Diabetes

Pancreatic beta cell-derived IP-10/CXCL10 isletokine mediates early loss of graft function in islet cell transplantation

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Abstract

Pancreatic islets produce and secrete cytokines and chemokines in response to inflammatory and metabolic stress. The physiological role of these "isletokines" in health and disease is largely unknown. We observed that islets release multiple inflammatory mediators in patients undergoing islet transplants within hours of infusion. The proinflammatory cytokine interferon gamma-induced protein 10 (IP-10/CXCL10) was among the highest released, and high levels correlated with poor islet transplant outcomes. Transgenic mouse studies confirmed that donor islet-specific expression of IP-10 contributed to islet inflammation and loss of beta-cell function in islet grafts. The effects of islet-derived IP-10 could be blocked by treatment of donor islets and recipient mice with anti-IP-10 neutralizing monoclonal antibody. *In vitro* studies showed induction of the IP-10 gene was mediated by calcineurin-dependent NFAT signaling in pancreatic beta cells in response to oxidative or inflammatory stress. Sustained association of NFAT and p300 histone acetyltransferase with the IP-10 gene required p38 and JNK MAP kinase (MAPK) activity, which differentially regulated IP-10 expression and subsequent protein release. Overall, these findings elucidate an NFAT-MAPK signaling paradigm for induction of isletokine expression in beta-cells and reveal IP-10 as a primary therapeutic target to prevent beta-cell-induced inflammatory loss of graft function after islet cell transplantation.

Islet endocrine cells have been shown to produce cytokines under conditions of physical, inflammatory, and metabolic stress (1-6). Expression of IL-1β has been observed in beta cells exposed to hyperglycemic conditions and in type 2 diabetes patients (7). IL-1β signaling further promotes cytokine expression in beta cells (3,5,6,8-10). Acute effects of IL-1β to enhance insulin expression and induce proliferation in beta cells indicate a physiological role of cytokines to enable islets to adapt to inflammatory stress and metabolic demand (11-16). However, inappropriate expression of cytokines by islets are associated with endoplasmic reticulum (ER) and oxidative stress responses that promote beta-cell death and dysfunction (17-20). Overexposure of islets to osmotic, metabolic, oxidative, or inflammatory stress induces p38 and JNK MAP kinase (MAPK) signaling (21-24). This leads to downstream effects of NF-κB to upregulate genes that promote apoptosis (17,25).

During islet transplantation, islets are exposed to multiple physical and chemical stresses throughout islet cell isolation and infusion procedures that induce expression of cytokines. These inflammatory mediators are secreted by islets and persist for days within culture. Upon transplantation of islets, an innate immune response is observed that is largely responsible for a loss of up to 50% or more of the initial islet graft mass during the immediate post-transplant period. This phenomenon was first described as an instant blood mediated inflammatory reaction (IBMIR) that is characterized by a heparin-sensitive, platelet-mediated activation of the complement cascade (26 ,27). We hypothesized that release of islet-derived cytokines by stressed islets contributes to the early inflammatory loss of islet cell tissue upon transplantation.

In this study, we monitored circulating inflammatory mediators in patients immediately following islet transplantation and identified interferon gamma-induced protein 10 (IP-10/CXCL10) as a major chemokine released immediately upon islet infusion that adversely

affects transplant outcomes. Protection of islet grafts with beta-cell specific deletion of IP-10 in an islet transplant model confirmed a role of donor islet-specific IP-10 to contribute to early inflammatory loss of islet graft function. Further analysis of IP-10 in beta cells indicated that NFAT and stress-activated MAPK signaling directly induced expression of the IP-10 gene in response to oxidative or inflammatory stress. Moreover, high glucose stimulated release of IP-10 protein from stressed beta cells. Finally, a monoclonal antibody (mAb) directed toward IP-10 could prevent early loss of islet grafts in transplanted mice. These findings highlight IP-10 as a key isletokine that can be targeted to prevent islet inflammation and improve islet cell transplant outcomes.

Research Design and Methods

Cell and tissue samples. Blood samples were collected from 34 patients undergoing islet transplants at Baylor University Medical Center at time intervals post islet infusion in accordance with IRB-approved protocols. Isolated human islets from multiple donors (>90% purity) were provided by the Integrated Islet Distribution Program at City of Hope and from the cGMP Islet Cell Processing Laboratory at Baylor University Medical Center. C57BL/6 mouse pancreatic islets were isolated as described below. Isolated islets were cultured 1-2 days prior to use. Isolated islets, FACs-purified beta cells, and MIN6 beta-cell line were cultured in RPMI 1640 or Krebs-Ringer bicarbonate HEPES buffer media at 37°C in 5% CO_2 humidified air.

Mouse islet isolation. Islets were isolated from wildtype and IP-$10^{-/-}$ C57BL/6 9-12 week old male mice (The Jackson Laboratory). Pancreata were removed from mice after collagenase (type V, C9263, Sigma-Aldrich) perfusion and digested for 18 min at 37°C and washed with HBSS. Islets were separated from digested pancreas preparations by centrifugation on a 1.077 and 1.100g/mL ficoll gradient solution. The separated islet tissue layer was collected and washed

with HBSS. Intact islets were hand-selected from the purified preparation by pipette and cultured overnight at 37°C.

Reagents and recombinant DNA constructs. Antibodies were used as follows: NFATc2, p300, and HDAC3 (Santa Cruz Biotechnology, Inc) and ERK1/2, p38, and JNK (Cell Signaling Technology). The mouse IP-10 promoter-reporter vector containing *Gaussia* luciferase reporter was obtained from GeneCopoeia. Anti-IP-10 mAb was obtained from the Baylor Research Institute Biotechnology Core. Vectors expressing GFP and mouse NFATc2 or sequence-scrambled shRNA were obtained from OriGene. Expression vectors containing dominant negative NFAT PxIxIT motif (dnNFAT) and mutated dnNFAT AxAxAA motif (dnNFATm) were previously described (5,28).

DNA transfection and promoter assays. MIN6 cells were transfected with plasmids and shRNA constructs by Lipofectamine 2000 24 h prior to purification and cell treatments. Cells were harvested with 1× Reporter Lysis buffer and Luciferase/Renilla enzyme activity was detected by the Dual-Luciferase reporter assay system (Promega) using a TD20/20 bioluminometer.

Flow cytometry. Human islets and MIN6 cells were subjected to fluorescent activated cell sorting (FACS) on a MoFlo high speed sorter. Beta cells were purified based on zinc-sensitive fluorescence of Newport Green (NG) DCF diacetate as previously described (5). NG-stained beta-cell fractions were screened for exclusive expression of insulin by immunoassay. MIN6 cells transfected with shNFAT-GFP and shScramble-GFP were trypsinized and purified based on relative GFP fluorescence.

Immunoassays. Proteins from human serum collected from patients undergoing islet transplants and supernatant samples from isolated human islets (300 IEQ in 1 mL medium) were measured by multiplex assays with magnetic beads using the Luminex platform system (EMD Millipore). Enzyme-linked immunosorbent assays (ELISA) were performed to measure IP-10 and insulin using a Cytation 5 microplate reader (BioTek).

Immunofluorescent staining. Formalin-fixed and paraffin-embedded sections of isolated human islets were incubated with primary antibody (1:250) in PBS containing 0.1% Triton X-100 and 4% BSA overnight at 4°C. After washing, samples were subjected to FITC and Cy3 conjugated secondary antibody (1:500) and imaged by an Olympus BX61 TRF Fluorescent Microscope.

Islet transplantation. Wildtype C57BL/6, IP-$10^{-/-}$ C57BL/6, and nude mice (Envigo) (9-12 week old males) were subjected to 200 mg/kg body weight of streptozotocin (STZ). Mice with blood glucose >400 mg/dL for 2 or more consecutive days were selected as diabetic recipients for islet transplantation. For mouse islet recipients, 200 islets were infused to C57BL/6 recipients via the portal vein. For human islet recipients, 2000 IEQ were infused to subcapsular kidney sites in nude mice and kidneys were removed after 6 h for analysis of islet graft cytokine gene expression.

Glucose Measurements. Blood glucose and body weight were monitored twice a week for up to 30 d. After 30 d, mice were fasted for 8 hours prior to intraperitoneal administration of 20% glucose solution at 2 g glucose/kg body weight. Blood glucose was measured at 30 min intervals for IPGTT analyses.

Nuclear run-on assay. MIN6 cells and were treated and lysed in Tris buffer containing 0.5% Triton X-100. Nuclei were washed and isolated by centrifugation on a 0.6 M sucrose cushion at 600g in a table top centrifuge. Intact fractionated nuclei were incubated at 30°C for 30 min in Tris buffer containing 2-U RNasin recombinant ribonuclease inhibitor (Promega), and 30% glycerol in the presence of 200 μM of rNTPs. RNA was isolated using TRI reagent and transcribed to cDNA. Relative changes in gene targets and 18S mRNA were quantified by qPCR.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed as previously described (6). Briefly, MIN6 cells were fixed and chromatin DNA-protein was cross-linked with 1% formaldehyde for 10 min, then quenched with 125 mM glycine for 5 min and washed with PBS. Fixed cells were sonicated with a Bioruptor 200 (Diagenode) and lysed in RIPA buffer containing protease inhibitor cocktail. DNA-protein complexes were immunoprecipitated with indicated antibodies or IgG isotype controls and extensively washed. Cross links were reversed at 65°C for 4 h in Tris buffer containing 5 M NaCl and 0.5 M EDTA. DNA was extracted by phenol/CHCl3 and precipitated with ethanol followed by proteinase K and RNase A treatment. Precipitated DNA and 1% control inputs were analyzed by QPCR.

