Disruption of Hepatocyte Growth Factor/c-Met Signaling Enhances Pancreatic β-Cell Death and Accelerates the Onset of Diabetes

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OBJECTIVE—To determine the role of hepatocyte growth factor (HGF)/c-Met on β-cell survival in diabetogenic conditions in vivo and in response to cytokines in vitro.

RESEARCH DESIGN AND METHODS—We generated pancreas-specific c-Met-null (PancMet KO) mice and characterized their response to diabetes induced by multiple low-dose streptozotocin (MLDS) administration. We also analyzed the effect of HGF/c-Met signaling in vitro on cytokine-induced β-cell death in mouse and human islets, specifically examining the role of nuclear factor (NF)-κB.

RESULTS—Islets exposed in vitro to cytokines or from MLDS-treated mice displayed significantly increased HGF and c-Met levels, suggesting a potential role for HGF/c-Met in β-cell survival against diabetogenic agents. Adult PancMet KO mice displayed normal glucose and β-cell homeostasis, indicating that pancreatic c-Met loss is not detrimental for β-cell growth and function under basal conditions. However, PancMet KO mice were more susceptible to MLDS-induced diabetes. They displayed higher blood glucose levels, marked hypoinsulinemia, and reduced β-cell mass compared with wild-type littermates. PancMet KO mice showed enhanced intraislet infiltration, islet nitric oxide (NO) and chemokine production, and β-cell apoptosis. c-Met-null β-cells were more sensitive to cytokine-induced cell death in vitro, an effect mediated by NF-κB activation and NO production. Conversely, HGF treatment decreased p65/NF-κB activation and fully protected mouse and, more important, human β-cells against cytokines.

CONCLUSIONS—These results show that HGF/c-Met is critical for β-cell survival by attenuating NF-κB signaling and suggest that activation of the HGF/c-Met signaling pathway represents a novel strategy for enhancing β-cell protection. ***Diabetes*** **60:525–536, 2011**

Type I diabetes is an autoimmune disease that results from cellular cytotoxicity leading to selective and progressive destruction of insulin-secreting cells (1–3). Many growth factors known to control cell growth and survival in physiologic and pathologic conditions are expressed in the pancreas and could potentially participate in an autocrine/paracrine fashion in the final fate of β-cells in an autoimmune environment. Overexpression of IGF-1, transforming growth factor-β, or granulocyte macrophage-colony stimulating factor ameliorates islet infiltration and β-cell death in mouse models of increased islet inflammation and diabetes (4–6). However, the role of endogenous pancreatic growth factors in type I diabetes has not been examined. Because growth factors can locally affect β-cell survival, neogenesis, and regeneration, and modulate chemokine production and immune responses, alterations in the level/activation of growth factor signaling pathways might contribute to the delay/acceleration of the onset of diabetes.

Hepatocyte growth factor (HGF)/c-Met signaling pathway participates in the control of multiple biological functions, including development, proliferation, survival, regeneration, and branching morphogenesis (7). HGF binds with high affinity to, and induces the dimerization of, c-Met, its transmembrane tyrosine kinase receptor (8). Deletion of exon 16 of the *c-Met* gene, which encodes Lys^{1108} (ATP binding site), essential for the kinase activity of this receptor, in knockout mice results in embryonic lethality (9). These mice display a phenotype identical to HGF knockout mice (10). Both HGF and c-Met are expressed in the pancreas; HGF localizes to endothelial, islet, and mesenchymal cells, and c-Met is expressed in islet, ductal, and pancreatic progenitor cells (11–14). Conditional ablation of the *c-Met* gene in mouse β-cells using RIP-Cre and lox-c-Met mice leads to deficient insulin secretion without alteration of β-cell mass (12,13). On the other hand, HGF overexpression in the β-cell of transgenic mice increases β-cell replication, mass, and function (15,16). Furthermore, HGF improves islet graft survival in animal models of diabetes (17–19). HGF positively influences autoimmune responses, reducing the severity of autoimmune myocarditis and arthritis (20,21). HGF also downregulates airway and kidney inflammation, and inflammatory bowel disease (22–24). Whether HGF plays a role in autoimmune diabetes is unknown.

To address the function of c-Met in the development, growth, and maintenance of β-cells under physiologic conditions, as well as its role in β-cell survival and response to injury in vivo, we generated pancreas-specific c-Met-null (PancMet KO) mice. We report that although c-Met is dispensable for normal β-cell growth and function under basal conditions, it is critically important for β-cell survival in diabetogenic conditions. β-Cell survival is dramatically worsened in the absence of HGF/c-Met signaling, resulting in accelerated diabetes onset. These observations also apply to human β-cells, underscoring a therapeutic

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opportunity for the HGF/c-Met signaling pathway in human diabetes.

RESEARCH DESIGN AND METHODS

Generation of c-Met conditional knockout mice in the pancreas. Mice homozygous for the floxed *c-Met* allele (25) were crossed with Pdx-Cre transgenic mice (26). The resultant double-heterozygous mice were then crossed with c-Met$^{lox/lox}$ mice, resulting in c-Met$^{lox/lox}$, Pdx-Cre (PancMet KO) mice, and their wild-type (WT) littermates c-Met$^{lox/lox}$ or c-Met$^{lox/+}$ without Pdx-Cre transgene. Genotyping and assessment of deletion efficiency were analyzed by PCR on genomic DNA obtained from tails or pancreas (12,26). All the studies were performed with the approval of, and in accordance with, guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

Glucose homeostasis in adult PancMet KO mice in basal conditions. Blood obtained by retro-orbital bleed was analyzed for glucose by a portable glucometer (Medisense, Bedford, MA), and plasma insulin was analyzed by radioimmunoassay (Linco Research, St. Louis, MO) (15). Intraperitoneal glucose tolerance test was performed in 16–18-h fasted mice injected intraperitoneally (IP) with 2 g glucose/kg body wt, and insulin sensitivity tests were performed in mice in the random-fed state injected IP with 0.75 units bovine insulin/kg body wt (12). Insulin content in islets or pancreas, and glucose-stimulated insulin secretion in isolated islets were measured as reported (12).

