flow cytometry analysis showed a strong increase of ICAM-1 on the surface of β -cells after CD40 activation (Fig. 7). Quantitative real-time PCR showed a statistically significant CD40L-mediated transcriptional activation of ICAM-1 ($P < 0.001562$, $n = 7$), analyzed by Wilcoxon's signed-rank test. Induction of ICAM-1 transcripts was sharply decreased by pretreatment with the NF- κ B pathway inhibitor Bay 117082.

DISCUSSION

Advances in islet isolation methods and immunosuppressive regimens are leading to expanded clinical trials to develop islet transplantation as a therapeutic option for patients with type 1 diabetes (31). However, the isolation process is still potentially harmful to the islets (32) because it may expose them to damaging factors that induce a general proinflammatory state. Moreover, after transplantation, islets are subject to hypoxia and early nonspecific inflammatory events, mostly mediated by the

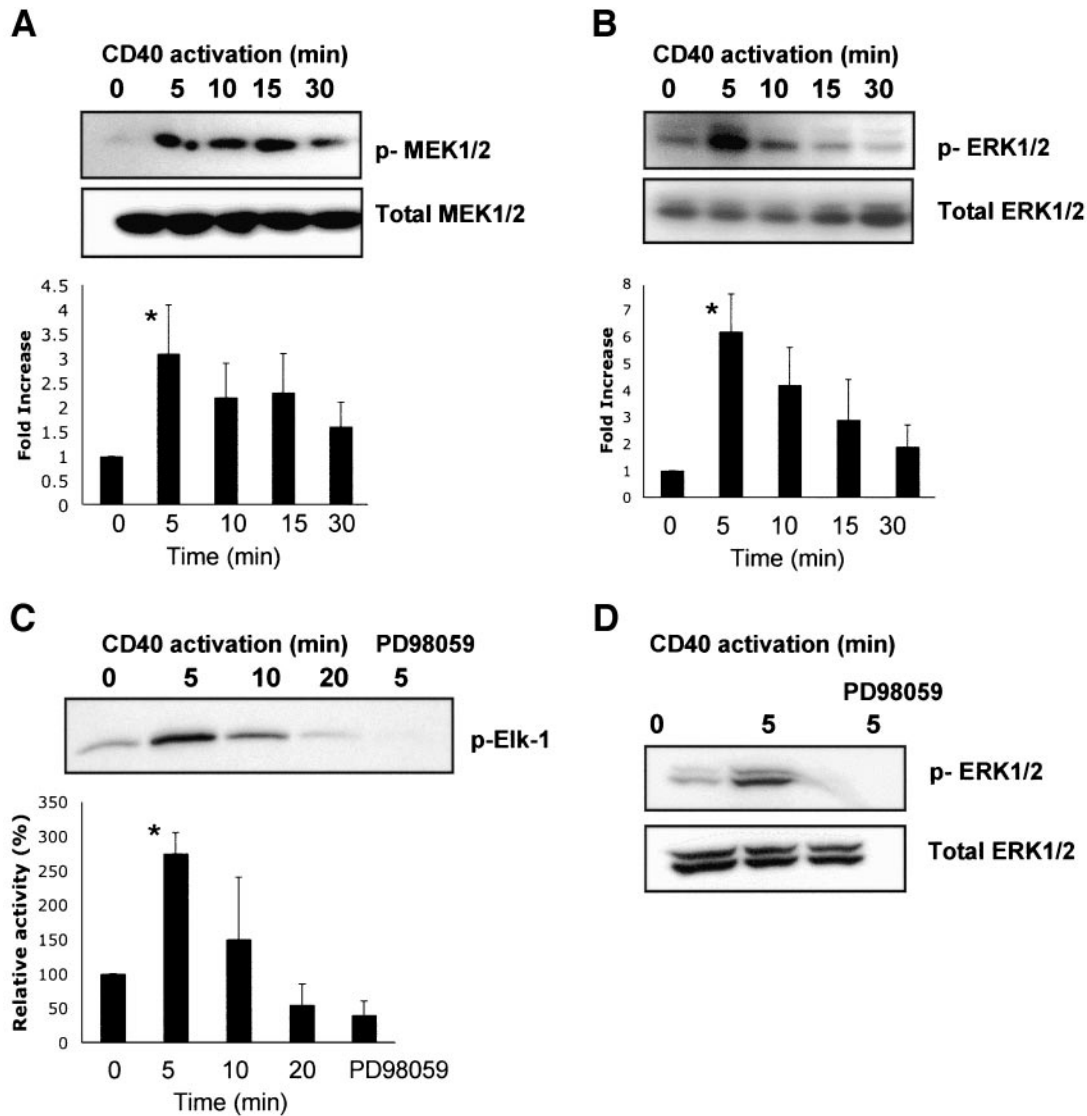


FIG. 5. CD40 activates the Raf/MEK/ERK pathway. *A* and *B*: Western blots of phosphorylated and total (phosphorylated plus nonphosphorylated) MEK1/2 and ERK1/2. Densitometries: ratios of phosphorylated to total MEK1/2 or ERK1/2. *C*: Enzymatic activity of ERK1/2 and densitometry. Data in *A–C* are the means \pm SD ($n = 3$). * $P < 0.05$. *D*: Cells treated with PD98059. The Western blot images are representative of three independent experiments.

recipient's immune cells, that can compromise β -cell viability and function. Locally secreted chemoattractants and proinflammatory molecules might recruit and activate immune cells to the transplant site, mediating irreparable damage to the islet graft. Indeed, it is often reported that islets from more than one donor are required to achieved insulin independence, even when an acceptable islet mass was transplanted in the first infusion. CD40-CD40L interactions