Real-time QPCR. QPCR was performed using the Bio-Rad CFX Connect system with RT^2 SYBR Green qPCR Mastermix. RT² qPCR Primer Assays were used to detect specific cDNA-converted mRNA transcripts for 18S and target genes (Qiagen). Primer sequences used to detect input and immunoprecipitated DNA sequences from -266 to -122 of the 5′-flank mouse IP-10 promoter region were 5’- TCTGCAAGGCACTCATCTGAT and 5’- CGAGGGCATTGCTTGTGTTT.

Results

High IP-10 released from islets upon transplantation is associated with poor clinical transplant outcomes. We analyzed clinical samples for inflammatory mediators present in the blood plasma circulation immediately after islet infusion. Pre- and post-infusion samples from islet transplant patients were analyzed by Luminex multiplex protein screening to identify early inflammatory mediators present upon infusion into the portal vein. Several isletokines were present in the blood circulation within hours of islet transplantation (Fig. 1A), with particularly high levels of IP-10, MCP-1, and MDC (>100 pmol/mL). Notably, IP-10 showed the largest increase in expression (~4 fold) in the circulation within 1 h of islet infusion relative to pre-transplant controls.

To identify isletokines directly produced by islets under inflammatory conditions, we exposed isolated human islets (3000 IEQ) to a proinflammatory cytokine cocktail (CC) containing 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 50 ng/mL IFN-γ, and for 2 h, washed extensively, and analyzed proteins released into the medium 6 h posttreatment. The analysis showed that chemokines IP-10, MCP-1, and MIP-1β had the highest rates of release (>5 pmol/IEQ/h) (Fig. 1B). Large inductions (>5 fold) were observed for IFN- γ, IL-1β, IL-10, IP-10, MCP-1, MIP-1β, and TNF-α (Fig. 1C). Among all cytokines tested, IP-10 had the highest increase in induction and amount of protein released into the circulation after islet infusion or into the medium *in vitro* after islet exposure to inflammatory stress.

To investigate the role of IP-10 in innate inflammatory reactions immediately following islet transplantation in isolation from adaptive immune responses associated with allotransplantation, we analyzed autologous islet transplant patient serum samples (n=26) collected at time intervals immediately following infusion and correlated them to transplant outcomes assessed more than 6 months post transplantation. Transplant patients were grouped by

IP-10 area under the curve plasma levels over 24 h (AUC_{0-24h}) of low (< 2 pg·h/IEQ/kg, n=10), medium (2-4 pg·h/IEQ/kg, n=9), and high (>4 pg·h/IEQ/kg, n=7) (Fig. 1D). The analyses showed that patients in the group having higher levels of IP-10 had significantly reduced islet graft function assessed by C-peptide (Fig. 1E). Collectively, these data indicate that IP-10 plays a role in the inflammatory response and loss of islet function observed during the immediate peritransplant period. They also suggest that that lowering IP-10 released by islets could improve transplant outcomes.

Effects of IP-10 on islet transplant outcomes is donor-islet specific and can be reversed by anti-IP-10 mAb. To determine the direct contributions of islet-derived IP-10 on transplant outcomes, we used transgenic IP-$10^{-/-}$ mice to compare effects of IP-10 gene deletion in islet donor tissue and in islet recipients on graft function. STZ-induced diabetic mice were recipients of marginal doses of 200 isolated mouse donor islets intraportally. Blood glucose was monitored in recipients up to 30 d post transplantation and glucose profiles were assessed by IPGTT (Fig. 2A). Hyperglycemia was reversed in those receiving IP-$10^{-/-}$ donor islets up to 30 d after transplantation. IPGTTs showed reversal of blood glucose to normal concentrations within 2 h of glucose challenge. In contrast, recipients of wild type donor islets remained diabetic and exhibited poor glucose tolerance 30 d after transplantation. The results indicate that IP-10 specifically derived from beta cells of donor islets contributes to loss of islet graft endocrine function in mouse islet transplants.

Both clinical and animal transplant data from these studies suggested that lowering or ablating IP-10 could improve islet transplant outcomes. Therefore, we sought to neutralize effects of IP-10 with an anti-IP-10 mAb in a syngeneic mouse islet transplant model. Donor islet preparations were pretreated for 10 min with 100 μg of anti-IP-10 or IgG1K isotype control mAb

and then transplanted intraportally to STZ-diabetic mouse recipients. Diabetes was reversed in the IP-10 mAb-treated group of mice receiving marginal doses of islets, and the rate of normoglycemia was significantly improved compared to the mAb isotype control group (Fig. 2B). IP-10 mAb treated mice also displayed improved blood glucose profiles with AUC glucose concentrations significantly lower than those of controls (Fig. 2C). These data indicate that IP-10 production by donor islets is detrimental to islet graft function and that ablation or neutralization of donor islet IP-10 can improve islet transplantation.

IP-10 is expressed in islet beta cells in response to stress-induced calcineurin and MAPK signaling. The results from mouse islet transplant experiments suggested that targeting IP-10 expression by stressed islets may also provide a point of intervention to preserve islet graft function. To identify stress-mediated signaling responses that may induce expression of IP-10 in islets during transplantation procedures, we treated human islets *in vitro* with proinflammatory CC as well as hypoxia and hydrogen peroxide to generate oxidative stress. Exposure to cytokines resulted in a large induction of IP-10, primarily in insulin-producing pancreatic beta cells within isolated islets treated for 24 h before fixation and immunofluorescent antibody staining (Fig. 3A). Further analysis by ELISA indicated that IL-1β produced the largest effect on IP-10 protein expression in human islets compared to TNF-α, IFN-γ, hypoxia, and H_2O_2 within 6 h exposure (Fig. 3B). In each case, induction of IP-10 protein was suppressed by the selective protein phosphatase 2B calcineurin inhibitor FK506 (1 μM). This suggests that inflammatory and oxidative stress induces IP-10 expression in islets by calcineurin-dependent signaling pathways.

We further investigated requirements of MAPKs ERK1/2, p38 and JNK to induce IP-10 by the use of selective MAPK signaling inhibitors U0126 (10 μM), SB203580 (10 μM), and SP600125 (10 μM), respectively. Whereas p38 and JNK inhibitors prevented induction of IP-10

protein expression, inhibiting ERK1/2 signaling sustained, and in some cases, enhanced, the response with 6 h of IL-1β treatment (Fig. 3C). These results indicate that stress-activated MAPKs p38 and JNK have selective if not reciprocal roles in the induction of intracellular IP-10 protein expression in human islets in response to IL-1β.

We also examined conditions that could influence IP-10 release from stressed islets. We observed that human islets treated with IL-1β for 6 h had significantly increased rates of IP-10 release (>40 pmol/IEQ/h) when exposed to high glucose (Fig. 3D). The effect of glucose on release of IP-10 from islets was likely independent of insulin action, as exogenous insulin up to 100 nM could not directly stimulate IP-10 secretion alone or in combination with glucose ((Supplementary Fig. 1)). Glucose alone was not sufficient to induce IP-10 expression, but it was required for IP-10 release after 6 h induction with IL-1β. Moreover, glucose-stimulated IP-10 protein release was significantly reduced in human islets subjected to IL-1β in the presence of p38 and JNK but not ERK1/2 inhibitor. Notably, glucose-stimulated insulin secretion was not affected by MAPK inhibitors (Supplementary Fig. 2). Taken together, these data indicate that calcineurin signaling and MAPKs p38 and JNK are required for IP-10 expression and that glucose can stimulate its subsequent release from human islets. Importantly, the results demonstrate that IP-10 signaling pathways can be targeted for suppression without affecting beta-cell function.

Calcineurin and MAPKs regulate NFAT-mediated isletokine gene transcription in pancreatic beta cells. The previous experiments demonstrated that human islets could express IP-10 in response to stress-activated signaling pathways. To determine if islets expressed isletokines during islet transplantation, we transplanted isolated human islets into the kidney capsules of diabetic nude mice and removed the islet grafts by scalpel dissection after 6 h to

evaluate expression of isletokine genes. Gene expression assays showed significant induction of mRNA in human islet xenografts for all isletokines tested (Fig. 3E). In each case, isletokine mRNA was blocked or largely reduced by pretreatment of islets with calcineurin and MAPK p38 and JNK inhibitors. These results indicate that IP-10 and other isletokines are induced in human islets upon transplantation at the transcriptional level via a common mechanism involving calcineurin and MAPK signaling pathways. Similarly, IP-10 mRNA was induced in isolated human and mouse islets *in vitro* within 2 h of exposure to IL-1β and suppressed by both calcineurin and MAPK p38 and JNK inhibitors (Fig 4A and B). These responses were also observed in FACS-purified human beta cells and MIN6 beta-cell lines (Fig 4C and D). The results were further corroborated using a different set of highly selective MAPK inhibitors in MIN6 cells (Supplementary Fig. 3). Moreover, calcineurin and MAPKs p38 and JNK were required to induce IP-10 gene promoter activity in MIN6 cells as detected by luciferase promoter-reporter assays (Fig 4E). These experiments collectively demonstrate that IP-10 induction is regulated by calcineurin and MAPKs in pancreatic islet beta cells during islet transplantation and under conditions of inflammatory stress.

