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Human pancreatic duct cells can produce tumour necrosis factor-α that damages neighbouring beta cells and activates dendritic cells

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Abstract

Aims/hypothesis. In the human pancreas, a close topographic relationship exists between duct cells and beta cells. This explains the high proportion of duct cells in isolated human islet preparations. We investigated whether human duct cells are a source of TNFα-mediated interactions with beta cells and immune cells. This cytokine has been implicated in the development of autoimmune diabetes in mice.
Methods. Human duct cells were isolated from donor pancreases and examined for their ability to produce TNFα following a stress-signalling pathway. Duct-cell-released TNFα was tested for its in vitro effects on survival of human beta cells and on activation of human dendritic cells.
Results. Exposure of human pancreatic duct cells to interleukin-1β (IL-1β) induces TNFα gene expression, synthesis of the 26,000 M_r TNFα precursor and conversion to the 17,000 M_r mature form, which is rapidly released. This effect is NO-independent and involves p38 MAPK and NF-κB signalling. Duct-cell-released TNFα contributed to cytokine-induced apoptosis of isolated human beta cells. It also induced activation of human dendritic cells.
Conclusions/interpretation. Human pancreatic duct cells are a potential source of TNFα that can cause apoptosis of neighbouring beta cells and initiate an immune response through activation of dendritic cells. They may thus actively participate in inflammatory and immune processes that threaten beta cells during development of diabetes or after human islet cell grafts have been implanted.

Keywords Apoptosis · Diabetes mellitus · Insulin · Islets of Langerhans · Pancreas

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Abbreviations: CK-19, Cytokeratin 19 · DuC, duct cell · iNOS, inducible nitric oxide synthetase · JNK, jun N-terminal kinase · MAPK, mitogen activated protein kinase · NF-κB, nuclear factor kappa B · NOD mouse, non-obese diabetic mouse

Introduction

Inflammatory infiltration of pancreatic islets is considered a major pathogenic event in the development of Type 1 diabetes. Insulitis is noticed at clinical onset of the disease [1], particularly in patients younger than 15 years [2], and found to contain a mixture of reactive lymphocytes [3]. A form of insulitis was also detected in rodents developing autoimmune diabetes [4]. In (pre)diabetic non-obese diabetic mice (NOD), infiltrating lymphocytes and macrophages were shown to produce cytokines [5, 6, 7, 8] that can destroy pancreatic beta cells in vitro [9, 10]. Cytokines were therefore proposed as potential mediators of beta cell death in vivo. Studies in NOD mice have suggested that TNFα is involved in the in vivo process leading to

beta cell death [11, 12, 13, 14, 15, 16]. While studying the influence of cytokines on human pancreatic cell preparations, we noticed that several effects were primarily exerted on duct cells instead of on beta cells; this was the case for their induction of MHC-class II expression [17], and of nitric oxide synthetase expression leading to NO production [18, 19]. This led to the view that duct cells might be actively involved in immune and inflammatory processes that surround beta cells during development of diabetes and after islet cell transplantation [19]. The human pancreas is indeed characterised by a close anatomic association of duct cells and islet beta cells [20], which explains why isolated human islet cell preparations contain relatively large proportions of duct cells [21, 22] that are attached to beta cells (unpublished observations). It is therefore conceivable that in situ as well as in preparations used in vitro or for transplantation, a fraction of beta cells is directly exposed to duct cell products [19]. Our study demonstrates this notion and shows that TNFα qualifies as one of these products, with potential effects on islet beta cells and on dendritic cells.

Materials and methods

Reagents and cytokines. Human interleukin-1β (IL-1β) was kindly provided by Dr Reynolds (NCI-FCRDC Frederick, Md., USA), human interferon gamma (IFN-γ), IL-6 and TNFα was purchased from Peprotech (Rocky Hill, N.J., USA), human IL-4 from Brucells (Brussels, Belgium), GM-CSF from Novartis (Basel, Switzerland), mouse recombinant TNFα (mTNFα) and anti-human TNFα neutralising antibody from R&D systems (Minneapolis, Minn., USA), cycloheximide (CHX), actinomycin D (ACTD), Brefeldin A, SB203580 and PGE2 from Sigma-Aldrich (St. Louis, Mo., USA), SP600125 and MG-132 from Biomol Research Laboratories (Plymouth Meeting, Pa., USA).

Duct cell isolation and culture. Human pancreatic duct cells were prepared from donor organs that were procured by transplant departments affiliated to Eurotransplant Foundation (Leiden, the Netherlands). The organs were sent to the Human Beta Cell Bank in Brussels for preparation of beta cell grafts to be used in a clinical trial [22]. The use of donor organs and of isolated fractions followed the guidelines of Eurotransplant, and of protocols that were approved by the ethics committee of Brussels Free University-VUB. The techniques for preparation of duct cells have been described [17]. Briefly, after collagenase digestion of the pancreas and Ficoll gradient centrifugation of the digest, the non-endocrine fraction is recovered and cultured as suspension in serum-free medium for 3 to 7 days. Virtually all acinar cells disappeared during this period. The preparation was then further cultured in 24 or 6-well tissue culture plates (Falcon, Becton Dickinson, N.J., USA), with—respectively—2×10^5 or 10^6 cells per well in HAM's F-10 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 7.5 mmol/l glucose (Merck, Darmstadt, Germany), 0.5% bovine serum albumin (Roche Diagnostics, Mannheim, Germany), 0.1 mg/ml streptomycin (Sigma Chemical, St Louis, Mo., USA), 0.075 mg/ml penicillin (Continental Pharma, Brussels, Belgium) and 0.3 mg/ml L-glutamine (Gibco BRL, Life Technologies, Paisley, UK). Fetal calf serum was present during the first 4 days (10% heat inactivated; Gibco BRL, Life Technologies, Paisley, UK) in order to facilitate monolayer formation. Once monolayers were established, the cells were washed and experiments were performed in serum-free medium. Duct cell supernatant used for beta cell viability and dendritic cell experiments was obtained from duct cell monolayers after a 24 h culture with or without IL-1β 30 U/ml. Supernatants were collected, centrifuged and stored at −20 °C prior to use.