contribute not only to physiological cell-mediated responses, but also to immune pathological conditions such as autoimmune and vascular diseases, leading to a chronic inflammatory state. We have reported previously that the CD40 receptor molecule is expressed in human pancreatic β -cells and that expression can be upregulated by incubation with a cocktail of proinflammatory cytokines (22,30).

Here, we demonstrate that CD40 signaling in β -cells upregulates secretion of IL-6, IL-8, MCP-1, and MIP-1 β . We found statistically significant transcription upregulation for IL-8 and MIP-1 β but not for IL-6 and MCP-1. This contrasts with the observed CD40L-mediated induction of

secretion (Fig. 3). One of the possible explanations is that the transcription for IL-6 and MCP-1 peaked at an earlier time than 24 h, which was the length of CD40L treatment.

Our observation that pancreatic islets secrete inflammatory mediators postisolation, without any stimulation, is in agreement with previous reports and emphasizes the consequences of the traumatic injury that pancreatic islets suffer during isolation and purification (17,18,32,33). Proinflammatory cytokines can induce or accelerate the recurrence of autoimmune process (16,34,35), whereas the inhibition of inflammatory pathways suppresses cytokine secretion and improves islet graft function (36). The relevance of our *in vitro* studies to the clinical islet transplant setting is supported by studies from Piemonti et al. (18) showing an inverse correlation between MCP-1 protein expression in isolated human islets and clinical outcomes. MCP-1 is a chemokine involved in mononuclear infiltration and is known to be secreted by pancreatic β -cells (17). Transgenic expression of MCP-1 in NOD mice induces insulinitis (37) and is associated with overexpression of NF- κ B (30). Correspondingly, a chemical inhibitor of I κ B (38) down-