It is well established that calcineurin activates downstream target NFAT-family transcription factors to directly regulate cytokine genes in immune cells (29-39). Interfering with the ability of calcineurin to bind to and dephosphorylate NFAT-family member substrates prevents NFAT from regulating cytokine gene transcription. Thus, we used an RNAi knockdown approach to test the requirement of NFAT in the induction of IP-10 promoter activity in MIN6 cells. Overexpression of NFATc2 shRNA reduced induction of IP-10 promoter activity in MIN6 cells treated for 6 h with IL-1β compared to scrambled shRNA controls. Additionally, overexpression of a dominant negative NFAT (dnNFAT) PxIxIT motif to interfere with NFAT

interaction with calcineurin suppressed IL-1β -induced IP-10 promoter activity compared to an alanine-substituted AxAxAA mutant (dnNFATm) control (Fig. 5F). These results indicated that NFAT is an essential component for the activation of the IP-10 gene promoter by calcineurin in pancreatic beta cells.

To determine if NFAT directly interacts with the IP-10 gene in beta cells, we performed chromatin immunoprecipitation assays to examine the association of NFATc2 with the 5' flanking IP-10 gene promoter in MIN6. Time-course analyses indicated that the IP-10 promoter was enriched with NFATc2 up to 40 fold within 10 min of IL-1β exposure (Fig. 5A). The association of NFATc2 with the IP-10 promoter was largely sustained for up to 60 min. In contrast, FK506 abolished NFATc2 binding. MAPK inhibitors had little effect on acute (10 min) association of NFATc2 with the IP-10 gene, but showed a significant reduction in a sustained (60 min) response. Histone acetylase p300 showed a biphasic association with the IP-10 promoter. However, whereas ERK1/2 inhibition had no effect on p300 association, inhibitors of p38 and JNK prevented a sustained response. The biphasic kinetics of p300 was inverse to what was observed with HDAC3 association where p38 and JNK inhibition enhanced the response to IL-1β. This inverse relationship indicates competitive MAPK signaling requirements for p300 and HDAC3 to regulate the IP-10 promoter. The results also suggest that calcineurin signaling is required for relative changes of association of p300 and HDAC3 with the IP-10 promoter to occur.

MAPKs have previously been shown to translocate to the nucleus and associate with DNA-protein complexes within gene promoters (13,40,41). Thus, we also examined effects of IL-1β to induce MAPK association with the IP-10 promoter. Both p38 and JNK associated with the IP-10 promoter with similar kinetics as observed with p300 (Fig. 5B). This contrasted with

ERK1/2, which were transiently enriched within 10 min but sharply subsided by 60 min. In each case, FK506 prevented MAPKs from associating with the IP-10 promoter. MAPK inhibitors had little effect on acute accumulation of MAPKs on the promoter, but selectively inhibited late-phase responses. Together, these results suggest that calcineurin signaling is required for NFAT, p300, HDAC3, and MAPK association with the IP-10 promoter, and MAPK activity is required for sustained formation of the protein-DNA chromatin complex.

The transient association of transcription factors versus sustained complex formation on the DNA promoter may impart differences in transcriptional activity. We therefore performed nuclear run-on assays to measure transcriptional output of nuclei from MIN6 cells exposed to conditions to simulate transcriptional complexes described above. The results showed a calcineurin-dependent induction of IP-10 mRNA output for both 10 min and 60 min stimulations (Fig. 5C). MAPK inhibitors had no effect on 10 min output when all factors were observed on the gene promoter. However, inhibition of p38 and JNK resulted in diminished IP-10 mRNA output by 60 min. These conditions correlated with the loss of p300, p38, and JNK on the IP-10 promoter (Fig. 5A and B). U0126 had no effect on 60 min promoter output when ERK1/2 were absent, confirming that ERK1/2 are not required for IP-10 sustained p300 binding and IP-10 transcription (Fig. 5B and C). Overall, these data suggest that calcineurin and NFATc2 signaling is required for recruitment of enzymes p300 and MAPKs to regulate the IP-10 promoter and that p38 and JNK MAPK-selective activity is required for DNA-protein complex stability to promote a sustained transcriptional response.

Discussion

The current study reveals a role of islet-derived cytokines in mediating acute inflammatory damage to islet grafts during the early stages of islet transplantation. High levels of circulating

IP-10 observed in transplant patients within hours of islet infusion correlates with poor long-term outcomes in graft function. The source of IP-10 released from islets was confirmed in a mouse transplant model where beta-cell specific ablation of IP-10 in donor islets could significantly improve glucose tolerance in STZ-induced diabetic recipients compared to wild type donor islets. This was further highlighted by improvements in islet transplant outcomes by the use of anti-IP-10 mAb to neutralize IP-10 secreted from islets transplanted into diabetic mouse recipients. The acute detrimental effects of locally released IP-10 on transplanted islets is likely indirect as direct treatment of isolated islets with concentrations of to 100 ng/mL of recombinant IP-10 did not affect beta-cell function or apoptosis within 24 h exposure (Supplementary Fig. 4A and B).

These results provide proof-of-principle for the potential use of anti-cytokine therapy to protect islets during clinical islet transplantation procedures. Indeed, promising results have been observed with the use of anti-inflammatory recombinant protein antagonists to neutralize cytokines during islet transplantation. Our center has successfully used etanercept and anakinra to block TNF-α and IL-1β, respectively, to improve islet transplant outcomes (42). These observations were corroborated in a controlled mouse transplant study that demonstrated combined effects of etanercept and anakinra to improve islet cell function and engraftment in immunodeficient mice (43). Based on these results, it is conceivable that a combinatorial approach to targeting circulating inflammatory mediators such as IP-10, TNF-α, and IL-1β could have synergistic effects to improve islet transplant outcomes or reduce islet equivalent tissue required. The use of neutralizing protein antagonists to selectively target key isletokines released upon islet infusion to improve transplant outcomes warrants further exploration for clinical use.

The study also identifies upstream molecular targets to block isletokine production by islet beta cells. Both *in vitro* and *in vivo* results indicated that there are a set of common signaling components that are activated by oxidative or inflammatory stress, which induces expression of isletokine genes in beta cells. One common signaling component is the calcium-dependent calcineurin/NFAT pathway. The selective calcineurin inhibitor FK506 was shown to suppress multiple cytokines in human islet grafts transplanted in mice. Further analysis showed that the downstream transcriptional target of calcineurin, NFATc2, and histone acetylase p300 associated with the IP-10 promoter in response to IL-1β.

The other identified common signaling component involves stress-activated MAPKs. The activity of chromatin-bound p38 and JNK promoted the association of both NFATc2 and p300 while opposing the accumulation of HDAC3. Transcriptional activity of IP-10 in beta cells correlated closely with kinetics of promoter-bound NFATc2 and p300, indicating that p38 and JNK were required for gene expression. ERK1/2, on the other hand, had little effect on the IP-10 gene promoter, which highlights a kinase-selective role of stress-activated p38 and JNK in islet inflammation. Together, these results indicate that multiple isletokines expressed during islet transplantation can be blocked by perturbing calcineurin/NFATc2 and p38/JNK signaling.

It is well established that NFAT interacts with basic helix-loop-helix (bZIP) family proteins to regulate cytokine gene promoters (44-50). These bZIP proteins can enhance or repress promoter activity, depending on the cell signaling and gene promoter context. We previously showed that NFAT requires bZIPs c-Jun and ATF2 to activate the TNF-α promoter in beta cells when exposed to high glucose and IL-1β (5). However, under normal conditions, beta-cell specific bZIP MafA interacts with NFAT to both regulate the insulin gene in response to glucose conditions and maintain the TNF-α gene in a silenced state (5,6). Further studies are

required to determine specific NFATc2-interacting cofactors that bind to and regulate IP-10 and other isletokine promoters in beta cells during islet cell transplantation.

Lastly, proinflammatory cytokines are systemically elevated during the progression of type 1 as well as type 2 diabetes. Although inflammatory cells largely contribute to this inflammatory state, the role of isletokines in the context of diabetes has not been fully explored. Considering that islets can evoke an inflammatory response when stressed and transplanted, it is conceivable that beta cells could contribute to inflammatory responses during a metabolic or inflammatory disease state. Thus, beta cells could play a role in the initiation or exacerbation of their own immune destruction by release of isletokines when damaged or distressed. In this broader context, targeting isletokines may have potential to prevent or delay progression of metabolic diseases propagated by islet inflammation and beta-cell targeted immune destruction.