Analysis of TNFα and of nitrite formation. Monolayers of human pancreatic duct cells were incubated for 1.5 to 72 h in serum-free medium with or without cytokines. Nitrites and TNFα were assayed in the supernatants. The hTNFα levels were measured by ELISA (BioSource International, Camarillo, Calif., USA) using calibration with the international standard preparation (87/650-NIBC, Hertfordshire, EN6 3QG—1 μg equals 40,000 U). Nitrites were determined spectrophotometrically at 546 nm after a Griess reaction [23]. For immunoblotting studies cells were lysed in 50 mmol/l Tris (pH 7.5), 150 mmol/l sodium chloride, 1% deoxy cholic acid (wt/vol), 1% Igepal CA-630 (vol/vol), 0.1% SDS (wt/vol), 2 mmol/l EDTA, phosphatase inhibitors (50 mmol/l sodium fluoride, 10 mmol/l sodium orthovanadate, 10 mmol/l β-glycerophosphate, 10 mmol/l p-nitophenylphosphate, 1 mmol/l sodium pyrophosphate) and proteinase inhibitors (leupeptine, antipain, benzamidine, trypsin inhibitor; chymostatin, pepstatin A). Samples were frozen in liquid nitrogen and kept at −80 °C until processed. Before analysis, thawed samples were sonicated and cleared by centrifugation. Protein concentration was measured by a commercial colorimetric assay (Pierce, Rockford, Ill., USA). For immunoblotting, samples with 50 μg protein were mixed with an equal volume of two times concentrated sample buffer [10% SDS (wt/vol), 10% β-mercaptoethanol (vol/vol), 160 mmol/l Tris-HCl (pH 6.8), 10 mmol/l EDTA, 20% glycerol (vol/vol), and 1 mmol/l phenylmethylsulphonyl fluoride] and run on 15% SDS-polyacrylamide together with the Benchmark prestained molecular weight marker (Life Technologies, Paisley, UK). After electrophoresis, samples were electroblotted to nitrocellulose filters (Protran, Schleicher and Schuell, Keen, NH). Blots were incubated, first for 1 h at room temperature in 5% non-fat dry milk/Tris buffered saline (TBS) and then overnight at 4 °C with anti-human TNFα (R&D Systems, Minneapolis, Minn., USA), anti-phospho-c-jun and anti-β actin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), anti-human iNOS (Transduction Laboratories, Lexington, Ky., USA), anti-Phospho-JNK, anti-Phospho-p38 (New England Biolabs, Beverly, Mass., USA). Horseradish peroxidase-linked anti- (goat, rabbit or mouse) IgG (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used as second antibody for 1 h at room temperature and the peroxidase activity was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK) and photosensitive film (Biomax ML; Kodak, Rochester, N.Y., USA).

Real time RT-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen Corporation/Life Technologies, Carlsbad, Calif., USA), and cDNA prepared by reverse transcription; 5 μmol/l Oligo $(dT)_{16}$ (Applied Biosystems, Foster City, Calif., USA) was added to 0.5 μg of total RNA, heated to 72 °C for 10 min and then cooled on ice. Next, 100 units of Superscript II (Invitrogen Corporation/Life Technologies) were added to RNA-oligo (dT) mixture, together with 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 5 mmol/l dNTPs, and incubated at 42 °C for 80 min. Real time PCR was performed using the ABI prism 7700 SDS (Applied Biosystems) in combination with TaqMan chemistry. Primers and probe sequences for human TNFα were as described [24]. Hypo-

xanthine phosphoribosyltransferase 1 (HPRT) was used as housekeeping gene to normalise TNFα values. HPRT primers and probe sequences are as follows: F 5′-TGTAGGATATGCCCTTGACTATA-3′ R 5′-CAATAGGACTCCAGATGTTTCCA-3′ P 5′-TGGAAAAGCAAAATACAAAGCCTAAGATGAG-3′. PCR amplifications were carried out in duplicate in a total volume of 25 µl containing 0.5 µl cDNA sample, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 10 mmol/l EDTA, 60 nmol/l Passive Reference, 1200 µmol/l dNTPs, 3 to 9 mmol/l $MgCl_2$, 100 to 900 nmol/l of each primer, 100 mmol/l of TaqMan probe and 0.625 U AmpliTaqGold (Applied Biosystems). Thermal conditions: 10 min at 94 °C, followed by 45 two temperature cycles (15 s at 94 °C and 1 min at 60 °C). cDNA plasmid standards were used for each target to quantify relative expression [24].

Immunocytochemistry. For TNFα and CK-19 staining, duct cell monolayers were fixed at room temperature in 4% buffered formaldehyde and then permeabilised with Triton-X100 before incubation in 10 mmol/l EDTA at 70 °C for 10 min. Non-specific binding sites were blocked by 10% normal goat serum or 2% normal donkey serum prior to, respectively, TNFα and CK19 immunostaining using mouse anti-human TNFα mAb (HyCult biotechnology b.v., The Netherlands) and sheep anti-human CK19 Ab (The Binding Site, Birmingham, UK). The cells were then washed in PBS and incubated with Texas Red conjugated secondary donkey anti-mouse mAb or Cy2-conjugated secondary donkey anti-sheep Ab (both from Jackson ImmunoResearch Laboratories, West Grove, Pa., USA). After washing, the preparations were mounted, covered by Dako fluorescence mounting medium (Dako Corporation, Carpinteria, Calif., USA) and analysed by a Leica TCS SP confocal laser-scanning microscope (CLSM). The CLSM is equipped with Ar/HeNe-lasers and Leica TCS NT software (version 1.6.587). Fluoresbrite grade microspheres, (∅ 3.0 µm, Polylab BVBA, Belgium) were used to calibrate the magnification. Images were transferred to Adobe PhotoShop 5.5 software for multicolour channel analysis and figure assembly. For subcellular localisation of the P65 subunit of NF-κB, monolayers were fixed and permeabilised by ice-cold acetone before incubation with primary rabbit anti-human NF-κB-P65 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). After washing in PBS, the preparations were incubated with Cy3-conjugated secondary donkey anti-rabbit Ab (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA), washed again, mounted and analysed in a Axioplan 2 fluorescence microscope (Carl Zeiss Jena, Jena, Germany) equipped with Photometrics SenSys 1401 digital Camera (vysys, France) and Smart Capture VP (version 1.4) software (Digital Scientific, UK).