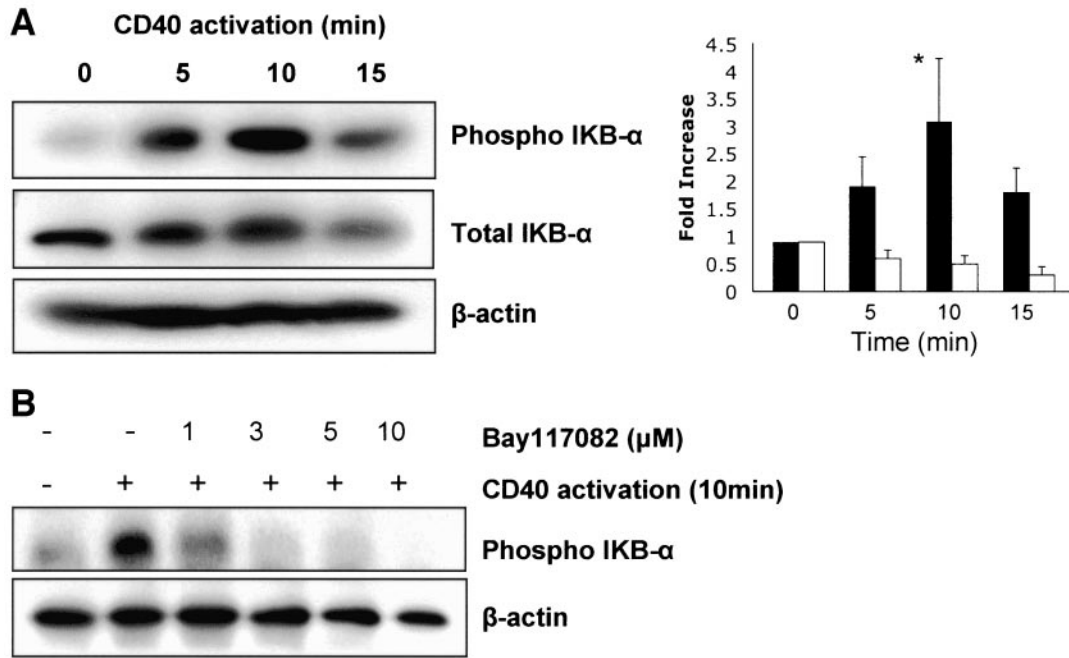


FIG. 6. CD40 activates the NF-κB pathway. **A**: Blots were probed with antibodies against phosphorylated IκBα, total IκBα, or β-actin and quantified by densitometry. ■, ratio of phosphorylated IκBα to actin; □, ratio of total IκBα to actin. Data are the means ± SD (n = 3). *P < 0.05. **B**: Cells were incubated with Bay 117082.

regulates MCP-1. In this regard, the use of specific blockers immediately after the isolation process could improve islet viability and perhaps clinical success rates.

We are the first to report that islet β-cells secrete MIP-1β. We did not detect MIP-1β in pancreatic tissue sections, suggesting that the expression is stimulated by the stress of isolation. MIP-1β is a chemokine of the C-C subfamily, previously known to be secreted only by activated monocytes (39). MIP-1β coordinates inflammatory responses by recruiting and activating proinflammatory hematopoietic cells. The MIP-1β receptor selectively stim-

ulates dendritic cells and T-helper 1 (Th1) lymphocytes (40) through engagement of CCR5 (CC chemokine receptor 5) to secrete Th1-type proinflammatory cytokines associated with insulinitis (41).

Previous reports have shown that a significant number of genes are induced by CD40 in nonimmune cells, including genes associated with costimulation signals (42), cytokine secretion (10,20,43), apoptosis induction (44), cell survival (45), and inflammation (46). Earlier studies showed that CD40 signaling results in the activation of the MEK/NF-κB pathways in monocytes/macrophages (28,43,47)