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guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References

1. Chen MC, Schuit F, Eizirik DL: Identification of IL-1beta-induced messenger RNAs in rat pancreatic beta cells by differential display of messenger RNA. Diabetologia 1999;42:1199-1203

2. Cardozo AK, Kruhoffer M, Leeman R, Orntoft T, Eizirik DL: Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. Diabetes 2001;50:909-920

3. Kutlu B, Cardozo AK, Darville MI, Kruhoffer M, Magnusson N, Orntoft T, Eizirik DL: Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells. Diabetes 2003;52:2701-2719

4. Jacobsen ML, Ronn SG, Bruun C, Larsen CM, Eizirik DL, Mandrup-Poulsen T, Billestrup N: IL-1beta-induced chemokine and Fas expression are inhibited by suppressor of cytokine signalling-3 in insulin-producing cells. Diabetologia 2009;52:281-288

5. Lawrence MC, Naziruddin B, Levy MF, Jackson A, McGlynn K: Calcineurin/nuclear factor of activated T cells and MAPK signaling induce TNF-alpha gene expression in pancreatic islet endocrine cells. The Journal of biological chemistry 2011;286:1025-1036

6. Lawrence MC, Borenstein-Auerbach N, McGlynn K, Kunnathodi F, Shahbazov R, Syed I, Kanak M, Takita M, Levy MF, Naziruddin B: NFAT targets signaling molecules to gene promoters in pancreatic beta-cells. Molecular endocrinology 2015;29:274-288

7. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY: Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. The Journal of clinical investigation 2002;110:851-860

8. Cardozo AK, Proost P, Gysemans C, Chen MC, Mathieu C, Eizirik DL: IL-1beta and IFN-gamma induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice. Diabetologia 2003;46:255-266

9. Taylor-Fishwick DA, Weaver JR, Grzesik W, Chakrabarti S, Green-Mitchell S, Imai Y, Kuhn N, Nadler JL: Production and function of IL-12 in islets and beta cells. Diabetologia 2013;56:126-135

10. Lopes M, Kutlu B, Miani M, Bang-Berthelsen CH, Storling J, Pociot F, Goodman N, Hood L, Welsh N, Bontempi G, Eizirik DL: Temporal profiling of cytokine-induced genes in pancreatic beta-cells by meta-analysis and network inference. Genomics 2014;103:264-275

11. Comens PG, Wolf BA, Unanue ER, Lacy PE, McDaniel ML: Interleukin 1 is potent modulator of insulin secretion from isolated rat islets of Langerhans. Diabetes 1987;36:963-970

12. Maedler K, Schumann DM, Sauter N, Ellingsgaard H, Bosco D, Baertschiger R, Iwakura Y, Oberholzer J, Wollheim CB, Gauthier BR, Donath MY: Low concentration of interleukin-1beta induces FLICE-inhibitory protein-mediated beta-cell proliferation in human pancreatic islets. Diabetes 2006;55:2713-2722

13. Lawrence MC, Shao C, McGlynn K, Naziruddin B, Levy MF, Cobb MH: Multiple chromatin-bound protein kinases assemble factors that regulate insulin gene transcription. Proceedings of the National Academy of Sciences of the United States of America 2009;106:22181-22186

14. Roep BO, Kleijwegt FS, van Halteren AG, Bonato V, Boggi U, Vendrame F, Marchetti P, Dotta F: Islet inflammation and CXCL10 in recent-onset type 1 diabetes. Clinical and experimental immunology 2010;159:338-343

15. Arous C, Ferreira PG, Dermitzakis ET, Halban PA: Short term exposure of beta cells to low concentrations of interleukin-1beta improves insulin secretion through focal adhesion and actin remodeling and regulation of gene expression. The Journal of biological chemistry 2015;290:14491

16. Hajmrle C, Smith N, Spigelman AF, Dai X, Senior L, Bautista A, Ferdaoussi M, MacDonald PE: Interleukin-1 signaling contributes to acute islet compensation. JCI insight 2016;1:e86055

17. Ortis F, Pirot P, Naamane N, Kreins AY, Rasschaert J, Moore F, Theatre E, Verhaeghe C, Magnusson NE, Chariot A, Orntoft TF, Eizirik DL: Induction of nuclear factor-kappaB and its downstream genes by TNF-alpha and IL-1beta has a pro-apoptotic role in pancreatic beta cells. Diabetologia 2008;51:1213-1225

18. Gurzov EN, Ortis F, Cunha DA, Gosset G, Li M, Cardozo AK, Eizirik DL: Signaling by IL-1beta+IFN-gamma and ER stress converge on DP5/Hrk activation: a novel mechanism for pancreatic beta-cell apoptosis. Cell Death Differ 2009;16:1539-1550

19. Hasnain SZ, Borg DJ, Harcourt BE, Tong H, Sheng YH, Ng CP, Das I, Wang R, Chen AC, Loudovaris T, Kay TW, Thomas HE, Whitehead JP, Forbes JM, Prins JB, McGuckin MA:

Glycemic control in diabetes is restored by therapeutic manipulation of cytokines that regulate beta cell stress. Nature medicine 2014;20:1417-1426

20. Gorasia DG, Dudek NL, Veith PD, Shankar R, Safavi-Hemami H, Williamson NA, Reynolds EC, Hubbard MJ, Purcell AW: Pancreatic beta cells are highly susceptible to oxidative and ER stresses during the development of diabetes. Journal of proteome research 2015;14:688-699

21. Paraskevas S, Aikin R, Maysinger D, Lakey JR, Cavanagh TJ, Hering B, Wang R, Rosenberg L: Activation and expression of ERK, JNK, and p38 MAP-kinases in isolated islets of Langerhans: implications for cultured islet survival. FEBS Lett 1999;455:203-208

22. Nishiki Y, Adewola A, Hatanaka M, Templin AT, Maier B, Mirmira RG: Translational control of inducible nitric oxide synthase by p38 MAPK in islet beta-cells. Molecular endocrinology 2013;27:336-349

23. Noguchi H, Nakai Y, Ueda M, Masui Y, Futaki S, Kobayashi N, Hayashi S, Matsumoto S: Activation of c-Jun NH2-terminal kinase (JNK) pathway during islet transplantation and prevention of islet graft loss by intraportal injection of JNK inhibitor. Diabetologia 2007;50:612-619

24. Fukuda K, Tesch GH, Yap FY, Forbes JM, Flavell RA, Davis RJ, Nikolic-Paterson DJ: MKK3 signalling plays an essential role in leukocyte-mediated pancreatic injury in the multiple low-dose streptozotocin model. Laboratory investigation; a journal of technical methods and pathology 2008;88:398-407

25. Abdelli S, Ansite J, Roduit R, Borsello T, Matsumoto I, Sawada T, Allaman-Pillet N, Henry H, Beckmann JS, Hering BJ, Bonny C: Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes 2004;53:2815-2823

26. Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O: Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. Upsala journal of medical sciences 2000;105:125-133

27. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B: Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. Diabetes 2002;51:1779-1784

28. Chow CW, Rincon M, Davis RJ: Requirement for transcription factor NFAT in interleukin-2 expression. Molecular and cellular biology 1999;19:2300-2307

29. Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR: Identification of a putative regulator of early T cell activation genes. Science 1988;241:202-205

30. Emmel EA, Verweij CL, Durand DB, Higgins KM, Lacy E, Crabtree GR: Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science 1989;246:1617-1620

31. Bierer BE, Mattila PS, Standaert RF, Herzenberg LA, Burakoff SJ, Crabtree G, Schreiber SL: Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. Proceedings of the National Academy of Sciences of the United States of America 1990;87:9231-9235

32. Flanagan WM, Corthesy B, Bram RJ, Crabtree GR: Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 1991;352:803-807

33. Clipstone NA, Crabtree GR: Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 1992;357:695-697

34. Cockerill PN, Shannon MF, Bert AG, Ryan GR, Vadas MA: The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. Proceedings of the National Academy of Sciences of the United States of America 1993;90:2466-2470

35. Park J, Yaseen NR, Hogan PG, Rao A, Sharma S: Phosphorylation of the transcription factor NFATp inhibits its DNA binding activity in cyclosporin A-treated human B and T cells. The Journal of biological chemistry 1995;270:20653-20659

36. Wesselborg S, Fruman DA, Sagoo JK, Bierer BE, Burakoff SJ: Identification of a physical interaction between calcineurin and nuclear factor of activated T cells (NFATp). The Journal of biological chemistry 1996;271:1274-1277

37. Loh C, Shaw KT, Carew J, Viola JP, Luo C, Perrino BA, Rao A: Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. The Journal of biological chemistry 1996;271:10884-10891

38. Luo C, Burgeon E, Carew JA, McCaffrey PG, Badalian TM, Lane WS, Hogan PG, Rao A: Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. Molecular and cellular biology 1996;16:3955-3966

39. Okamura H, Aramburu J, Garcia-Rodriguez C, Viola JP, Raghavan A, Tahiliani M, Zhang X, Qin J, Hogan PG, Rao A: Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Molecular cell 2000;6:539-550

40. Lawrence MC, McGlynn K, Shao C, Duan L, Naziruddin B, Levy MF, Cobb MH: Chromatin-bound mitogen-activated protein kinases transmit dynamic signals in transcription complexes in beta-cells. Proceedings of the National Academy of Sciences of the United States of America 2008;105:13315-13320

41. Nelson JD, LeBoeuf RC, Bomsztyk K: Direct recruitment of insulin receptor and ERK signaling cascade to insulin-inducible gene loci. Diabetes 2011;60:127-137

42. Matsumoto S, Takita M, Chaussabel D, Noguchi H, Shimoda M, Sugimoto K, Itoh T, Chujo D, SoRelle J, Onaca N, Naziruddin B, Levy MF: Improving efficacy of clinical islet transplantation with iodixanol-based islet purification, thymoglobulin induction, and blockage of IL-1beta and TNF-alpha. Cell transplantation 2011;20:1641-1647

43. McCall M, Pawlick R, Kin T, Shapiro AM: Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in immunodeficient mice. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2012;12:322-329

44. Jain J, McCaffrey PG, Miner Z, Kerppola TK, Lambert JN, Verdine GL, Curran T, Rao A: The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature 1993;365:352-355

45. Northrop JP, Ullman KS, Crabtree GR: Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. The Journal of biological chemistry 1993;268:2917-2923

46. Tsai EY, Jain J, Pesavento PA, Rao A, Goldfeld AE: Tumor necrosis factor alpha gene regulation in activated T cells involves ATF-2/Jun and NFATp. Molecular and cellular biology 1996;16:459-467

47. Sun LJ, Peterson BR, Verdine GL: Dual role of the nuclear factor of activated T cells insert region in DNA recognition and cooperative contacts to activator protein 1. Proceedings of the National Academy of Sciences of the United States of America 1997;94:4919-4924

