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ARTICLE

Interferon regulatory factor-1 is a key transcription factor in murine beta cells under immune attack

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Abstract
Aims/hypothesis IFN-γ, together with other inflammatory cytokines such as IL-1β and TNF-α, contributes to beta cell death in type 1 diabetes. We analysed the role of the transcription factor interferon regulatory factor (IRF)-1, a downstream target of IFN-γ/signal transducer and activator of transcription (STAT)-1, in immune-mediated beta cell destruction.
Methods Islets from mice lacking *Irf-1* (*Irf-1*$^{-/-}$) and control C57BL/6 mice were transplanted in overtly diabetic NOD mice. Viability and functionality of islets were evaluated in vitro. Chemokine expression by *Irf-1*$^{-/-}$ islets and INS-1E cells transfected with *Irf-1* short interfering RNA (siRNA) was measured by real-time PCR as well as in functional assays in vitro.
Results IRF-1 deletion in islets was associated with higher prevalence of primary non-function (63% vs 25%, $p \leq 0.05$) and shorter functioning graft survival (6.0±2.6 vs 10.4± 4.8 days, $p \leq 0.05$) in contrast to similar skin graft survival. Although *Irf-1*$^{-/-}$ islets were resistant to cytokine-induced cell death, insulin secretion by them was lower than that of control C57BL/6 islets under medium and cytokine conditions. IL-1 receptor antagonist partly restored the cytokine-induced secretory defect in vitro and completely prevented primary non-function in vivo. Cytokine-exposed *Irf-1*$^{-/-}$ islets and INS-1E cells transfected with *Irf-1* siRNA showed increased expression of *Mcp-1* (also known as *Ccl2*), *Ip-10* (also known as *Cxcl10*), *Mip-3α* (also known as *Ccl20*) and *Inos* (also known as *Nos2*) mRNA and elevated production of monocyte chemoattractant protein-1 (MCP-1) and nitrite compared with controls. In vivo, *Irf-1*$^{-/-}$ islets displayed a higher potential to attract immune cells, reflected by more aggressive immune infiltration in the grafted islets.
Conclusions/interpretation These data indicate a key regulatory role for IRF-1 in insulin and chemokine secretion by pancreatic islets under inflammatory attack.

Keywords Beta cell death · Chemokines · IFN-γ · IRF-1 · Nitric oxide · NOD mice · Type 1 diabetes

Abbreviations

IL-1Ra	IL-1 receptor antagonist
IRF	Interferon regulatory factor
MCP	Monocyte chemoattractant protein
NF-κB	Nuclear factor κB
SI	Stimulation index
siRNA	Short interfering RNA
STAT	Signal transducer and activator of transcription

Introduction

Type 1 diabetes is caused by immune destruction of the pancreatic beta cells. Several lines of evidence point towards a central role for T lymphocytes in this destruction

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process [1, 2], with activated CD8+ T lymphocytes killing beta cells by contact-dependent cytotoxicity [3–5]. In addition, T lymphocytes, together with macrophages, secrete inflammatory cytokines such as IL-1β, IFN-γ and TNF-α [6, 7]. There is a growing understanding of how cytokines contribute to beta cell death in vitro and key transcription factors mediating cytokine-induced beta cell death have been identified. Two such transcription factors are nuclear factor κB (NF-κB) and signal transducer and activator of transcription (STAT)-1, crucial regulators of IL-1β and IFN-γ signalling respectively [7, 8]. Activation of the classical anti-apoptotic transcription factor, NF-κB, leads to apoptosis in beta cells [9], whereas silencing or elimination of STAT-1 prevents apoptosis of beta cells in vitro [10]. In vivo, the picture becomes more complex, since it is the interplay between beta cells and immune system that eventually decides beta cell fate during insulitis. Micro-array analyses of cytokine-exposed beta cells have demonstrated that complex networks of genes, downstream of NF-κB and STAT-1, are activated or silenced, leading to beta cell apoptosis [9–11]. Interestingly, beta cells exposed to cytokines paradoxically react to such a cytokine assault by producing molecules such as chemokines, which have active roles in the communication between beta cells and immune system [11–13]. This phenomenon is also observed in vivo, where chemokines are expressed by beta cells in islets of spontaneously diabetic NOD mice before massive infiltration is present, and also by allografted mouse and human beta cells [13–15]. These chemokines may play a crucial role in attracting immune cells and in the final demise of the beta cell [16].

In the present study we investigated the role of another transcription factor with a central position in the signalling cascades triggered by inflammatory cytokines, interferon regulatory factor (IRF)-1. IRF-1 is expressed constitutively at a low level in almost every cell type. It is typically induced by IFN-γ via binding of STAT-1 to the IFN-γ-activation site in the IRF-1 promoter, but other cytokines and hormones can also trigger its expression [17]. In the case of IL-1β or TNF-α, this induction is mediated through binding of NF-κB on the κB site of the IRF-1 promoter [18, 19]. In other cell types, IRF-1 plays a physiological role in host defence against pathogens, tumour prevention and development of the immune system [20], but its exact role in the beta cells remains elusive.

Our group has previously shown that islets, but not sorted beta cells, from *Irf-1*$^{-/-}$ mice are resistant to cell death when exposed to a mixture of cytokines in vitro [21]. In the present study, we demonstrate, using islets from *Irf-1*$^{-/-}$ mice and short interfering RNA (siRNA) knockdown of *Irf-1* in INS-1E cells, that IRF-1 is involved in insulin secretion and, especially, in the modulation of chemokine expression by beta cells. Lack of islet-cell *Irf-1* aggravates local inflammation and contributes to cell loss in an autoimmune setting.

Methods

Animals *Irf-1* knockout (*Irf-1*$^{-/-}$) mice were obtained from T.W. Mak (Ontario Cancer Institute, University of Toronto, ON, Canada) and have been back-crossed to C57BL/6 mice six times [22]. C57BL/6 mice were used as controls and were obtained from stocks purchased from Harlan (Horst, the Netherlands). NOD mice, inbred in our animal facility (Proefdierencentrum Leuven, Leuven, Belgium) since 1989, were used as diabetes-prone animals, with diabetes detected and defined as described [23]. All mice were housed under semi-barrier conditions. The institutional review committee for animal experiments approved all the procedures for mouse care and animal killing.