Beta cell toxicity assay. Human beta cell preparations were obtained from the Human Beta Cell Bank. Methods for isolation, dissociation, purification and culture have been described elsewhere [21, 22]. The cultured endocrine fraction was dissociated and enriched in single beta cells by flow cytometry (FACS sorting) according to forward scatter and autofluorescence intensity at 530 nm [25]; the degree of purity is lower than that for rat beta cells but routinely exceeds 60%. The purified beta cell preparation was cultured in micro titer cups at 4000 cells per well in a HAM F-10 basis of unconditioned or duct cell (DuC) conditioned medium with replacement of half the volume every 2 days. Cells cultured in unconditioned HAM F10 without cytokines served as controls. The effect of IL-1β was also tested in unconditioned medium with or without recombinant human TNFα added. An anti-human TNFα neutralising antibody was added in two conditions. Each beta cell preparation was incubated with conditioned media of three different duct cell preparations and each condition was carried out in duplicate. After 10 days of culture, the percentages of apoptotic and necrotic cells were determined as described previously [26]. The apoptosis index was calculated as: (% apoptotic cells in test−% apoptotic cells in control condition)/(100−% apoptotic cells in control condition)×100. The necrosis index was calculated by replacing the percentage of apoptotic cells by the percentage of necrotic cells [27].

Dendritic cell activation assay. Dendritic cells were isolated as described [28] with minor modifications to increase dendritic cell yields [29]. Briefly, peripheral blood mononuclear cells were isolated from buffy coat preparations of healthy donors by gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). The cells were seeded in RPMI 1640 (Gibco, Invitrogen, Merelbeke, Belgium) containing 1% human AB serum (PAA Laboratories, Linz, Austria), and incubated for 2 h to allow adherence of monocytes. After washout of non-adherent cells, adherent cells were further cultured for 5 days in the presence of GM-CSF (1000 U/ml) and IL-4 (100 U/ml). They were then transferred to culture conditions in DuC-conditioned medium or with a cytokine mixture that is known to activate dendritic cells [30] namely IL-1β (100 U/ml), IL-6 (1000 U/ml), TNFα (100 U/ml) plus Prostaglandin E2 (PGE2, 1 µg/ml). Dendritic cell activation was analysed by flow cytometry [31] after staining with PE conjugated monoclonal antibodies against CD25, CD80 and CD83 or isotype controls (BD Pharmingen, Erembodegem, Belgium).

Statistical methods. Results are expressed as means ± SEM of *n* independent experiments each using cells from a different donor. Statistical analysis was done using the SPSS computer program. Student's *t* test was used to compare means of two groups, one way ANOVA with post hoc LSD to compare means of three or more groups, and Friedman test and Mann-Whitney U test to compare results that were normalised to the control. Significant differences were based on a *p* value of less than 0.05.

Results

IL-1β-induction of TNFα release from human duct cells. Human duct cell monolayers released marginally detectable TNFα levels, i.e. 100 to 200 pg TNF $10^6 \cdot cells^{-1} \cdot 72\ h^{-1}$. Addition of IL-1β (30 U/ml) increased TNF-levels 20 fold (Fig. 1a), while no stimulation was seen with human IFN-γ (1-1000 U/ml, data not shown). IL-1β induced TNFα release is detected from 60 min on, proceeds linearly during the first 6 h and then levels off to slower increment rates (Fig. 1a). In the period between 12 and 72 h, the rate of TNFα release was only 20% of that during the first 12 h. The half-maximal effect was reached after 5 h exposure (Fig. 1a). A similar curve was obtained with 3 U/ml IL-1β with values that were, on average, 40% lower than those at 30 U/ml (Fig. 1a). The lower TNFα increment beyond 12 h of IL-1β exposure was not caused by inactivation of the stimulus, since adding fresh IL-1β every 12 h did not further increase TNFα levels in the medium. Nor was it caused by degrada-

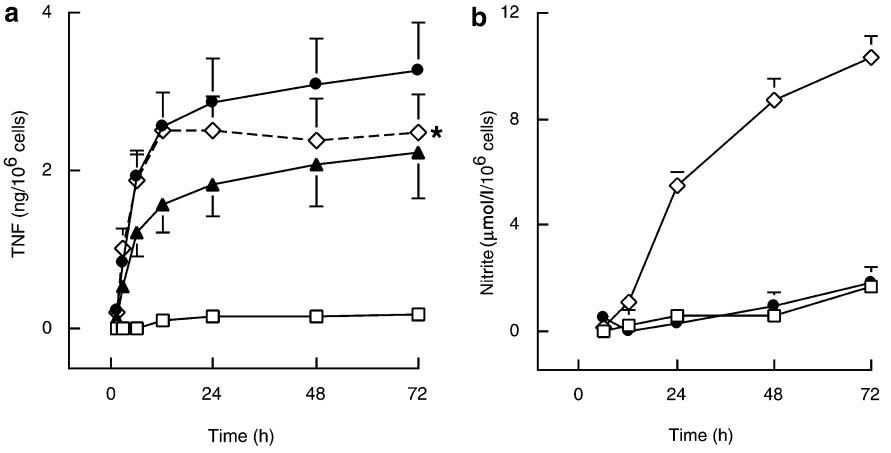


Fig. 1. Effects of IL-1β and IFN-γ on TNFα secretion and NO production by human pancreatic duct cells. TNFα (**a**) and nitrite (**b**) production by human pancreatic duct cells (2×10^5/ml) exposed to IL-1β at 3 U/ml (▲), 30 U/ml (●) or 30 U/ml of IL-1β plus IFN-γ (100 U/ml) (◇ - - ◇). Control cells were cultured in medium only (□). At indicated time points, media were retrieved and TNFα and nitrites determined as described in Materials and methods. Values are means of four independent experiments ± SEM. * $p<0.05$ vs IL-1β alone

tion of released TNFα during prolonged culture, since medium replacement every 12 h did not increase TNFα levels (data not shown).