48. Yang TT, Chow CW: Transcription cooperation by NFAT.C/EBP composite enhancer complex. The Journal of biological chemistry 2003;278:15874-15885

49. Falvo JV, Lin CH, Tsytsykova AV, Hwang PK, Thanos D, Goldfeld AE, Maniatis T: A dimer-specific function of the transcription factor NFATp. Proceedings of the National Academy of Sciences of the United States of America 2008;105:19637-19642

50. Walters RD, Drullinger LF, Kugel JF, Goodrich JA: NFATc2 recruits cJun homodimers to an NFAT site to synergistically activate interleukin-2 transcription. Molecular immunology 2013;56:48-56

Figure Legends

FIGURE 1. Analysis of inflammatory mediators released from human islets. (A) Screening of inflammatory mediators present in patient blood serum samples (n=34) after islet transplantation. (B) Release of inflammatory mediators from isolated human islets upon 2 h exposure to cytokine cocktail (CC). (C) Fold release of inflammatory mediators from isolated human islets exposed to CC. (D) IP-10 serum levels detected in patients undergoing autologous islet transplantation. Data is represented by $AUC_{0\text{-}24h}$ in groups of low (< 2 pg·h/IEQ/kg, n=10),

medium (2-4 pg·h/IEQ/kg, n=9), and high (>4 pg·h/IEQ/kg, n=7). (E): Correlation of IP-10 levels released in blood circulation within 24 h of islet transplantation (AUC_{0-24h}) to C-peptide output (pg/mL) as a measure of graft function. Asterisk denotes statistical significance (*$p < 0.05$) in mean values based on a two-way ANOVA and Tukey's multiple comparison test. Data shown are representative results from three independent experiments using three replicate assays for human islet and patient samples.

FIGURE 2. Effect of IP-10 gene deletion and antibody neutralization on islet transplant outcomes (A) Blood glucose profiles (left) and intraperitoneal glucose tolerance tests (IPGTT) (right) comparing STZ-diabetic recipients of wild type (WT) and IP-10 knockout ($IP10^{-/-}$) donor islets up to 30 d post transplantation. (B) Transplant outcomes comparing blood glucose profiles (left) and rate of achieving normal glucose (<200 mg/dL) (right) between STZ-diabetic recipients of WT donor islet preparations with IP-10 mAb or IgG1K isotype mAb control. (C) IPGTT time course (left) and AUC_{0-2h} (right) comparing IP-10 mAb treated mice and controls 30 d post transplantation. Asterisks denote statistical significance (*$p < 0.05$, **$p < 0.01$) in mean values compared to controls based on two-tailed Student's t-test. Data shown are representative of results from at least three independent experiments using three replicate assays with 4-7 mice per treatment group.

FIGURE 3. Expression and release of IP-10 by human islets in response to stress signaling. (A) Immunofluorescent co-staining of IP-10 and insulin in isolated human islets exposed to CC for 24 h with 5x magnification inset. (B) Induction of IP-10 protein in response to 6 h treatment with hypoxia, H_2O_2, IL-1β, TNF-α, and IFN-γ in the presence of DMSO control (left) and FK506 (right). (C) Effect of MAPK inhibitors U0126, SB203580, and SP600125 on IL-1β-induced IP-10 protein expression. (D) Glucose stimulated release of IP-10 protein from islets pretreated with

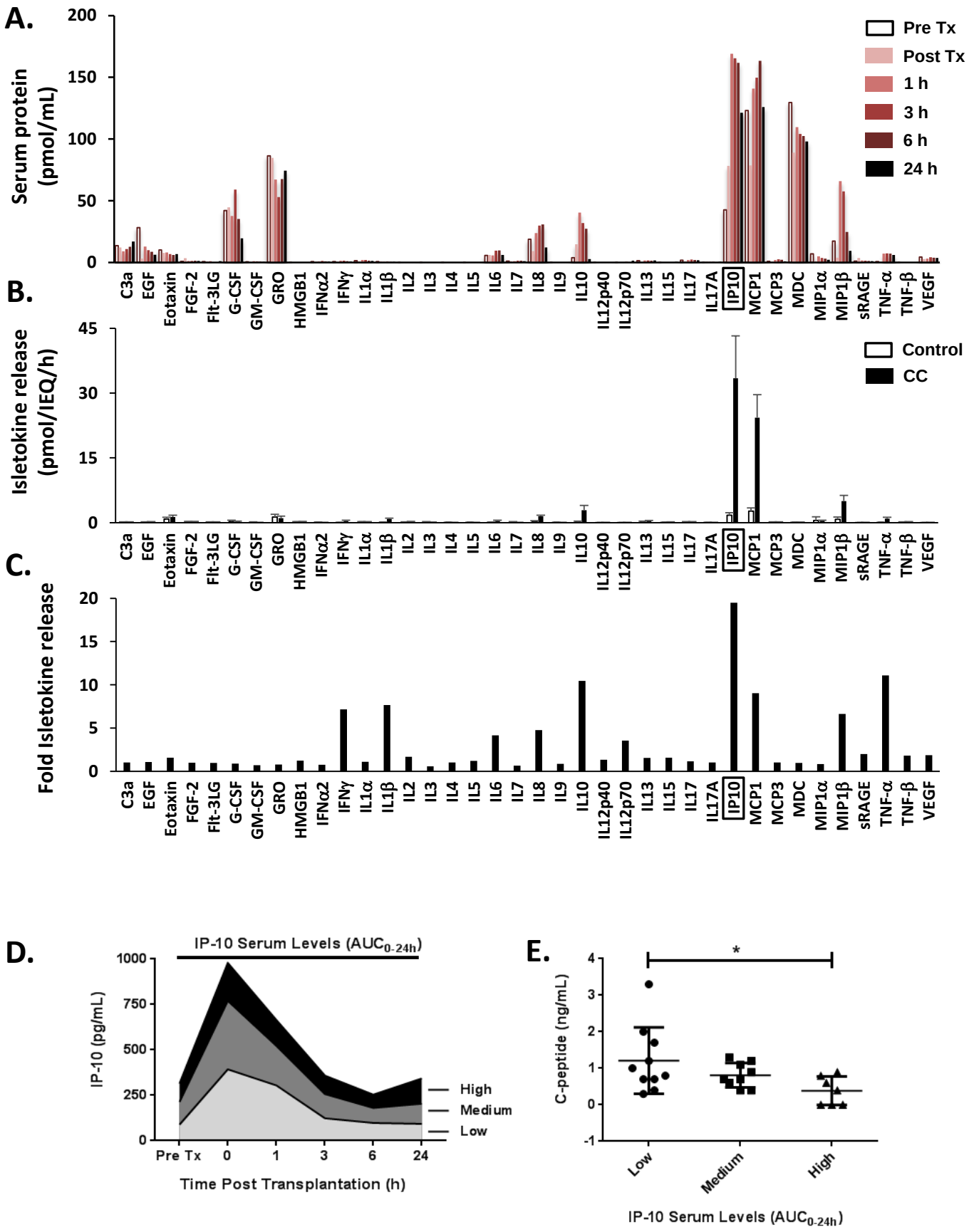
IL-1β and inhibitors or DMSO control. (E) Induction of isletokine mRNA in human islet grafts transplanted into the mice treated with inhibitors or DMSO vehicle control relative to untransplanted islets. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) in mean values for treatments compared to untreated control (* above bar) or condition indicated by lines (* above line) as determined by (B and E) Student's t-test and two-way ANOVA with (C) Dunnett's and (D) Sidak's multiple comparison test. Data shown are representative of results from at least three independent experiments using three replicate assays.

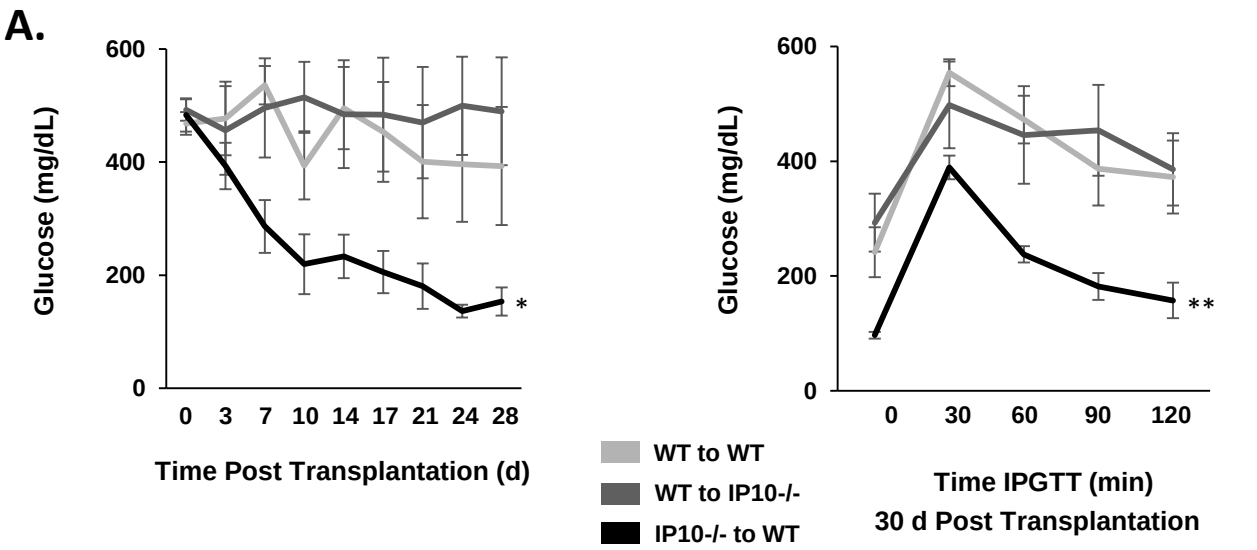
FIGURE 4. Induction of the IP-10 gene by MAPK and NFAT signaling in pancreatic beta cells. (A) Fold induction of IP-10 mRNA expression after 2 h exposure to IL-1β in (A) human islets, (B) mouse islets, (C) purified human beta cells, and (D) MIN6 beta-cell line. Induction of the mouse IP-10 gene promoter in MIN6 cells and blockade by (E) calcineurin and MAPK pharmacological inhibitors and (F) NFAT shRNAi knockdown and dominant negative (dn) NFAT. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) in mean values for treatments compared to untreated control (* above bar) or condition indicated by lines (* above line) as determined by two-way ANOVA and Dunnett's multiple comparison test. Data shown are representative of results from at least three independent experiments using three replicate assays.