Islet isolation, culture and treatment To obtain pancreatic islets, pancreases from *Irf-1*$^{-/-}$ or control C57BL/6 mice were removed and islets were isolated by collagenase digestion [23]. Batches of 100 islets were collected and cultured overnight in RPMI 1640 medium (with GlutaMAX–I), containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS [vol./vol.] (Invitrogen, Merelbeke, Belgium). Thereafter, islets were kept for 1 or 3 days in culture medium in the absence or presence of inflammatory cytokines as follows: recombinant human IL-1β (50 U/ml; kind gift of C.W. Reynolds, National Cancer Institute, Bethesda, MD, USA) in combination with recombinant mouse IFN-γ (1,000 U/ml; PeproTech, London, UK). In some experiments, islets were pre-incubated for 30 min with recombinant human IL-1 receptor antagonist (IL-1Ra; Kineret; Amgen, Thousand Oaks, CA, USA) at a concentration of 500 ng/ml [24].

Islet viability and function Islet viability was evaluated using Hoechst 342 (20 μg/ml)/propidium iodide (10 μg/ml) (Molecular Probes, Invitrogen) as described [25, 26].

In vitro function of pancreatic islets was assessed by glucose-stimulated insulin release. Islets from *Irf-1*$^{-/-}$ or control C57BL/6 mice were washed twice with KRB (115 mmol/l NaCl, 24 mmol/l $NaHCO_3$, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$ and 25 mmol/l HEPES, pH7.4). After 30 min of sedimentation in KRB at 37°C, islets were incubated first at low (3 mmol/l) and then at high (20 mmol/l) concentrations of glucose in culture medium. At the end of incubation, supernatant fractions were assayed using an insulin ELISA kit (Mercodia, Uppsala, Sweden). Stimulation index (SI) was calculated by dividing the insulin release upon high glucose stimulation by the insulin release upon low glucose stimulation.

For glucose tolerance tests, mice were fasted overnight and received an intraperitoneal glucose load (2 g/kg body weight). Before and at 15, 30, 60, 90 and 120 min after glucose administration, glucose levels were measured in venous blood using a glucose meter (AccuChek Aviva; Roche Diagnostics Belgium, Vilvoorde, Belgium).

Monocyte chemoattractant protein-1 and nitrite measurement Culture supernatant fractions from *Irf-1*$^{-/-}$ or control C57BL/6 islets were collected at 1 and 3 days after treatment with or without cytokines. The concentration of monocyte chemoattractant protein (MCP)-1 in the supernatant fraction was measured using a commercial kit (mouse MCP-1 ELISA; eBioscience, Immunosource, Halle-Zoersel, Belgium). Nitrite production was determined by Griess assay (Sigma-Aldrich, Bornem, Belgium).

Leucocyte chemotaxis Cell migration was evaluated using a classical chemotaxis assay [27, 28]. Briefly, islets from *Irf-1*$^{-/-}$ or control C57BL/6 mice were cultured for 3 days in serum-free synthetic medium, using BioWhittaker Ultraculture medium (Lonza, Verviers, Belgium), supplemented with GlutaMAX–I (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin, in absence or presence of recombinant human IL-1β (50 U/ml) plus recombinant mouse IFN-γ (1,000 U/ml). Chemotaxis assay was performed for 1 h at 37°C using Transwell filter membranes (5 µm pore size; Costar, Boston, MA, USA) containing 1×10^6 leucocytes, isolated from spleens of 10- to 12-week-old female NOD mice, in 100 µl assay buffer (Hanks' buffered salt solution supplemented with 20 mmol/l HEPES and 0.2% bovine serum albumin [wt/vol.]) in the upper compartment and 600 µl of test solution in the lower compartment. The migrated cells were collected and counted in a flow cytometer (FACSort; BD Biosciences, Erembodegem, Belgium). The number of migrated cells represents the number of counts registered during a 2 min acquisition. The chemotactic index was calculated as the number of leucocytes attracted by test solution (supernatant fraction of islet preparations) divided by the number of leucocytes attracted by medium alone (negative control). Recombinant mouse MCP-1 (PeproTech) at concentrations of 10 and 50 ng/ml was used as reference chemoattractant.

Culture and transfection of INS-1E cells with siRNA against Irf-1 Rat insulin-producing INS-1E cells (a kind gift of C. Wollheim, Center Medical Universitaire, Geneva, Switzerland) were cultured as described [29]. Two different siRNAs against rat *Irf-1* were purchased from Invitrogen and designed using a commercial software (BLOCK-iT RNAi Express/Stealth Select; Invitrogen): si-IRF-1#1 (5′-CCCUGGCUAGAGAUGCAGAUUAAUU-3′) and si-IRF-1#2 (5′-GCCCUCCAUUCAGGCUAUUCCUUGU-3′). Allstars negative control siRNA (Qiagen Benelux, Venlo, The Netherlands) was used as a control for siRNA transfection. Transfection of siRNAs in INS-1E cells was done using the lipid carrier DharmaFECT (Dharmacon, Chicago, IL, USA) as described previously [29]. Lipid–RNA complexes were formed in Optimem in a proportion of 0.7 µl of DharmaFECT to 150 nmol/l of siRNA at room temperature for 20 min. The complex was added to cells for overnight transfection in antibiotic-free medium at a final concentration of 30 nmol/l siRNA. The transfection efficiency was ≥90% as measured using an FITC-conjugated siRNA (siGLO; Dharmacon). Afterwards, cells were cultured for a 24 h recovery period and subsequently exposed to recombinant human IL-1β (10 U/ml) and recombinant rat IFN-γ (100 U/ml; R&D Systems, Abingdon, UK) for 24 h.

Western blot experiments Western blot analysis of IRF-1 levels in cytokine-treated INS-1E cells was performed as described previously [30], using antibodies against IRF-1 (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or α-tubulin (dilution 1:5,000; Sigma-Aldrich) as primary antibodies and horseradish peroxidase-conjugated donkey anti-rabbit IgG as secondary antibodies (dilution 1:5,000; Lucron Bioproducts, De Pinte, Belgium). The protein-specific signals were detected using chemiluminescence Supersignal (Pierce, Rockford, IL, USA) and quantified using Aida1D analysis software (Fujifilm, London, UK).

Real-time PCR Mouse islets or INS-1E cells cultured for 1 day were used for RNA extraction as described [29, 31]. cDNA was created using Superscript II RT (Invitrogen) and quantitative PCR analysis was performed with a single colour real-time PCR detection system (MyiQ; Bio-Rad Laboratories, Hercules, CA, USA). Primer and probe sequences for the determination of rodent cDNAs for housekeeping genes (*Actb* and *Gapdh*), for chemokine genes *Mcp-1* (also known as *Ccl2*), *Ip-10* (also known as *Cxcl10*) and *Mip-3α* (also known as *Ccl20*), and for *Il-1β* (also known as *Il1b*) and *Inos* (also known as *Nos2*) were as described previously [31–33]. The target cDNA present in each sample was corrected for the respective *Actb* values in whole islets and for the respective *Gapdh* values in INS-1E cells.