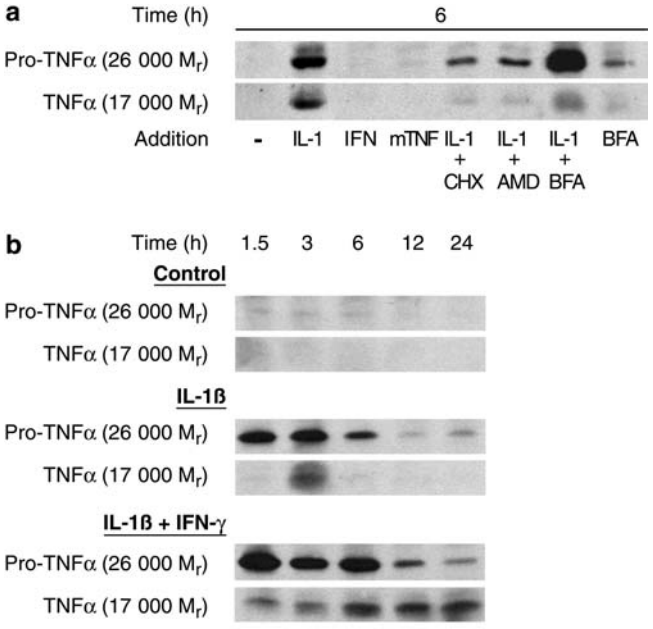
TNFα release depends on de novo synthesis and conversion of TNFα. IL-1β induced TNFα release was associated with de novo synthesis of TNFα as the 26,000 M_r precursor and its 17,000 M_r biologically active conversion product (Fig. 2a). No induction of TNFα expression was seen with IFN-γ or with mouse TNFα (Fig. 2a), and neither stimulated TNFα release (data not shown). Inhibitors of translation (cycloheximide) or transcription (actinomycin D) suppressed IL-1β-induced TNFα expression (Fig. 2a); they also inhibited TNFα release by more than 70% (Fig. 3). Their suppressive effect was not the result of cytodestruction as no increased cell death was measured during this culture period. The inhibitory effect of actinomycin D on TNFα production suggested that IL-1β can induce TNFα gene expression. This was confirmed by quantitative real time PCR (Fig. 2c). Adding Brefeldin A to the IL-1β condition selectively and strongly increased the 26,000 M_r band (Fig. 2a) which is compatible with its well known disrupting effect on intracellular transport and conversion processes [32]. This condition also blocked IL-1β-induced TNFα release (Fig. 3).

The IL-1β-induced expression of the TNFα precursor was strongest during the first 6 h (Fig. 2b), which is also the period of the highest release rate (Fig. 1a). The 26,000 M_r band became faint at 12 and 24 h, while the 17,000 M_r band disappeared. The lower release rates at these time points are thus not caused by an inhibition of conversion or release, but by a lower rate of biosynthesis.

IL-1β induced effects on TNFα were not associated with an induction of iNOS (data not shown) or an increase in nitrite release (Fig. 1b). The combination of IL-1β plus IFN-γ did induce iNOS expression within 6 h (data not shown) resulting in a subsequent increase in medium nitrite (Fig. 1b). Adding IFN-γ did not suppress IL-1β-induced TNFα expression (Fig. 2). On the contrary, it caused a time-dependent increase of the 17,000 M_r band over the 24-h study period, together with an increased and prolonged intensity of the 26,000 M_r band (Fig. 2b). The IFN-γ induced suppression of TNFα release beyond 12 h (Fig. 1a) can therefore not be attributed to a block in TNFα synthesis or conversion, but rather to an inhibition of TNFα discharge into the medium.

Immunocytological evidence for TNFα production by human duct cells. Immunocytochemistry was used to identify the cells responsible for TNFα production. We selected the condition with the highest intracellular TNFα expression, i.e. the combination of IL-1β and Brefeldin A (Fig. 2a). In these preparations of 70 to 90% CK-19-marked duct cells, more than 50% of the cells stained positively for TNFα (Fig. 4). Virtually all TNFα positive cells were positive for CK19 (Fig. 4). Their cytoplasmic staining pattern was similar to that described in other cell types [33]; control preparations exhibited only a faint staining. Thus we concluded that duct cells are responsible for TNFα production and not a contaminating cell type.

TNFα production mediated by p38 MAPK and the NF-κB signalling pathway. IL-1β induced phosphorylation of the MAP-kinases p38 and JNK and of c-jun within 15 min (Fig. 5). These activations preceded expression of pro-TNFα, which became clearly detectable after 60 min (Fig. 5). The activated state of p38 was maintained for 6 h and then disappeared. Phosphorylated forms of JNK and c-jun were also more prominent during this period. These activations were associated with an activation of NF-κB as indi-