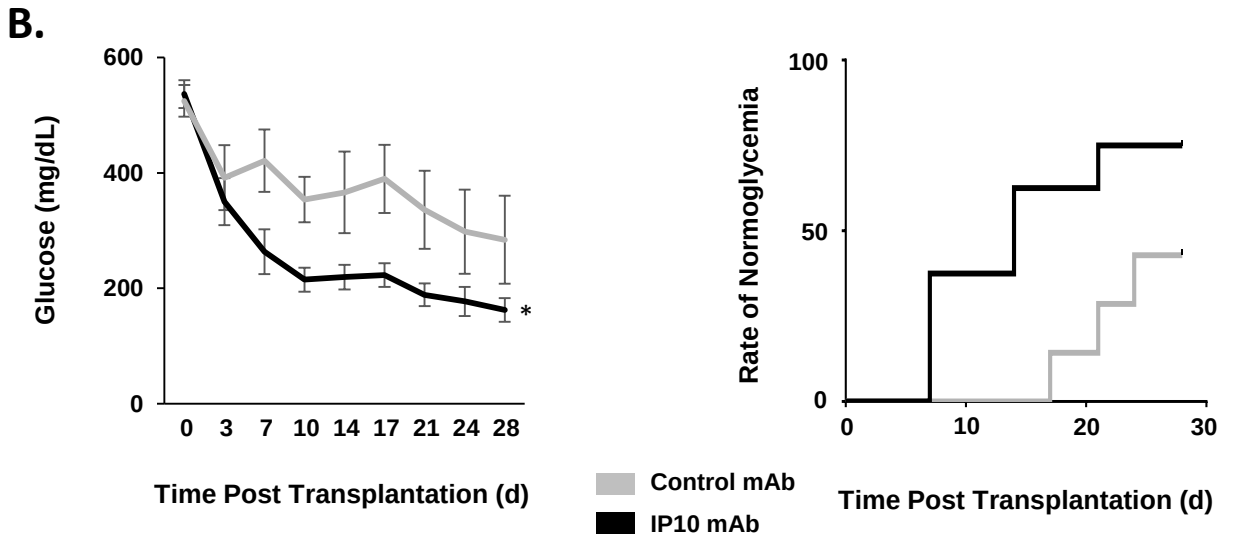
FIGURE 5. Signaling requirements for interaction of NFAT and associated factors with the IP-10 gene promoter. Chromatin immunoprecipitation timecourse analysis of promoter association of (A) NFAT, p300, and HDAC3 and (B) MAPKs p38, JNK, and ERK1/2 with the IP-10 promoter in MIN6 cells exposed to IL-1β in the presence of calcineurin and MAPK pharmacological inhibitors. (C) Nuclear run on assay of IP-10 mRNA released from nuclei isolated from MIN6 exposed to IL-1β for 10 min (left) and 60 min (right) in the presence of

calcineurin and MAPK pharmacological inhibitors. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) in mean values for treatments compared to untreated control (* above bar) or condition indicated by lines (* above line) as determined by two-way ANOVA with (A and B) Dunnett's and (C) Tukey's multiple comparison test. Data shown are representative of results from three independent experiments using (A and B) two or (C) three replicate assays.

Fig. 1


A.
Serum protein (pmol/mL)
200
150
100
50
0
Pre Tx
Post Tx
1 h
3 h
6 h
24 h
B.
Isletokine release (pmol/IEQ/h)
45
30
15
0
Control
CC
C.
Fold Isletokine release
20
15
10
5
0
C3a EGF Eotaxin FGF-2 Flt-3LG G-CSF GM-CSF GRO HMGB1 IFNα2 IFNγ IL1α IL1β IL2 IL3 IL4 IL5 IL6 IL7 IL8 IL9 IL10 IL12p40 IL12p70 IL13 IL15 IL17 IL17A IP10 MCP1 MCP3 MDC MIP1α MIP1β sRAGE TNF-α TNF-β VEGF
D.
IP-10 Serum Levels (AUC0-24h)
IP-10 (pg/mL)
1000
750
500
250
0
High
Medium
Low
Pre Tx 0 1 3 6 24
Time Post Transplantation (h)
E.
*
C-peptide (ng/mL)
4
3
2
1
0
-1
Low
Medium
High
IP-10 Serum Levels (AUC0-24h)


A.
Glucose (mg/dL)
600
400
200
0
0 3 7 10 14 17 21 24 28
Time Post Transplantation (d)
*
Glucose (mg/dL)
600
400
200
0
0 30 60 90 120
Time IPGTT (min)
30 d Post Transplantation
**
WT to WT
WT to IP10-/-
IP10-/- to WT


B.
Glucose (mg/dL)
600
400
200
0
0 3 7 10 14 17 21 24 28
Time Post Transplantation (d)
*
Rate of Normoglycemia
100
50
0
0 10 20 30
Time Post Transplantation (d)
Control mAb
IP10 mAb

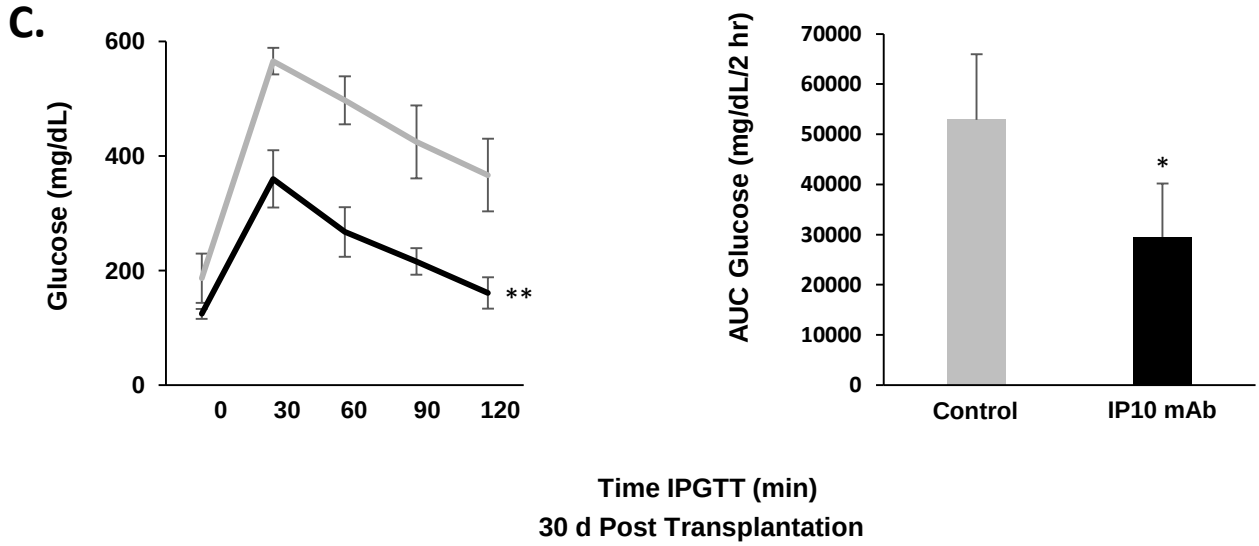
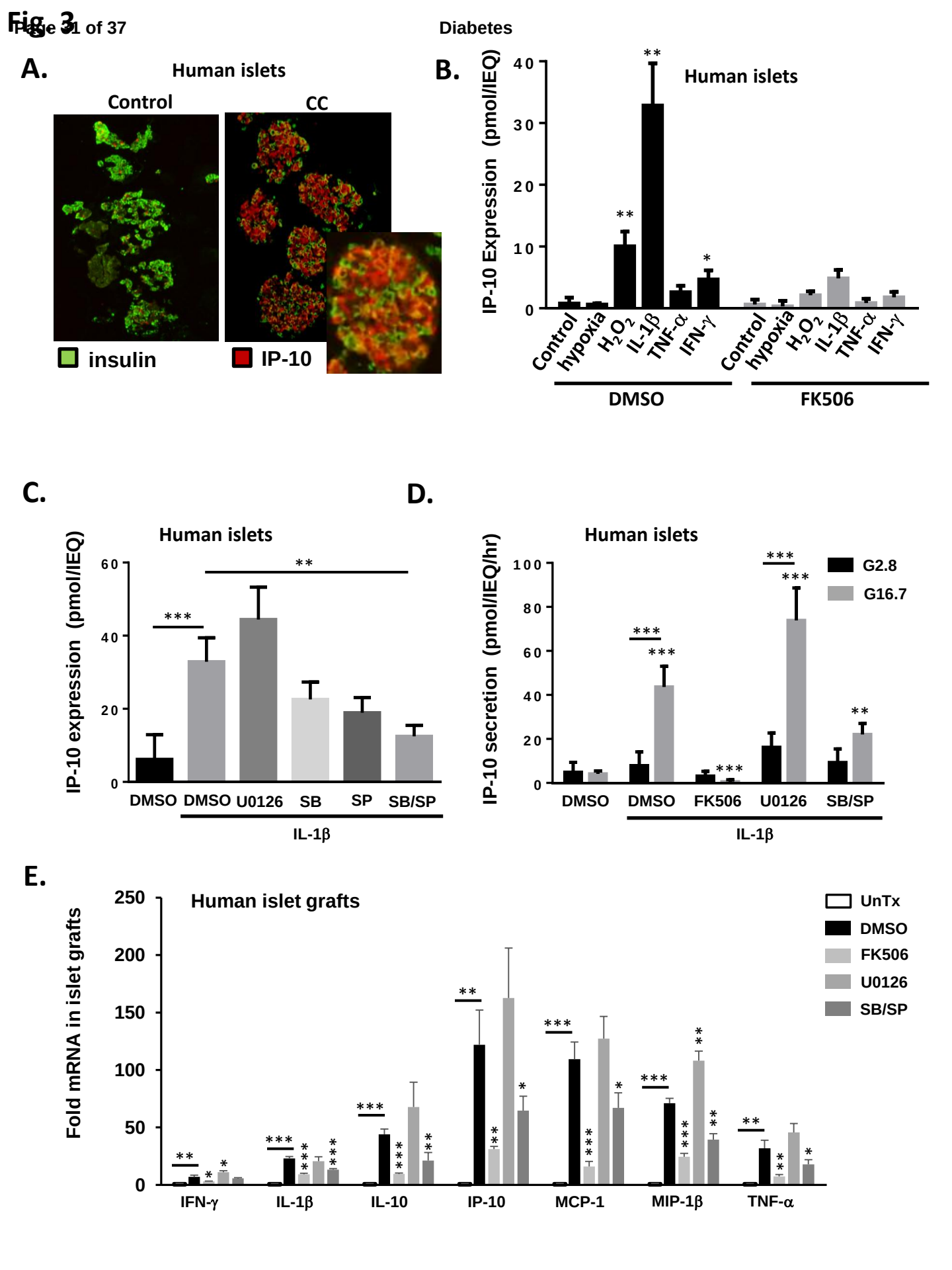