Islet transplantation and evaluation of graft function Freshly isolated *Irf-1*$^{-/-}$ or control C57BL/6 islets (n=500) were transplanted under the kidney capsule of overtly diabetic NOD mice as described previously [23]. Islet primary non-function was defined as blood glucose levels never reaching normoglycaemia within 48 h after islet transplantation, while graft rejection was defined as a return to hyperglycaemia (non-fasting glycaemic values ≥11.1 mmol/l in

three consecutive readings after initial normoglycaemia). Recipient mice were killed the day of graft rejection or in separate experiments for histological examination on days 3 and 5 post-transplantation. In a separate experiment, mice were treated with IL-1Ra (100 mg $kg^{-1}day^{-1}$) for 15 days, starting 1 day before islet transplantation as described [34].

Histology Paraffin-embedded kidneys containing islet grafts were sectioned, stained with haematoxylin and eosin, and analysed by light microscopy to assess the overall infiltration grade of the islet allografts. In addition, sections obtained from graft specimens were stained for insulin using guinea pig anti-insulin (dilution 1:100, A0564; Dako, Glostrup, Denmark), for T cells using rabbit anti-CD3 (dilution 1:200, A0452; Dako) and for macrophages using goat anti-F4/80 (dilution 1:500, sc-26642; Santa Cruz Biotechnology, Heidelberg, Germany) as described previously [10, 34]. All sections were visualised with a fluorescence microscope (AxioImager Z1; Carl Zeiss Micro Imaging, Oberkochen, Germany) using an EC Plan-Neofluar 20×/0.5 objective lens. Acquisition was done with AxioVision 4.6 software (Carl Zeiss Micro Imaging) and finally processed by ImageJ (US National Institutes of Health, Bethesda, MD, USA).

Skin transplantation Tail skins (2 cm^2) from *Irf-1*$^{-/-}$ and control C57BL/6 mice were placed in graft beds on the dorsum of allogeneic NOD mice. Grafts were scored by an observer blinded for source of skin graft and were considered rejected when less than 50% viable tissue was present.

Data analysis and statistical methods NCSS 2000 (Kaysville, UT, USA) software was used for statistical analysis. Data are expressed as mean±SEM. Peto's log-rank test was performed to compare two or more survival curves. χ^2 test was used to compare incidence of primary non-function. Student's *t* test and ANOVA were used for multiple comparisons, whenever appropriate. Significance was defined at the 0.05 level.

Results

Shorter graft survival of Irf-1$^{-/-}$ *islets transplanted into overtly diabetic NOD mice* Transplantation of *Irf-1*$^{-/-}$ islets (n=16) into overtly diabetic NOD mice resulted in a high rate of persistent hyperglycaemia, (primary non-function 63%) compared with control C57BL/6 islet grafts (25%, n=16, $p\leq0.05$) (Fig. 1a). Total graft survival was significantly lower in *Irf-1*$^{-/-}$ islet-transplanted NOD mice than in control C57BL/6 islet-transplanted NOD mice (2.0±3.2 vs 7.4±6.3 days, $p\leq0.005$) (Fig. 1b). Even after censoring for primary non-function, graft survival remained shorter in *Irf-1*$^{-/-}$ transplanted NOD mice (6.0±2.6 vs 10.4±4.8 days in control C57BL/6 grafts, $p\leq0.05$).

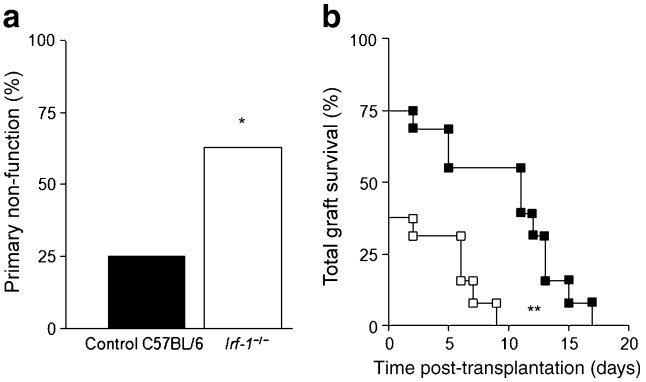

Fig. 1 Primary non-function and total allograft survival of *Irf-1*$^{-/-}$ islets transplanted into overtly diabetic NOD mice. **a** Percentage of primary non-function after transplantation of allogeneic islets of *Irf-1*$^{-/-}$ (n=16, white bars) and control C57BL/6 (n=16, black bars) mice into overtly diabetic NOD mice. Islet primary non-function was defined as failure of the grafts to normalise glycaemia within 48 h. **b** Total survival of allogeneic islets of *Irf-1*$^{-/-}$ (n=16, white squares) and control C57BL/6 (n=16, black squares) mice transplanted into overtly diabetic NOD mice. One superscript symbol $p\leq0.05$, two superscript symbols $p\leq0.01$: (*) compared with control C57BL/6 mice

Graft histology revealed that 3 days post-transplantation *Irf-1*$^{-/-}$ and control C57BL/6 islet grafts were almost free of host leucocyte infiltration and had clear insulin positivity (Fig. 2a, b, e, f). Few T cells were detected during the 3 days observation time (Fig. 2c, d). Interestingly, we observed increased numbers of macrophages (based on F4/80 immune labelling) in *Irf-1*$^{-/-}$ islet grafts (Fig. 2g, h). On the other hand, in grafts removed 5 days after transplantation, leucocyte infiltrate, consisting predominantly of CD3+ T cells, was strikingly denser in *Irf-1*$^{-/-}$ islet grafts than in control C57BL/6 islet grafts (Fig. 2i, l). This more pronounced cellular infiltration into the *Irf-1*$^{-/-}$ islet grafts coincided with a greater loss of insulin positivity in the grafts (Fig. 2m, n).

To investigate whether the difference in graft survival between *Irf-1*$^{-/-}$ and control C57BL/6 donors was an islet-specific phenomenon, *Irf-1*$^{-/-}$ and control C57BL/6 skin segments were transplanted into NOD mice. C57BL/6 (n=4) and *Irf-1*$^{-/-}$ skin grafts (n=4) were rejected at similar time-points (mean survival time 14.5±0.6 vs 15.7±1.2 days, p=NS).