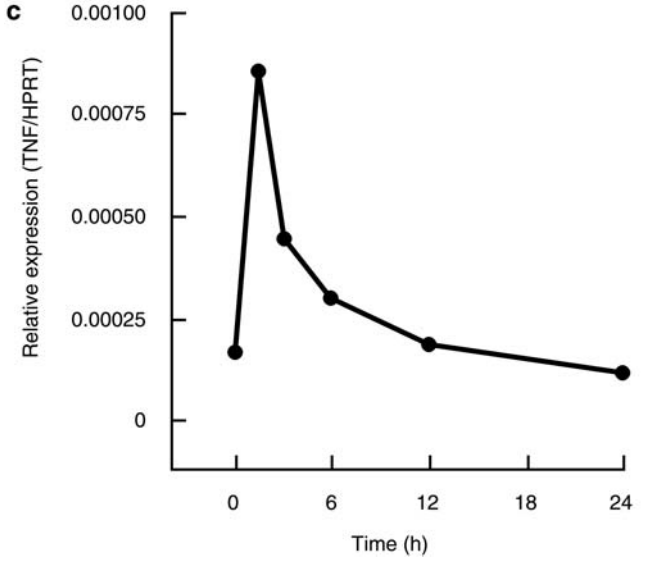


Fig. 2. Effects of cytokines on cellular expression of TNFα. Monolayers of human pancreatic duct cells were exposed for the indicated periods to IL-1β (IL-1; 30 U/ml), IFN-γ (IFN; 100 U/ml), or murine TNFα (mTNF; 100 U/ml) (**a**). The effect of IL-1β (30 U/ml) was examined in the absence and presence of cycloheximide (CHX; 5 μg/ml), actinomycin D (AMD; 1 μg/ml) or Brefeldin A (BFA; 5 μg/ml) (**a**), or IFN-γ (100 U/ml) (**b**). Whole cell lysates were separated by SDS-PAGE and immunoblotted with TNFα antibody. **c.** Real time PCR of TNFα expression after extraction of total RNA and reverse transcription to cDNA. TNFα values were normalised to HPRT levels. The figure represents three independent experiments

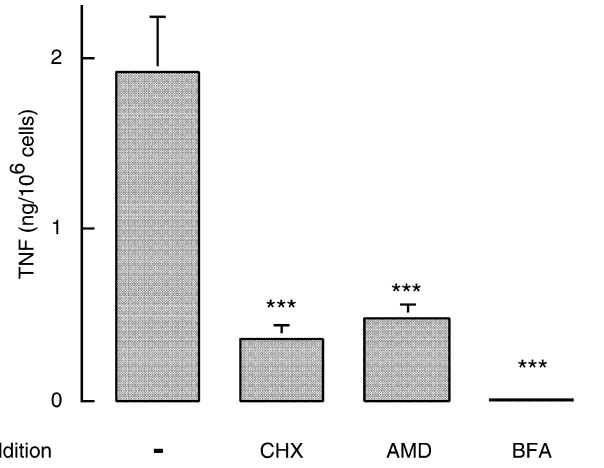


Fig. 3. Effects of cycloheximide (CHX), actinomycin D (AMD), and Brefeldin A (BFA) on IL-1β induced TNFα secretion. Monolayers of human pancreatic duct cells were exposed for 6 h to 30 U/ml of IL-1β with or without cycloheximide (5 μg/ml), actinomycin D (1 μg/ml) or Brefeldin A (5 μg/ml). Values are means ± SEM of four independent experiments. *** $p<0.001$ vs IL-1β alone

cated by its nuclear translocation after 15 to 30 min (data not shown). Adding SB203580, a specific inhibitor of p38 MAPK [34], suppressed IL-1β-induced TNFα expression and secretion by more than 75% (Fig. 6). TNFα release was also inhibited by MG-132, an inhibitor of IκB degradation and hence of NF-κB activation [35] (Fig. 6). On the other hand, SP600125, a specific inhibitor of JNK [36] did not decrease TNFα production (Fig. 6).

Cytotoxic effect of duct-cell-released TNFα on human beta cell preparations. Medium was collected from 24-h duct-cell cultures in the absence (DuC-Co) or presence of 30 U/ml of IL-1β (DuC-IL-1) (DuC, Fig. 7). The TNFα concentration in DuC-IL-1 varied between 300 and 600 pg/ml, while that in DuC-Co was either undetectable (<15 pg/ml) or lower than 50 pg/ml. These media were added to cultures of human beta cells to investigate their effect on cell survival. Parallel cultures were done in medium without DuC-supernatant added (no-DuC, Fig. 7), either with or without IL-1β (30 U/ml) or IL-1β (30 U/ml) plus human TNFα (400 pg/ml). The condition without supernatant and cytokines served as control. The cytotoxic effect of the test conditions was normalised to this control [26].

Duct cell medium (DuC-Co) did not exert a cytotoxic effect, as the percentages of apoptotic and necrotic cells were comparable to control values (Fig. 7). On the other hand, DuC-IL-1 medium induced apoptosis whereas this was not the case when only IL-1 was added (no DuC-IL-1, Fig. 7). This apoptotic effect was partially suppressed by a neutralising anti-TNFα antibody, suggesting its dependency on TNFα that is present in DuC-IL-1 medium. Apoptosis also occurred in control medium containing IL-1 plus TNFα at 400 pg/ml (no DuC-IL-1+TNF), the concentration that was measured in DuC-IL-1 medium. Apoptosis was partially suppressed by the anti-TNFα