C.
Glucose (mg/dL)
600
400
200
0
0 30 60 90 120
**
AUC Glucose (mg/dL/2 hr)
70000
60000
50000
40000
30000
20000
10000
0
*
Control
IP10 mAb
Time IPGTT (min)
30 d Post Transplantation

Fig. 3


A.
Human islets
Control
CC
insulin
IP-10
B.
Human islets
IP-10 Expression (pmol/IEQ)
Control
hypoxia
H2O2
IL-1β
TNF-α
IFN-γ
DMSO
FK506
C.
Human islets
IP-10 expression (pmol/IEQ)
DMSO
DMSO
U0126
SB
SP
SB/SP
IL-1β
D.
Human islets
IP-10 secretion (pmol/IEQ/hr)
G2.8
G16.7
DMSO
DMSO
FK506
U0126
SB/SP
IL-1β
E.
Human islet grafts
Fold mRNA in islet grafts
UnTx
DMSO
FK506
U0126
SB/SP
IFN-γ
IL-1β
IL-10
IP-10
MCP-1
MIP-1β
TNF-α

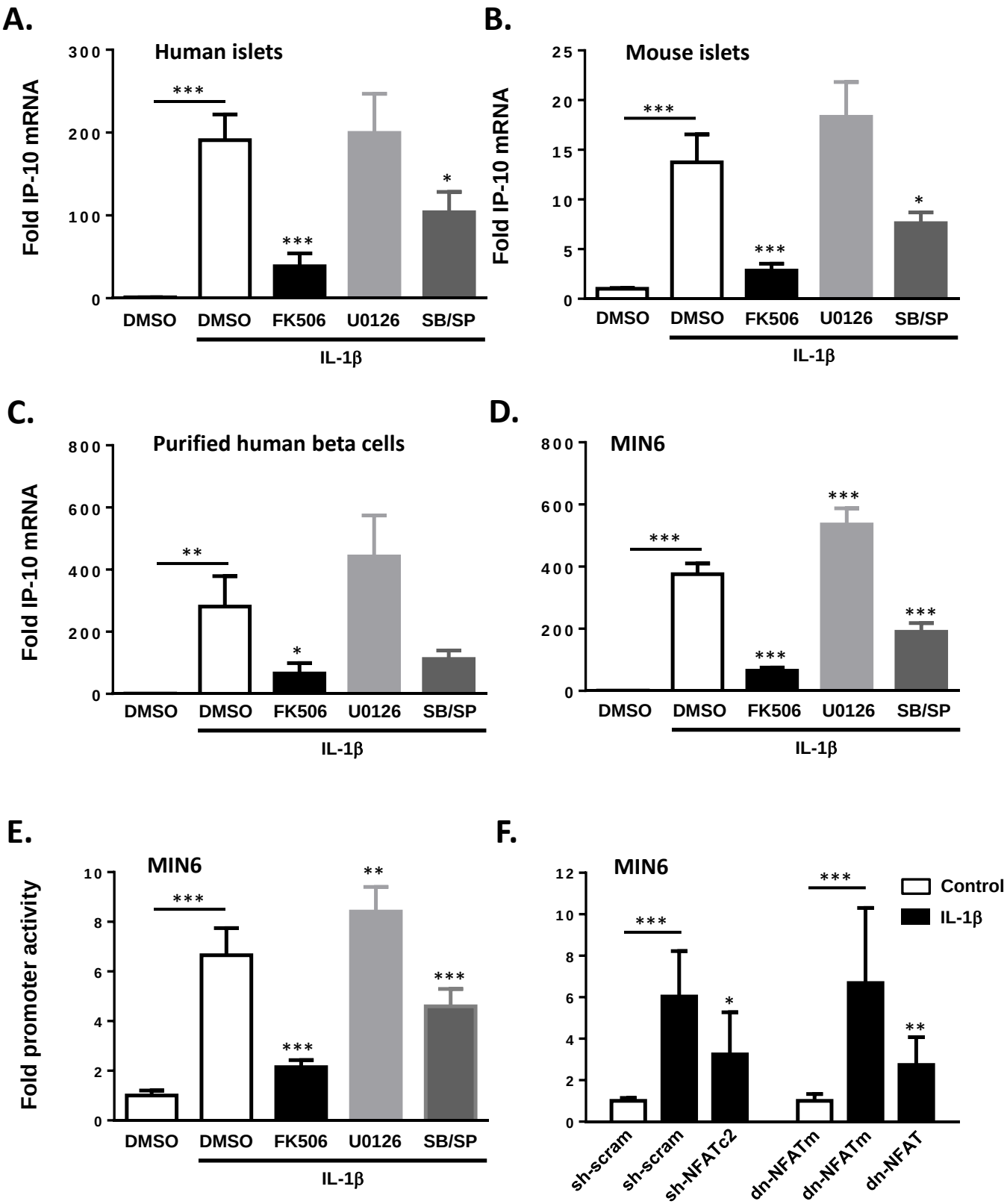
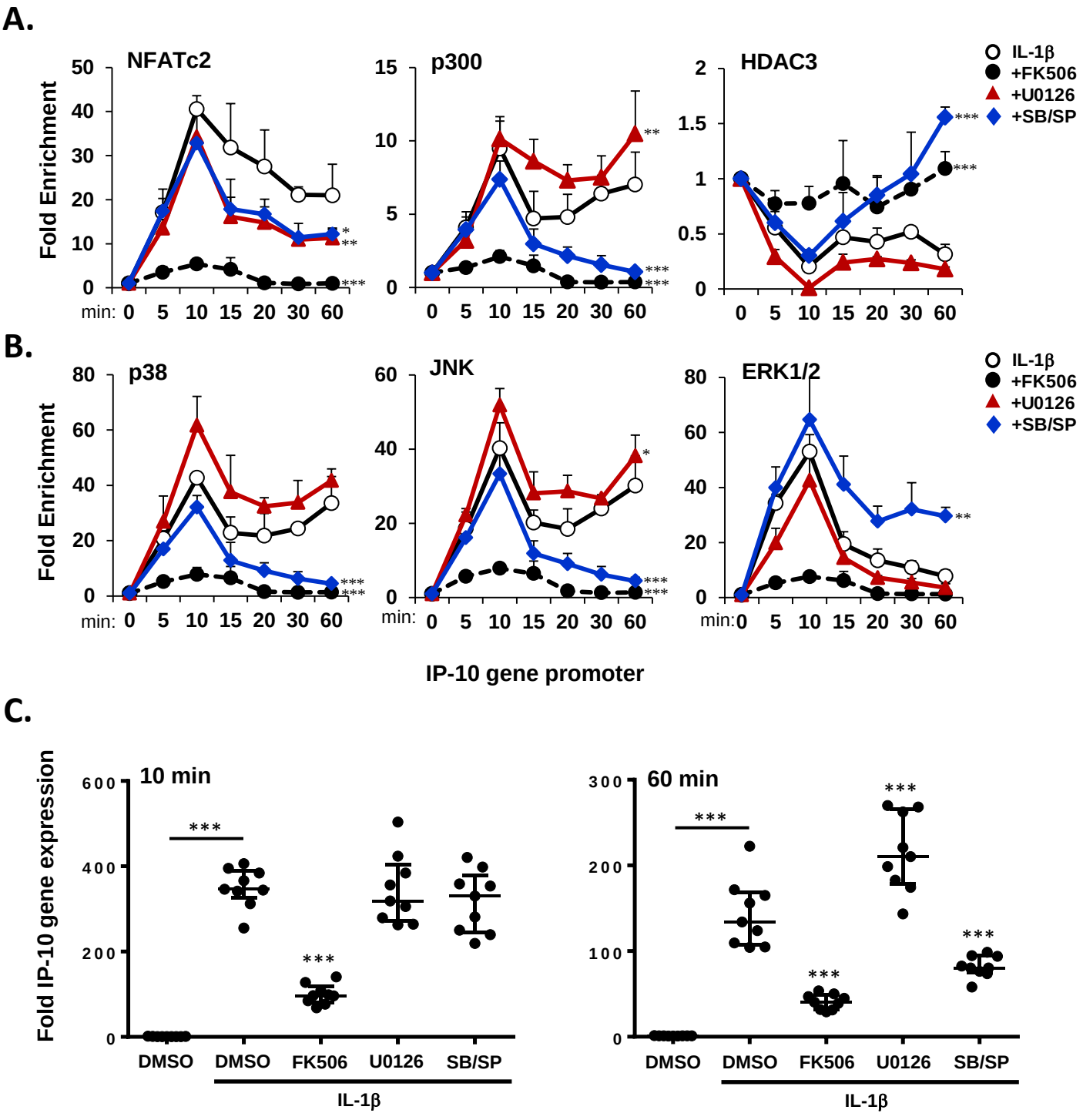
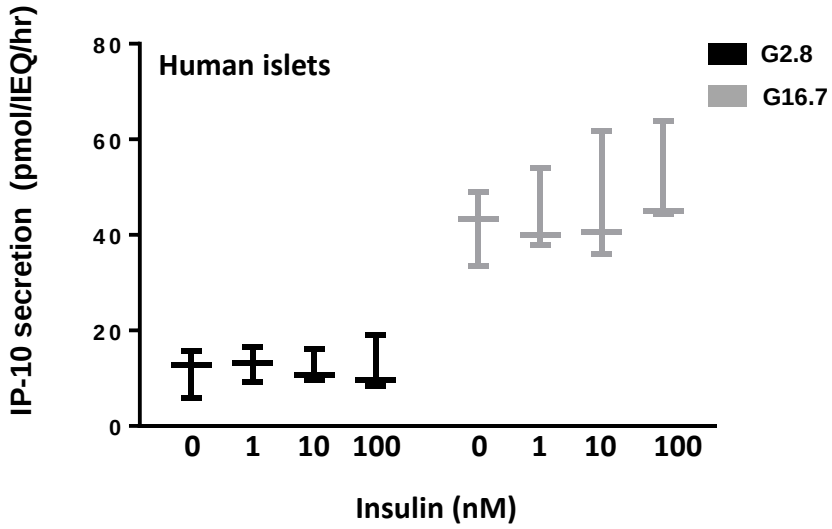