Impaired functionality, but resistance against cytokine-induced cell death of Irf-1$^{-/-}$ *islets in vitro—role of IL-1β* Deletion of *Irf-1* significantly protected islet cells against cytokine-induced cell death (Fig. 3a). However, glucose-induced insulin secretion was impaired in *Irf-1*$^{-/-}$ islets even in medium glucose conditions (SI 1.5±0.2 vs

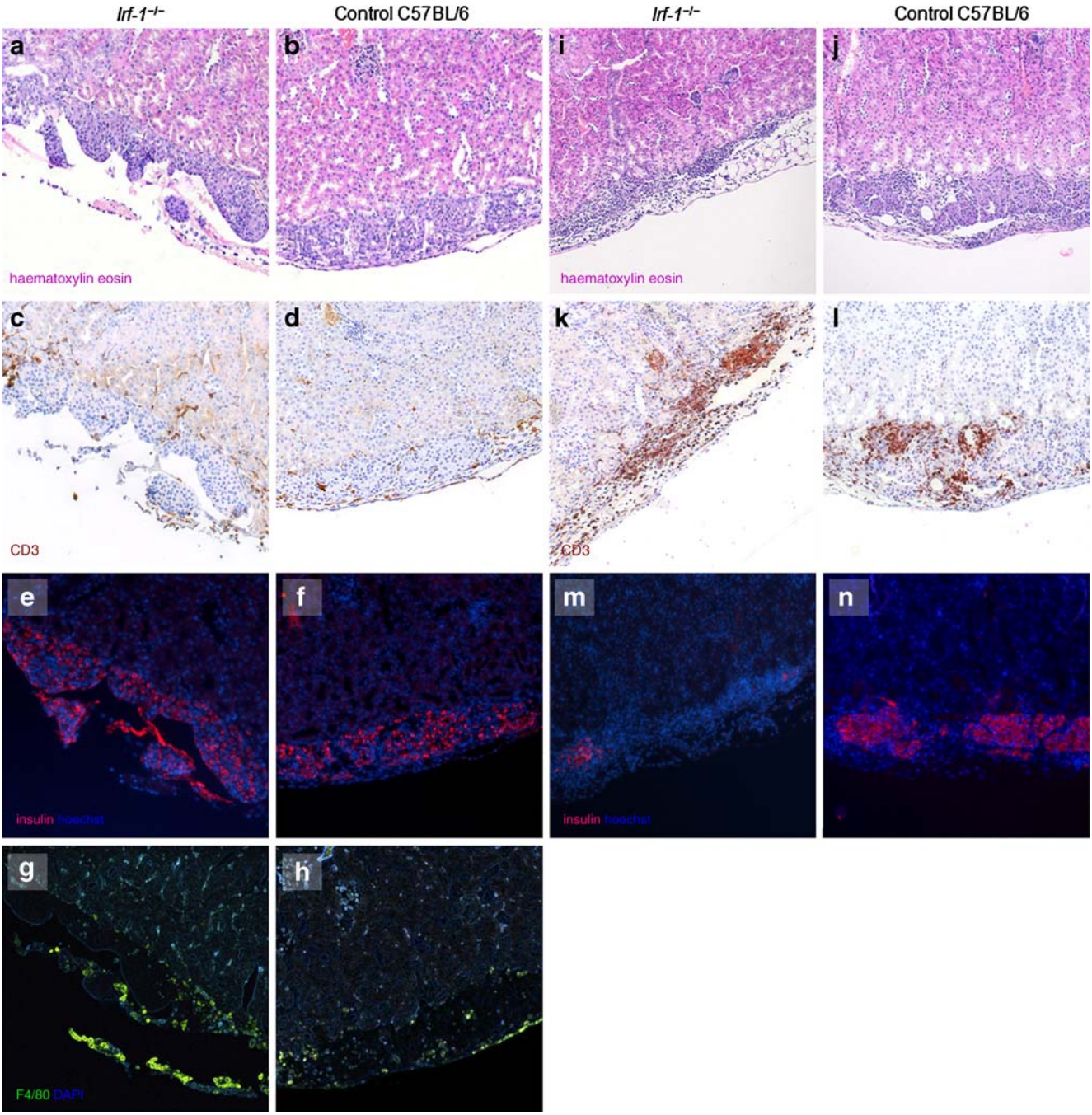

Fig. 2 Histological examination of *Irf-1*$^{-/-}$ and control C57BL/6 islets retrieved from NOD mice at 3 (**a**–**h**) and 5 (**i**–**n**) days after transplantation. *Irf-1*$^{-/-}$ islet grafts and control C57BL/6 were retrieved 3 and 5 days after transplantation in NOD mice, respectively and stained with haematoxylin and eosin (**a**, **b**, **i**, **j**) to assess graft architecture and infiltration grade of the graft. In addition, sections were immunostained for CD3+ T cells (brown) (**c**, **d**, **k**, **l**), insulin (red) (**e**, **f**, **m**, **n**) and macrophages (**g**, **h**) to determine the remaining insulin in the graft and the composition of the infiltrate. Samples were counterstained with haematoxylin, Hoechst 33258 (blue) (**e**, **f**, **m**, **n**) or DAPI (blue) (**g**, **h**), respectively. Magnification (all panels): 20×. Panels are representative of four to five mice under each experimental condition

4.0±0.3 in control C57BL/6 islets, $p \leq 0.01$). Exposure to cytokines for 24 h impaired insulin secretion in both groups, but the difference between *Irf-1*$^{-/-}$ and control C57BL/6 islets remained (SI 0.5±0.2 vs 1.6±0.4 in control C57BL/6 islets, $p \leq 0.05$) (Fig. 3b). Exposure to IL-1β alone did not induce cell death in *Irf-1*$^{-/-}$ or in control C57BL/6 islets, but did impair insulin secretion in both groups.