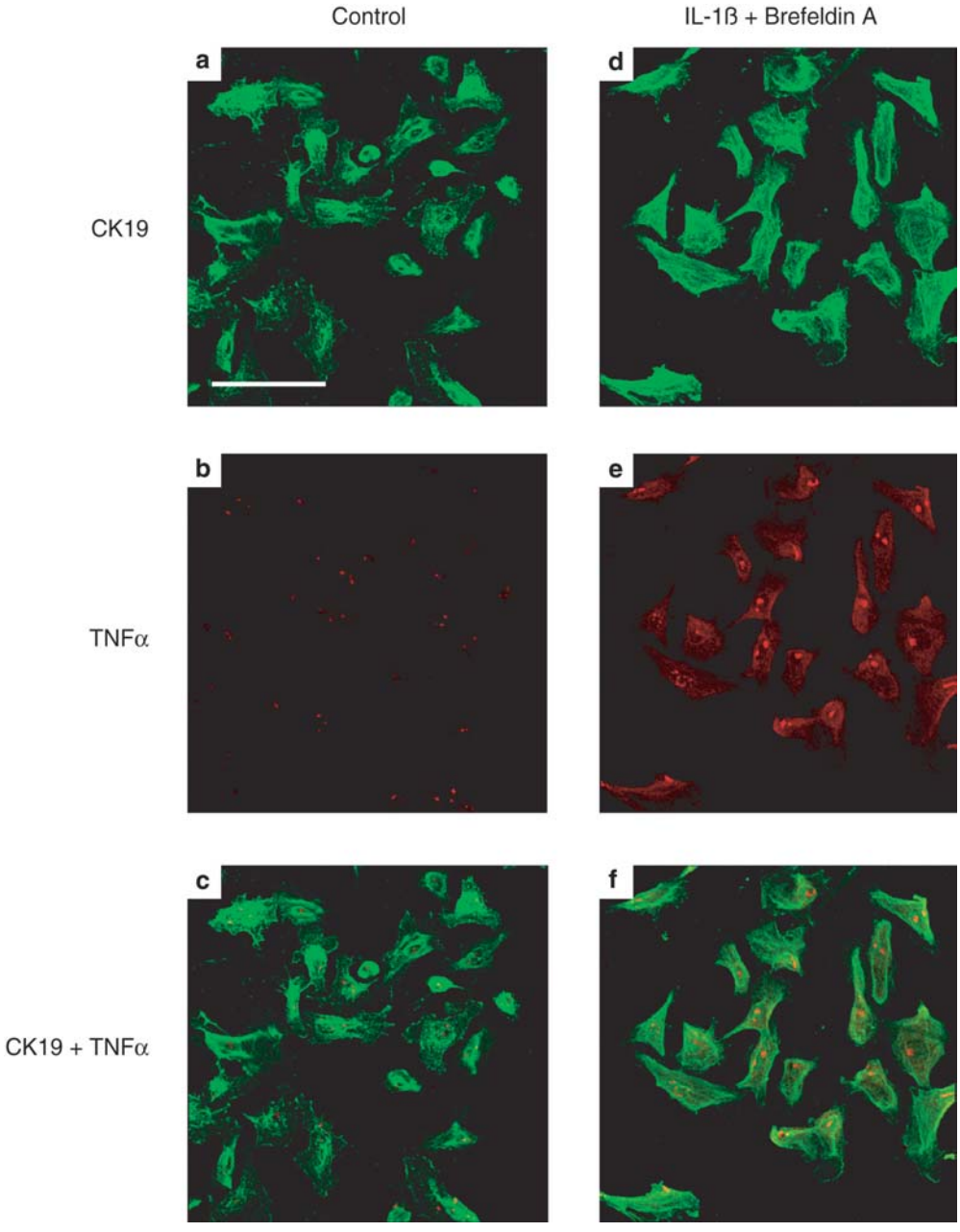


Fig. 4. Immunolocalisation of cellular Cytokeratin 19 (CK19) and TNFα by confocal laser scanning microscopy. Maximal projection images of human pancreatic duct cell monolayers incubated during 4 h with IL-1β (30 U/ml) plus Brefeldin A (5 µg/ml) **(d–f)** or medium alone (Control) **(a–c)**. Cells were stained for TNFα (Texas Red) (**b, c, e, f**) and CK19 (CY2: green) (**a, c, d, f**). Scale bar: 50 µm

antibody (Fig. 7). None of the conditions increased the percentage of necrotic cells (Fig. 7).

Dendritic cell activation by duct-cell-released TNF. We examined whether duct cell medium was able to induce dendritic cell activation, which is known to be TNFα-dependent. Immature dendritic cells were cultured for 24 h in DuC-IL-1 or DuC-Co and then evaluated for their expression of the activation markers CD25 (IL-2 receptor), CD80 (co-stimulatory molecule B7.1) and CD83 (maturation marker). Each experiment contained a positive control consisting of immature dendritic cells that were cultured with a mixture of cytokines (IL-1β, IL-6, TNFα and PGE2); this condition induced at least 30% positive cells above control for these three markers (data not shown). In medium without cytokines, less than 6% positive cells were detected. Culture of immature dendritic cells in DuC-Co did not result in their activation as judged from the percentage of CD25, CD80 and CD83 cells (<6%, NS vs negative control). On the other hand, DuC-IL-1 medium induced a significant increase in CD25, CD80 and CD83 expressing cells (Fig. 8). This effect was significantly suppressed by the anti-TNFα antibody (Fig. 8).

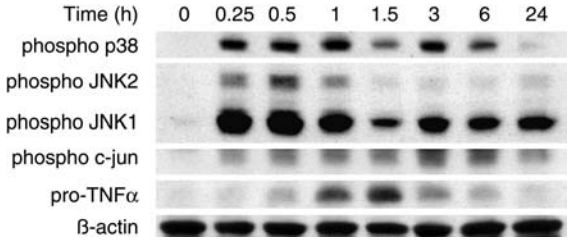


Fig. 5. Effect of IL-1β on MAP kinase phosphorylation. Monolayers of human pancreatic duct cells were incubated with IL-1β (30 U/ml) for the indicated times. Whole cell lysates were separated by SDS-PAGE and immunoblotted by the indicated antibodies. The figure represents three independent experiments

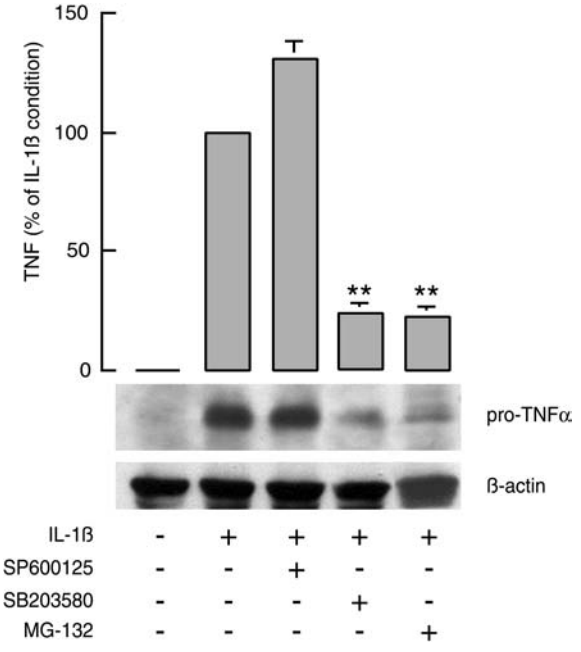


Fig. 6. Effects of SP600125, SB203580, and MG-132 on TNFα production and secretion. Monolayers of human pancreatic duct cells were pre-treated for 1 h with SP600125 (10 µmol/l) or SB203580 (5 µmol/l) or for 30 min with MG-132 (50 µmol/l) before exposure to IL-1β (30 U/ml) for 6 h. Whole cell lysates were separated by SDS-PAGE and immunoblotted by the indicated antibodies. Supernatants were harvested and assayed for TNFα. Data are expressed as percent of IL-1β condition and represent means ± SEM of three to six independent experiments. ** $p<0.01$ vs IL-1β alone