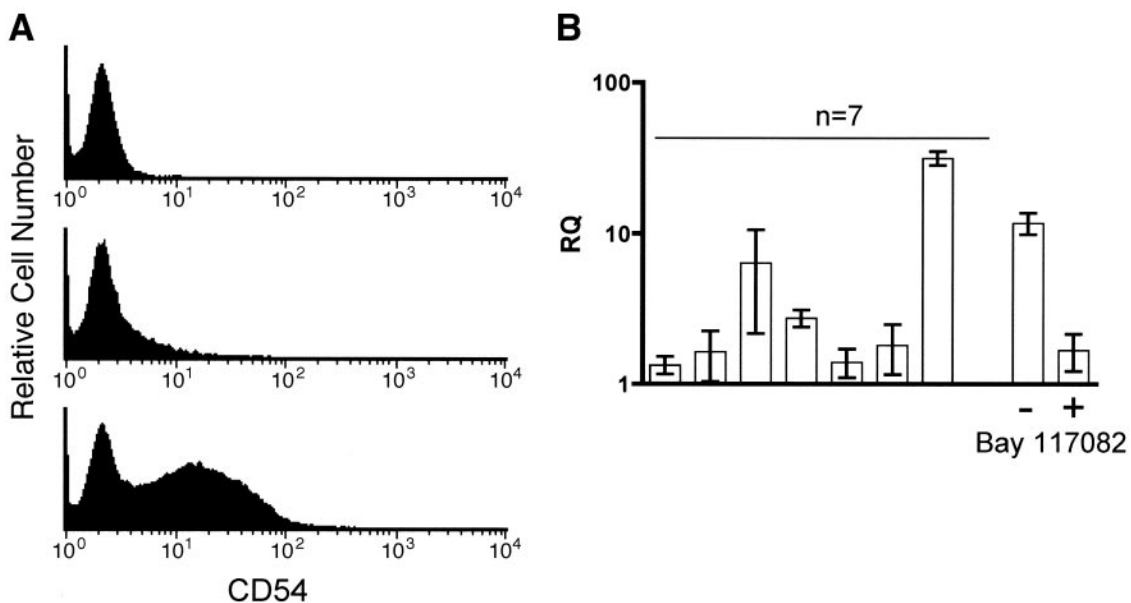


FIG. 7. CD40-CD40L interaction induced ICAM-1 expression in β-cells. **A**: FACS analysis of ICAM-1 expression in isotype (top panel), control (middle panel), and CD40L-treated cells (bottom panel) (n = 3 donors). **B**: Quantitative RT-PCR of depleted islet cells (n = 7 preparations). The last two columns in panel B show inhibition of CD40L-mediated activation of ICAM-1 by the NF-κB inhibitor Bay 117082. RQ, relative quantification.

and synovial (20) and epithelial (46) cells. Here, we show that the same pathways are activated in pancreatic β -cells. CD40 activation induces production of inflammatory soluble mediators through Raf/MEK/ERK and NF- κ B. The MEK1/2 and NF- κ B inhibitors PD98059 and Bay 117082, respectively, reduce cytokine production. Although PD98059 abrogates secretion of cytokines/chemokines activated purely by CD40, the effects of Bay 117082 (inhibitor of NF- κ B) were not limited to the signals mediated by CD40 (Table 1). This is in agreement with previous reports (30,32,38) showing that stress associated with pancreas harvesting and islet isolation may induce activation of NF- κ B and result in secretion of inflammatory mediators.

CD40L treatment of islets induced expression of ICAM-1 (Fig. 7). CD40-CD40L interaction upregulates ICAM-1 in proximal tubule renal cells as well (21). Transcription of the ICAM-1 gene was dependent on NF- κ B activation. These results are consistent with our findings that engagement of CD40 receptor in NIT-1 cells (Fig. 6) and islets (Table 1) activates NF- κ B, a pathway regulating ICAM-1 expression (30). Among adhesion molecules, ICAM-1 is an important mediator of inflammation, leading to extravasations of reactive lymphocytes to inflamed sites (48). The transgenic expression of IL-10 in pancreatic islets upregulated ICAM-1 in pancreatic endothelium and proved to be a critical molecule accelerating spontaneous diabetes in NOD mice because ICAM-1-deficient animals did not develop the disease (49). Our finding of ICAM-1 upregulation in β -cells after CD40 stimulation suggests that these two molecules may have synergistic proinflammatory effects that may be important in the development of autoimmune diabetes and in the context of islet transplantation.

In conclusion, our findings associate the induction of CD40 expression with ischemia of the pancreas and the islet isolation procedure. Signaling through the CD40 receptor elicits an intense proinflammatory response in both human and NHP β -cells that involves stress-mediated intracellular signaling pathways leading to the production of cytokines/chemokines involved in the amplification of inflammation and chemotaxis. Thus, CD40 expression may represent a response of islet cells to stress that could result in increased immunogenicity and cell damage.

In transplanted islets, the expression of CD40 stimulated by the ischemia and isolation procedure is likely to be consequential as inflammatory cells or soluble CD40L at the site of implant could predictably trigger signals through this receptor (50). The ensuing secretion of proinflammatory cytokines may have a negative effect on the function and survival of the transplanted islets, and it may possibly be a critical contributor to early and chronic graft loss. In addition, the inducible expression of a functional CD40 receptor on the surface of β -cells suggests that CD40 might also be involved in the autoimmune process leading to the spontaneous development of type 1 diabetes. Thus, the positive effects associated with CD40 signaling blockade in islet transplantation and models of spontaneous autoimmune diabetes may be exerted by interference at both the APC-T-cell interface and at the pancreatic β -cell level.

Strategies that specifically prevent or inhibit CD40 expression and its signaling pathways in β -cells may be of particular value during organ procurement and islet isolation procedures, and it may complement optimized strategies for successful long-term islet transplantation.

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