Use of IL-1Ra to block the effect of IL-1β prevented the cytokine-induced impairment of *Irf-1*$^{-/-}$ and control C57BL/6 islets. However, IL-1Ra did not restore the

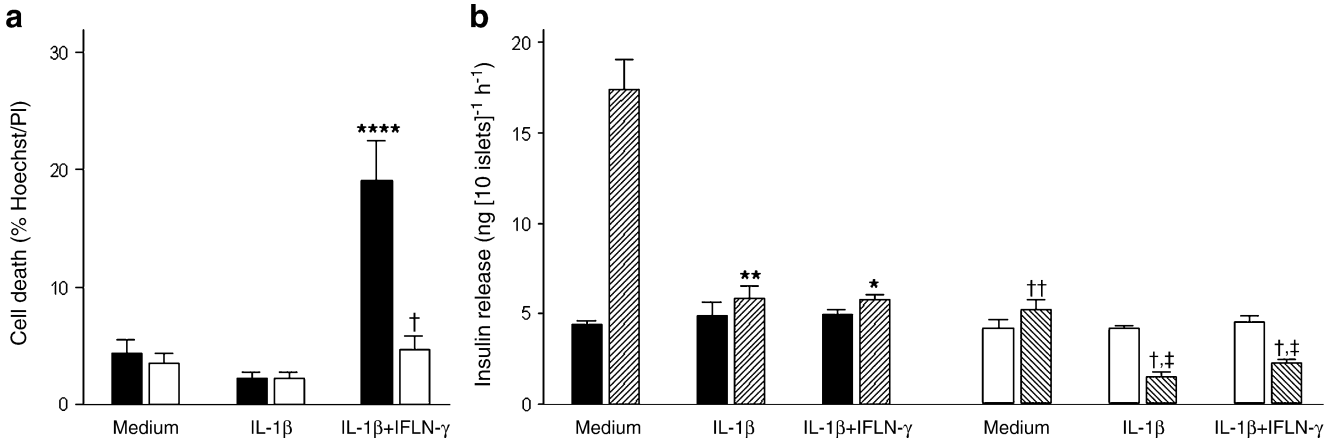

Fig. 3 Viability and functionality of *Irf-1*$^{-/-}$ and control C57BL/6 islets exposed to inflammatory cytokines. **a** Islet viability was determined after 1 day of exposure of whole islets that had been isolated from *Irf-1*$^{-/-}$ (white bars) and control C57BL/6 (black bars) mice to IL-1β (50 U/ml) with or without IFN-γ (1,000 U/ml). The percentage of cell death is expressed as mean±SEM from three to four independent experiments. Note that *Irf-1*$^{-/-}$ islets are protected against cell death when exposed to a mixture of cytokines in vitro. PI, propidium iodide. **b** Islet functionality was determined in *Irf-1*$^{-/-}$ islets (white bars) and control C57BL/6 islets (black bars) cultured for 1 day with or without IL-1β (50 U/ml) in combination with IFN-γ (1,000 U/ml). After 30 min of sedimentation, islets (n=10) were incubated with low glucose (3 mmol/l glucose, black or white bars) and subsequently with high glucose (20 mmol/l glucose, hatched bars) for 1 h and insulin release was measured by insulin ELISA. Assay was performed in triplicate from five independent experiments. One superscript symbol $p\leq0.05$, two superscript symbols $p\leq0.01$; three superscript symbols $p\leq0.001$, four symbols $p\leq0.0001$: (†) compared with control C57BL/6 islets (under similar conditions); (*) compared with control C57BL/6 islets (no cytokines added); (‡) compared with *Irf-1*$^{-/-}$ islets (no cytokines added)

secretory defect of *Irf-1*$^{-/-}$ islets under medium glucose conditions (Fig. 4a). To assess whether *Irf-1*$^{-/-}$ mice also had alterations in glucose-stimulated insulin secretion in vivo, glucose tolerance tests were performed. Blood glucose levels of *Irf-1*$^{-/-}$ mice were significantly higher than those of control C57BL/6 mice at nearly all time points after glucose administration (Electronic supplementary material [ESM] Fig. 1a). The AUC for the blood glucose response was significantly increased in *Irf-1*$^{-/-}$ mice compared with control C57BL/6 mice (ESM Fig. 1b), indicative of an impaired glucose tolerance.

In vivo, treatment with IL-1Ra slightly reduced the rate of primary non-function in NOD mice transplanted with control C57BL/6 islets (17% vs 25% in non-treated control C57BL/6 islet recipients; p=NS) and totally prevented the primary non-function in NOD mice transplanted with *Irf-1*$^{-/-}$ islets (0% vs 63% in non-treated *Irf-1*$^{-/-}$ islet recipients; $p\leq0.01$) (Fig. 4b). Moreover, total graft survival in *Irf-1*$^{-/-}$ islet recipients was now prolonged to the same time span as that in control C57BL/6 islet recipients, which was, however, similar to control islet survival in untreated NOD mice (Fig. 4b).

Increased chemokine expression and release by Irf-1$^{-/-}$ *islets and by insulin-producing INS-1E cells transfected with Irf-1 siRNA when exposed to cytokines* Considering the greater infiltrate present in *Irf-1*$^{-/-}$ grafts and the possible role of IRF-1 as a modulator of chemokine expression by cytokine-exposed beta cells, we measured expression of different chemokines. Although cytokines alone had clear effects on chemokine expression in control C57BL/6 islets, upon exposure to IL-1β and IFN-γ, expression of *Mcp-1*, *Ip-10* and *Mip-3α* increased in *Irf-1*$^{-/-}$ islets (Fig. 5a–c). Moreover, RNA levels of the inflammatory cytokine *Il-1β* and the enzyme *Inos* were increased in *Irf-1*$^{-/-}$ islets upon cytokine exposure (Fig. 5d, e).

siRNA targeting *Irf-1* in the rat insulinoma cell-line INS-1E led to a reproducible decrease in IRF-1 levels as judged by western blotting (Fig. 5f). The degree of knockdown achieved ranged from 75% to 95% depending upon the experiment. Mock transfection with a control siRNA failed to reduce IRF-1 levels. The effect of two different *Irf-1* siRNAs was specific in that it failed to knock down expression of the unrelated protein α-tubulin. Cells transfected with *Irf-1* siRNA cells showed a similar gene expression pattern to that observed in whole islets with *Irf-1* deletion (Fig. 5g–j). Expression of *Mcp-1*, *Ip-10*, *Mip-3α* and *Inos* after 24 h exposure to IL-1β plus IFN-γ in INS-1E cells transfected with *Irf-1* siRNA was higher than in non-transfected or siRNA control-transfected cells and reached significance for *Ip-10* and *Mip-3α* (Fig. 5g–j).