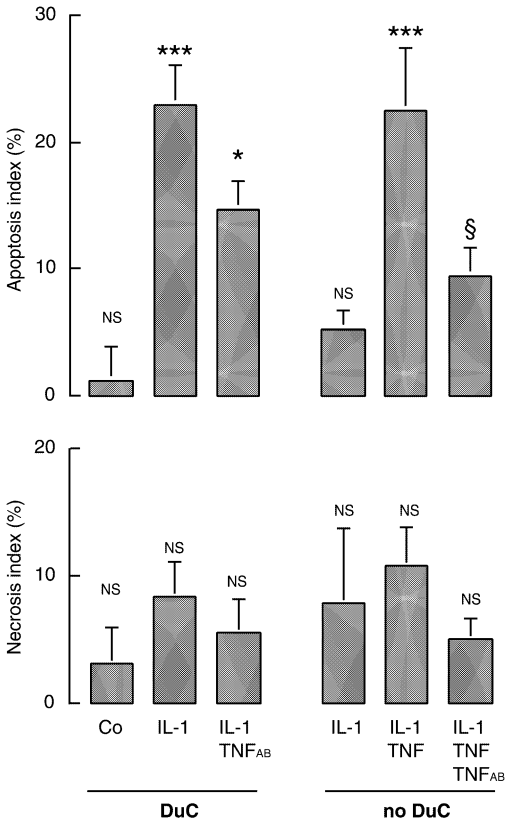


Fig. 7. Effect of duct cell medium on viability of cultured human pancreatic beta cells. FACS purified human pancreatic beta cells were cultured with medium (DuC) that was collected from human duct cell monolayers, which had been incubated for 24 h in the absence (Co) or presence of IL-1β at 30 U/ml (IL-1). After 10 days of culture the number of apoptotic and necrotic cells were counted and expressed relative to the numbers in control preparations cultured in unconditioned medium. For each condition an apoptosis and necrosis index was calculated as defined in reference [27]. The right panel shows data for cells cultured in unconditioned medium (no DuC) in the presence of IL-1β (30 U/ml) with or without TNFα (400 pg/ml). The role of TNFα was assessed by adding a TNFα neutralising antibody (TNF_{AB}) to the conditions DuC-IL-1 and IL-1β + TNFα. Data represent means ± SEM of three independent experiments. *** $p<0.001$ vs control, * $p<0.05$ vs DuC IL-1β, § $p<0.05$ vs IL-1β + TNFα

Discussion

In the developing pancreas, the juxtaposition of beta cells and duct cells is often noticed, and considered as indirect evidence for a ductal origin of beta cells. A similar topographic relationship exists in the adult human pancreas where 15% of insulin-producing cells occur as single units along ductules, and where beta cell aggregates are often directly juxtaposed to duct cells [20]. This anatomic characteristic explains why high numbers of duct cells co-migrate with endocrine cells during human islet isolation [21, 22]. The presence of 20 to 60% duct cells in human beta cell grafts led us to examine whether they might be involved in inflammatory and immune reactions around islet cell implants. We previously reported that human duct cells can respond to cytokines by expressing MHC-class II and iNOS with subsequent NO production [17, 18]. Our data show that these cells can also release TNFα at levels that affect survival of neighbouring beta cells and activate dendritic cells.

Duct cell release of TNFα was induced by interleukin-1ß. While this in vitro condition only serves as a model to demonstrate the existence and the mechanism of this duct cell property, it may occur in vivo