A.
Human islets
Fold IP-10 mRNA
300
200
100
0
***
***
*
DMSO
DMSO
FK506
U0126
SB/SP
IL-1β
B.
Mouse islets
Fold IP-10 mRNA
25
20
15
10
5
0
***
***
*
DMSO
DMSO
FK506
U0126
SB/SP
IL-1β
C.
Purified human beta cells
Fold IP-10 mRNA
800
600
400
200
0
**
*
DMSO
DMSO
FK506
U0126
SB/SP
IL-1β
D.
MIN6
800
600
400
200
0
***
***
***
***
DMSO
DMSO
FK506
U0126
SB/SP
IL-1β
E.
MIN6
Fold promoter activity
10
8
6
4
2
0
***
***
**
***
DMSO
DMSO
FK506
U0126
SB/SP
IL-1β
F.
MIN6
12
10
8
6
4
2
0
***
***
*
***
**
Control
IL-1β
sh-scram
sh-scram
sh-NFATc2
dn-NFATm
dn-NFATm
dn-NFAT

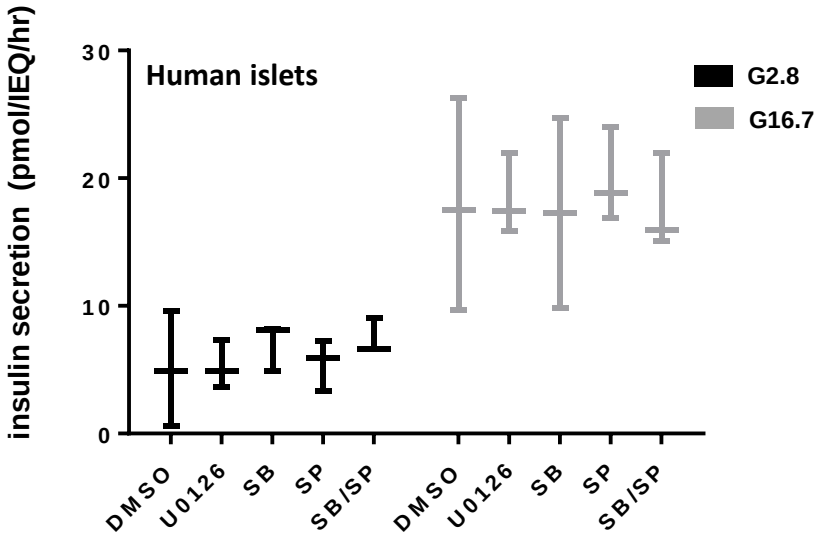
Fig. 5


A.
NFATc2
p300
HDAC3
IL-1β
+FK506
+U0126
+SB/SP
Fold Enrichment
min: 0 5 10 15 20 30 60
B.
p38
JNK
ERK1/2
IP-10 gene promoter
C.
10 min
60 min
Fold IP-10 gene expression
DMSO
DMSO
FK506
U0126
SB/SP
IL-1β

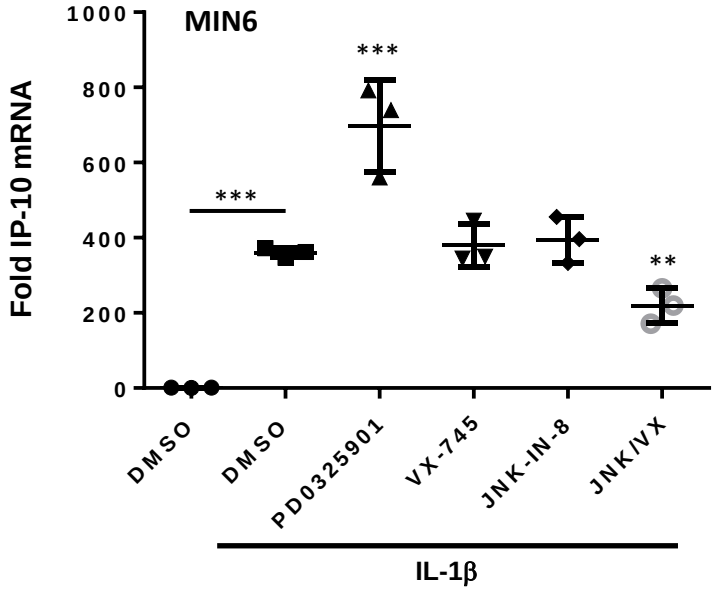
Supplementary Figure 1. Effect of insulin on IP-10 release in human islets. Isolated human islets were pretreated with IL-1β for 6 h before exposure to 1-100 nM recombinant insulin in the presence of low 2.8 mM and high 16.7 mM glucose and assayed for IP-10 release by ELISA. Data are represented as mean values with STD of 3 independent experiments.



Supplementary Figure 2. Effect of MAPK inhibitors on insulin secretion. Isolated human islets were pretreated for 15 min with 10 µM MAP inhibitors and placed in low 2.8 mM and high 16.7 mM glucose medium for static incubation for 2 h and assayed for insulin release by ELISA. Data are represented as mean values with STD of 3 independent experiments.



Supplementary Figure 3. Effect of MAPK inhibitors on IP10 gene expression. MIN6 cells were pretreated for 15 min with 1 μM ERK inhibitor PD0325901, p38 inhibitor VX-745, JNK inhibitor JNK-IN-8 , or combination of JNK and p38 inhibitors (JNK/VX) and placed in low 2.8 mM and high 16.7 mM glucose medium for 2 h. Cells were harvested for mRNA and converted to cDNA to be assayed for gene expression by QPCR. Asterisks denote statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001) in mean values compared to untreated control (* above bar) or condition indicated by lines (* above line) based on two-tailed Student's t-test. Data are represented as individual plots of means of replicates and mean values of 3 independent experiments are indicated by bars with STD.



Supplementary Figure 4. Effect of IP-10 on insulin secretion and apoptosis in islets and beta cells. (A) Isolated human islets were treated with 1-100 nM recombinant IP-10 in static incubation with low 2.8 mM and high 16.7 mM glucose for 2 h and assayed for insulin release by ELISA. (B) MIN6 cells were treated with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 50 ng/mL IFN-γ cytokines (CC) or 100 ng/mL for 24 h and assayed for apoptosis by Promega Caspase-Glo 3/7 assay. Asterisks denote statistical significance (*p < 0.05) in mean values based on one-way ANOVA. Data are represented as mean values with STD of 3 independent experiments.