To confirm the gene expression data, we also studied MCP-1 and nitrite release in the supernatant fraction of whole islets exposed to inflammatory cytokines. By ELISA, low levels of MCP-1 were detected in the culture supernatant fractions of *Irf-1*$^{-/-}$ and control islets in basal condition. Confirming the mRNA findings, secretion of

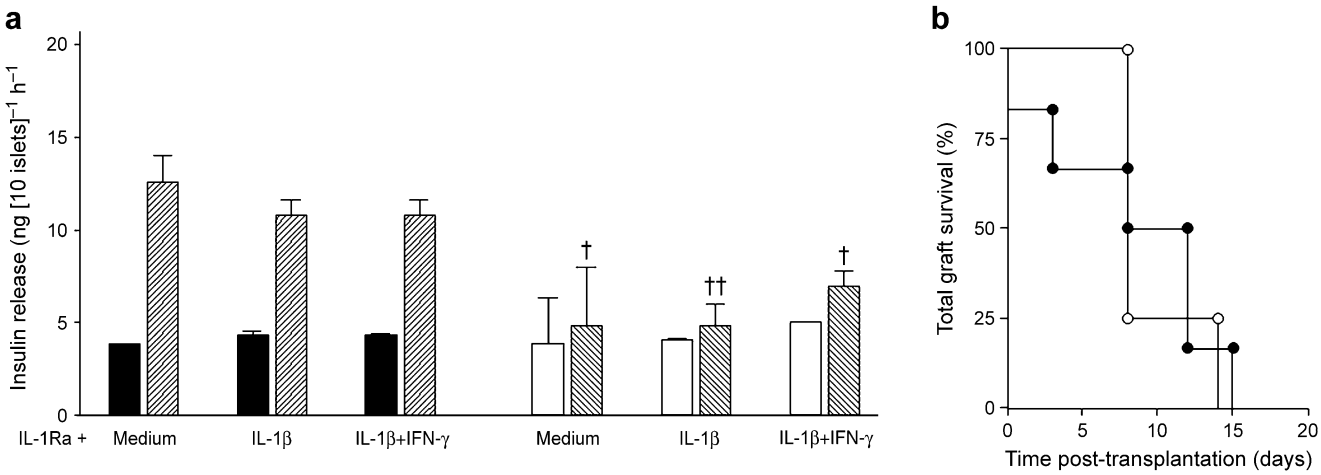

Fig. 4 Effect of IL-1Ra treatment on functionality of *Irf-1*$^{-/-}$ and control C57BL/6 islets in vitro and in vivo. **a** Glucose-stimulated insulin secretion of islets isolated from *Irf-1*$^{-/-}$ (white bars) and control C57BL/6 (black bars) mice was performed. Islets were pre-treated or not with 500 ng/ml recombinant human IL-1Ra for 30 min before addition of IL-1β (50 U/ml) with or without IFN-γ (1,000 U/ml). Low glucose (3 mmol/l, black or white bars) and stimulated insulin secretion (20 mmol/l glucose, hatched bars) during successive 1 h incubations was performed in duplicate from four independent experiments. One superscript symbol $p \leq 0.05$, two superscript symbols $p \leq 0.01$: (†) compared with control C57BL/6 islets (under similar conditions). **b** IL-1Ra treatment was initiated the day before transplantation and graft function of allogeneic islets of *Irf-1*$^{-/-}$ (n=6, white circles) and control C57BL/6 (n=6, black circles) mice transplanted into overtly diabetic NOD mice was evaluated daily after transplantation

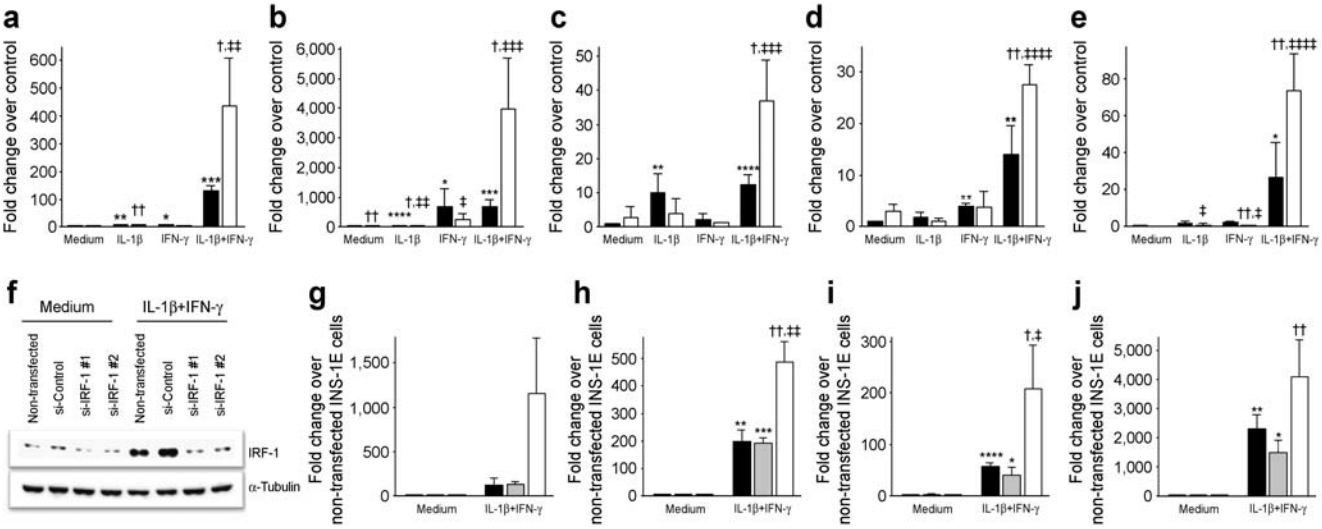

Fig. 5 Gene expression in whole islets and INS-1E cells exposed to inflammatory cytokines. mRNA expression of *Mcp-1* (**a**), *Ip-10* (**b**), *Mip-3α* (**c**), *Il-1β* (**d**) and *Inos* (**e**) from whole islets that had been isolated from *Irf-1*$^{-/-}$ (white bars) and control C57BL/6 (black bars) mice. Expression after 1 day exposure to IL-1β (50 U/ml), IFN-γ (1,000 U/ml) or combination of both was determined by real-time quantitative PCR analysis and is expressed as fold change over control (the ratio between the gene of interest and housekeeping gene *Actb*), means±SEM. Values are representative of four to five independent experiments. **f** INS-1E cells were transfected with 30 nmol/L IRF-1#1 or IRF-1#2 siRNA and after a recovery period of 24 h exposed or not for additional 24 h to IL-1β (10 U/ml) plus IFN-γ (100 U/ml). At that time, cells were processed for western blotting with anti-IRF-1 antibodies. The blots were subsequently stripped and re-probed with anti-α-tubulin as controls. **g–j** INS-1E cells were non-transfected (black bars) or transfected with control siRNA (grey bars) or with *Irf-1* siRNA (white bars). After 24 h of recovery, cells were exposed or not for 24 h to IL-1β (10 U/ml) plus IFN-γ (100 U/ml). mRNA levels of *Mcp-1* (**g**), *Ip-10* (**h**), *Mip-3α* (**i**) and *Inos* (**j**) were assayed by real-time PCR and are expressed as fold change over non-transfected INS-1E cells (ratio between gene of interest and housekeeping gene *Gapdh)*. Values are means±SEM of five to six experiments. One superscript symbol $p \leq 0.05$, two superscript symbols $p \leq 0.01$; three superscript symbols $p \leq 0.001$, four superscript symbols $p \leq 0.0001$: (†) compared with control C57BL/6 islets or non-transfected INS-1E cells (under similar conditions); (*) compared with control C57BL/6 islets or non-transfected INS-1E cells (no cytokines added); (‡) compared with *Irf-1*$^{-/-}$ islets or si-IRF-1-transfected INS-1E cells (no cytokines added)