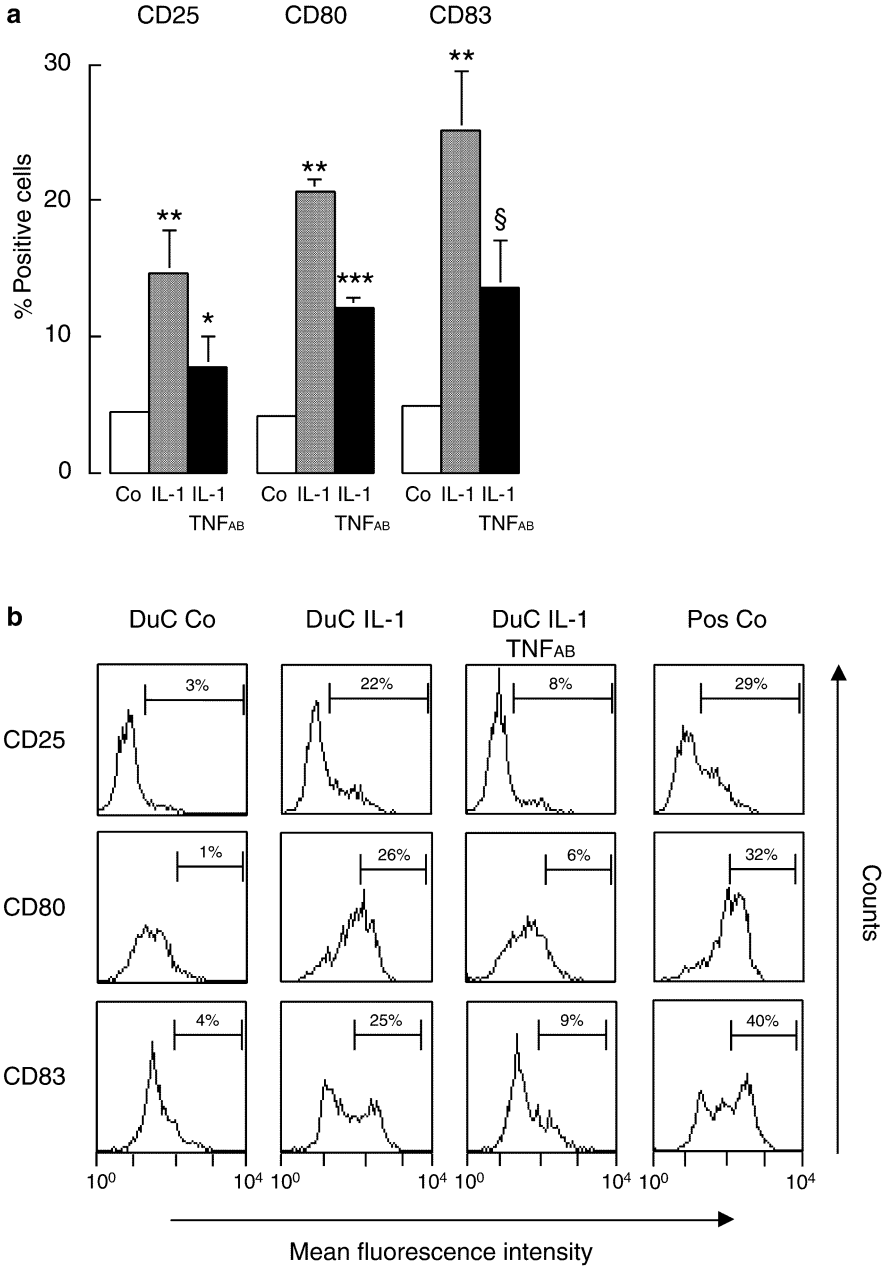


Fig. 8. Effect of duct cell medium on the activation of dendritic cells. Immature human dendritic cells were incubated for 24 h with medium that was collected from human duct cell monolayers, which had been incubated for 24 h in the absence (DuC-Co) or presence of IL-1β at 30 U/ml (DuC-IL-1). Their degree of activation was measured by FACS analysis of the percentage of CD25, CD80 and CD83 positive cells relative to the percentages in the negative control condition (**a**). Data represent means ± SEM for three to five independent experiments. ** $p<0.01$ compared to control; * $p<0.05$; *** $p<0.001$; § $p<0.05$ compared to DuC-IL-1. **b.** A representative flow diagram which also shows the degree of activation in the positive control condition (stimulation by IL-1β, IL-6, TNFα and PGE2)

when IL-1β is released by islet cells or by islet infiltrating cells [37]. TNFα release was dependent on the induction of TNFα precursor synthesis and conversion, rather than on discharge from a cellular pool of the converted mature form. The mechanism through which IL-1β induces TNFα-expression in human pancreatic duct cells is not fully understood. As in other cell types [38], this IL-1β effect depends on activation of MAP kinase p38 and seems mediated, at least in part, by NF-κB, which is known to bind to the promoter region of TNFα. On the other hand, it proceeds irrespective of iNOS induction or NO-production, and seems independent of JNK-activation, which contrasts with the mechanism in human CD4+ cells [36].

TNFα produced by human duct cells was shown to exert effects on neighbouring endocrine and immune

cells. The cytokine is released in a biologically active form and rapidly reaches in vitro concentrations in the range used in numerous in vitro studies [10, 39, 40]. Among the functions known to be TNFα sensitive, survival of beta cells [41, 42] and activation of dendritic cells [43, 44, 45] seem particularly relevant in the context of the pathogenesis of Type 1 diabetes and strategies to prevent or cure the disease. Medium containing duct-cell-released TNFα induced apoptosis in human beta cell preparations, probably in synergy with IL-1β that was added to stimulate TNFα production. Studies on rodent and human beta cells have indeed indicated that IL-1β alone does not cause apoptosis unless it is combined with IFN-γ and/or TNFα [42, 46]. A neutralising anti-TNFα antibody partially suppressed the apoptotic effect of the duct cell medium; the lack of complete suppression may result from insufficient neutralisation by the antibody or by another factor that is induced by IL-1β.

Pro-inflammatory cytokines such as IL-1β and TNFα are also known to stimulate migration and maturation of dendritic cells [43, 44, 45]. Our study shows that duct-cell-released TNFα can activate human dendritic cells, suggesting that these cells may play a role in the development of (auto)immune processes. Our data are compatible with earlier work showing TNFα involvement in the in vivo process of immune beta cell destruction. Neutralising TNFα antibodies were shown to protect NOD mice against diabetes, whereas treatment with recombinant TNFα accelerated the disease [11]. TNFα receptor 1-deficient NOD mice did not develop diabetes although insulitis was present [12], whereas induction of TNFα expression in islets accelerated progression to diabetes [13]. The latter effect was attributed to an immune modulation at an earlier age, following apoptosis of a few beta cells and subsequent activation of islet dendritic cells [14]. When islet TNF expression was regulated by a tetracycline-driven on–off switch, progression to diabetes depended on the duration of exposure to TNFα [15]. Another study showed the importance of timing islet-specific TNFα expression with respect to the autoimmune process [16]. These studies explain why the diabetogenic effects of TNFα were not observed when islet-specific TNFα expression was restricted to later stages [47]. It is also conceivable that local release of this cytokine alters the phenotype of neighbouring beta cells and thus varies their susceptibility to cytotoxic conditions as previously observed with interleukin-1 [48]. In the presence of other cytokines, TNFα may itself contribute to the death of beta cells [42]; in this respect, production of interleukin-1 by beta cells could be considered as a potential local trigger [37].

In conclusion, human pancreatic duct cells should be considered as potential participants in (auto)immune processes that occur during development of Type 1 diabetes or after islet transplantation. Their exposure to cytokines can induce expression of MHC-class II [17], iNOS [18] and/or TNFα precursor. It is still unknown whether these diverse responses can be generated by all duct cells or whether they characterise a particular subpopulation. Our in vitro observation that duct-cell-released TNFα can affect survival of human beta cells and activate human dendritic cells might bear clinical relevance, in particular since these different cell types are closely associated during development of Type 1 diabetes [1, 2] as well as during islet allograft reactivity.

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