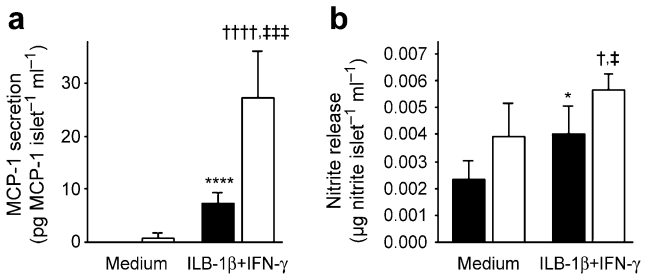

Fig. 6 MCP-1 (**a**) and nitrite production (**b**) of *Irf-1*$^{-/-}$ and control C57BL/6 islets exposed to inflammatory cytokines. **a** MCP-1 secretion or **b** nitrite production from whole islets that had been isolated from *Irf-1*$^{-/-}$ (white bars) and control C57BL/6 (black bars) mice. Values after 3 days exposure to IL-1β (50 U/ml) and IFN-γ (1,000 U/ml) were determined by ELISA and with Griess reagent protocol respectively, and are expressed as means±SEM from three to four independent experiments. One superscript symbol $p \leq 0.05$, two superscript symbols $p \leq 0.01$; three superscript symbols $p \leq 0.001$, four superscript symbols $p \leq 0.0001$: (†) compared with control C57BL/6 islets (under similar conditions); (*) compared with control C57BL/6 islets (no cytokines added); (‡) compared with *Irf-1*$^{-/-}$ islets (no cytokines added)

MCP-1 after 3 days of exposure to IL-1β and IFN-γ was higher in the supernatant fraction of *Irf-1*$^{-/-}$ islets than in that of control C57BL/6 islets under similar cytokine conditions (Fig. 6a). Moreover, cytokine-induced nitrite production was higher in the *Irf-1*$^{-/-}$ islets than in control C57BL/6 islets after 3 days of culture with IL-1β and IFN-γ (Fig. 6b).

Irf-1$^{-/-}$ *islets exposed to inflammatory cytokines induce immune-cell chemotaxis in vitro* Chemokines direct the migration of leucocytes via their interaction with chemokine receptors on cell surfaces of immune cells. As *Irf-1*$^{-/-}$ islets strongly express and secrete various chemokines, we investigated NOD leucocyte chemotaxis toward supernates of *Irf-1*$^{-/-}$ or control C57BL/6 islets exposed for 3 days to a mixture of inflammatory cytokines (IL-1β and IFN-γ). The chemotactic index for the cytokine-treated *Irf-1*$^{-/-}$ islets was higher than that for the corresponding conditions with control C57BL/6 islets (Fig. 7).

Discussion

Inflammatory cytokines like IL-1β, TNF-α and IFN-γ contribute to beta cell death. In vitro models demonstrate that interfering with transcription factors at central positions in the complex signalling cascades activated by these cytokines could render beta cells resistant to cytokine-induced destruction. However, in vivo data show a more complex picture [35]. In the present work, we demonstrate that islets in which *Irf-1* is knocked out (*Irf-1*$^{-/-}$) show more primary non-function and have a shorter overall survival when transplanted into spontaneously diabetic NOD mice.

This increase in primary non-function of *Irf-1*$^{-/-}$ islets was surprising given that (1) *Irf-1*$^{-/-}$ islets are partly protected against cytokine-induced cell death in vitro [36]; (2) *Irf-1*$^{-/-}$ NOD mice have decreased prevalence of insulitis and diabetes [37]; and (3) elimination of STAT-1, the transcription factor upstream of IRF-1 in the IFN-γ signalling cascade, results in complete protection against islet primary non-function in vivo [35]. To date, the pathophysiology of islet primary non-function is incompletely understood, but we have previously shown that it is of great importance in autoimmune hosts (spontaneously diabetic NOD mice) and seems to be associated with non-specific inflammation at the implantation site, with a major role being played by IL-1β and free radicals (nitric oxide) [23, 34]. This role of IL-1β in primary non-function also explains the difference in primary non-function between *Irf-1*$^{-/-}$ and *Stat-1*$^{-/-}$ islets transplanted into NOD mice, as IRF-1 is not only controlled by IFN-γ, but is also partially regulated by IL-1β, in contrast to STAT-1. The fact that IL-1Ra completely prevented primary non-function in *Irf-1*$^{-/-}$ islets again points to the excessively high local *Il-1β* levels at the transplant site in autoimmune NOD mice as reason for the increased prevalence of primary non-function in the present setting. In vitro as well as in vivo reports show that IL-1β primarily impairs functionality of the beta cell, with

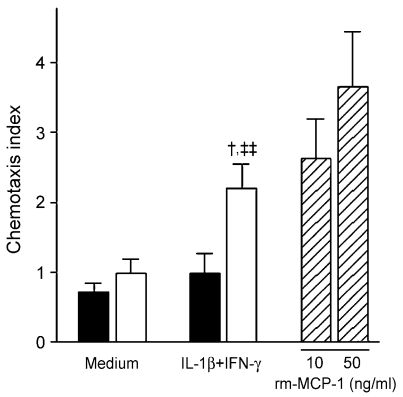

Fig. 7 Chemotaxis index from *Irf-1*$^{-/-}$ and control C57BL/6 islets exposed to inflammatory cytokines. Migration of leucocytes, derived from NOD spleens, towards supernates from *Irf-1*$^{-/-}$ (white bars) or control C57BL/6 (black bars) islets exposed for 3 days to IL-1β (50 U/ml) plus IFN-γ (1,000 U/ml) was counted during a 2 min acquisition with a flow cytometer. Chemotaxis index is defined as the ratio of the number of leucocytes attracted by test solution (supernatant fraction of islet preparations): number of leucocytes attracted by the negative control (medium alone). As positive control, we used recombinant mouse MCP-1 (hatched bars) at concentrations of 10 and 50 ng/ml. The chemotaxis index is expressed as means± SEM from three independent experiments. One superscript symbol $p \leq 0.05$, two superscript symbols $p \leq 0.01$: (†) compared with control C57BL/6 islets (under similar conditions); (‡) compared with *Irf-1*$^{-/-}$ islets (no cytokines added)

only minor effects on cell viability [6, 38]; they also show that blocking IL-1β action with IL-1Ra can prevent the deleterious effects [24, 34, 39–42]. Although this was confirmed in our study, we observed abnormal glucose-stimulated insulin release from *Irf-1*$^{-/-}$ islets under basal conditions that could not be corrected by IL-1Ra, indicating an independent defect in beta cell secretory machinery of *Irf-1*$^{-/-}$ islets. Moreover, *Irf-1*$^{-/-}$ mice had impaired glucose tolerance. The exact cause for these metabolic abnormalities remains unclear. Nevertheless, *Irf-1*$^{-/-}$ islets that were transplanted into chemically induced diabetic BALB/c mice had 100% islet function after transplantation [36]. Based on these observations, we do not claim that the secretory defect of *Irf-1*$^{-/-}$ islets is the major reason for the observed primary non-function of *Irf-1*$^{-/-}$ islets after transplantation into NOD mice, but instead hypothesise that the higher levels of the inflammatory cytokine *Il-1β* in *Irf-1*$^{-/-}$ islets under cytokine exposure may contribute to the high rate of islet primary non-function.

The complex role of IRF-1 in beta cell failure in vivo was further demonstrated by studying *Inos* gene expression and nitrite production in islets lacking *Irf-1*. Montolio et al. showed that increased expression of islet-derived inflammatory factors like *Il-1β* and *Inos* genes plays a predominant role in beta cell loss in the initial days after islet transplantation [43]. IL-1β causes pancreatic islet dysfunction and death at least in part through upregulation of *Inos* and production of nitric oxide [26, 44]. The higher *Il-1β* and *Inos* expression and nitrite production in cytokine-exposed *Irf-1*$^{-/-}$ islets, which was in contrast to the gene expression profile detected in *Stat-1*$^{-/-}$ islets exposed to the same cytokine mixture [10], point to a critical role for these inflammatory mediators in islet primary non-function. Interestingly, intense macrophage infiltration was observed in *Irf-1*$^{-/-}$ islet grafts at 3 days after transplantation. Kaufman et al. demonstrated that modulation of macrophages by administration of silica completely abolished islet allograft primary non-function [45]. We proposed that the release of IL-1β by islet-associated macrophages may induce expression of *Inos* by beta cells, resulting in inhibition of beta cell function by the production of nitric oxide. Analysis of *Inos* mRNA expression in INS-1E cells transfected with *Irf-1* siRNA confirmed the results obtained in *Irf-1*$^{-/-}$ islets. Our detection of elevated *Inos* gene expression and nitrite production in cytokine-exposed *Irf-1*$^{-/-}$ islets contrasts with previous findings showing lower expression of *Inos* in *Irf-1*$^{-/-}$ islets exposed to a mixture of cytokines [46]. Although some studies indicate that IRF-1 is required for full *Inos* gene expression in various cell types, including insulin-producing RINm5 cells, and although two adjacent IRF-1 response elements were identified in the *Inos* promoter [47–49], other data suggest that IRF-1 is not essential for *Inos* induction and nitric oxide production by mouse islets [50] or sorted rat beta cells [51].

In addition to the higher prevalence of primary non-function, we observed a shorter overall survival of the *Irf-1*$^{-/-}$ grafts than in controls, accompanied by a higher degree of inflammatory-cell infiltration in the grafts. This higher infiltration correlated with the increased expression and secretion of several chemokines by cytokine-exposed *Irf-1*$^{-/-}$ islets. *Irf-1* deficiency resulted in higher expression of *Mcp-1*, *Ip-10* and *Mip-3α*, and elevated MCP-1 production after exposure to cytokines in vitro, confirming results by other groups [47]. Moreover, chemokine mRNA expression analysis of INS-1E cells transfected with *Irf-1* siRNA confirmed the results obtained in whole *Irf-1*$^{-/-}$ islets. The higher chemokine expression and secretion also led to increased leucocyte migration when exposed to cytokine-treated *Irf-1*$^{-/-}$ islets. We therefore hypothesise that while *Irf-1*$^{-/-}$ islets as such are partially protected against cytokine-induced cell death, this is of little relevance in vivo, since exposure of these islets to inflammatory cytokines leads to enhanced functional impairment (via IL-1β and nitric oxide) and induces secretion by the beta cells themselves of chemokines that accelerate the influx of inflammatory and immune cells into the grafts, in turn leading to destruction of the beta cells via different pathways. Finally, these data are in contrast to the lower insulitis and diabetes prevalence in *Irf-1*$^{-/-}$ NOD mice [37]. A major criticism of this model is that *Irf-1* is knocked-out in the whole mouse, including the immune system, where it is crucial for IFN-γ signalling. Therefore, we believe that the lower insulitis and diabetes prevalence in *Irf-1*$^{-/-}$ NOD mice should be interpreted carefully, as these mice are relatively immune-deficient and the observation may be due to effects of *Irf-1* inactivation in the immune system rather than to the beta cell [20, 22].

In conclusion, IRF-1 is a central transcription factor in the modulation of cytokine-induced beta cell loss and may play a role in overall beta cell health. Its deletion leads to impairment of glucose-induced insulin secretion, glucose intolerance and impaired survival of transplanted allogeneic islets in spontaneously diabetic NOD mice. This impaired survival is due to increased primary non-function and more aggressive immune infiltration into the grafts. IRF-1 is crucial in controlling cytokine-induced *Il-1β* and *Inos* expression and chemokine production by beta cells. Elimination of *Irf-1* leads to increased chemokine secretion in vitro and to a more aggressive immune infiltration and more rapid destruction of grafted islets in vivo. These insights warn against too simplistic visions of the role of cytokines in beta cell destruction in vivo and should help create beta cell-

oriented interventions, aimed at rendering beta cells more resistant to beta cell attack.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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