Multiple low-dose streptozotocin-induced diabetes. Male mice aged 10–12 weeks were injected IP for 5 consecutive days with streptozotocin (STZ) (40 mg/kg) (27), starting at day 0, and nonfasting blood glucose was measured from snipped tails at different time points.

Immunohistochemistry and insulitis. Paraffin-embedded pancreatic sections were immunostained for insulin, glucagon, somatostatin, c-Met, and 5-bromo-2'-deoxyuridine (BrdU) as described (12,15). β-Cell mass and islet number were measured in three insulin-stained pancreas sections from each mouse using ImageJ (National Institutes of Health, Bethesda, MD) (12). BrdU incorporation in β and ductal cells was measured in pancreas sections of mice injected IP with BrdU (Amersham, Piscataway, NJ), killed 6 h later, and stained for insulin and BrdU (12). β-Cell death was determined in pancreas sections stained for insulin and using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method (Promega, Madison, WI). Sections were also stained with hematoxylin–eosin and anti-CD3 (Abcam, Cambridge, MA) for pathologic evaluation of islet insulitis (28).

Islet isolation and culture of pancreatic islets and βTC-3 cells. Mouse islets were isolated after injection of collagenase P through the pancreatic duct, as previously reported (12). Human islets were provided by the ICR and JDRF Basic Science Islet Distribution Programs. Individual mouse and human islets were hand-picked under a stereomicroscope, and 100–200 islets/mL were cultured in Roswell Park Memorial Institute medium in the presence or absence of recombinant mouse or human cytokines: interleukin (IL)-1β (5–500 units/mL), interferon (IFN)-γ (100–1,000 units/mL), and tumor necrosis factor (TNF)-α (100–1,000 units/mL), respectively.

Analysis of *c-Met*, *HGF*, inducible nitric oxide-synthase (*iNOS*), and *A20* mRNA expression in isolated islets was performed by real-time PCR using specific primers (Supplementary Table 1) (29). In a different set of real-time PCR experiments, mouse insulinoma βTC-3 cells were plated (50–100 × 10^3 cells/cm^2) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Twenty-four hours later, cells were serum depleted and treated with 1 mmol/L STZ or 50 units/mL IL-1β, 1,000 units/mL IFN-γ, and 1,000 units/mL TNF-α for 16 h before harvesting and RNA isolation.

Medium nitric oxide, monocyte chemoattractant protein-1, and monokine induced by γ-IFN concentration measurements. Medium (100 μL) from islet cultures containing 100 islets/mL was analyzed for nitric oxide (NO) by adding an equal volume of Greiss reagent (28). Monocyte chemotactic protein (MCP)-1 and monokine induced by γ-IFN (MIG) concentrations in medium were determined using a specific ELISA (R&D Systems, Minneapolis, MN).

Western blot analysis. Human and mouse islet extracts were separated on 7.5–10% SDS/PAGE, transferred to an Immobilon-P membrane (Millipore, Bedford, MA), blocked in 5% nonfat dry milk, and then incubated with primary antibodies against phospho-Ser536 p65, phospho-Ser32/36 IκBα, IκBα, phospho-Ser9 GSK3β, phospho-Ser473-AKT, phospho-ERK1/2, ERK1/2 (Cell Signaling, Beverly, MA), iNOS, p65, c-Met (Santa Cruz Biotechnology, Santa Cruz, CA), tubulin, and HGF (Calbiochem, La Jolla, CA). After several washes, blots were incubated with peroxidase-conjugated secondary antibodies followed by chemiluminescence detection (Amersham) (12).

Islet cell cultures and determination of β-cell death. Mouse and human islet cells were cultured as previously reported (30) and incubated with different doses of cytokines, STZ, or HGF for a period of 24 h and then fixed in 2% paraformaldehyde. β-Cell death was determined by TUNEL assay and insulin and DAPI staining. At least 2,000 β-cells per treatment were counted.

p65/NF-κB binding activity assay. Activation and binding of p65/NF-κB were quantified using an ELISA-based TransAM p65 kit (Active Motif, Carlsbad, CA). Briefly, protein extracts from human islets treated for 10 min with cytokines, HGF, or 10 nM Wortmannin were added to a 96-well plate with an immobilized oligonucleotide containing an NF-κB consensus binding site. Activated NF-κB homodimers and heterodimers contained in the islet extracts bind specifically to this oligonucleotide. p65 antibody was then added, followed by horseradish peroxidase-conjugated secondary antibody. Binding activity of p65/NF-κB was determined by measuring absorbance at 450 nm with a reference wavelength of 655 nm and expressed as –fold of untreated islets.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed using unpaired two-tailed Student *t* test, one-way ANOVA with Tukey's honestly significant difference post hoc test where indicated, Fisher exact test for the analysis of percent of hyperglycemic mice, and Pearson χ^2 test for analysis of insulitis. In all the tests, $P < 0.05$ was considered statistically significant.

RESULTS

HGF and c-Met expression increase in islets after multiple low-dose streptozotocin administration in vivo and after treatment with cytokines in vitro. The multiple low-dose streptozotocin (MLDS) model is a diabetogenic model in which hyperglycemia and diabetes are achieved after five daily injections of subdiabetogenic doses of STZ, leading to insulitis and selective β-cell loss (27). At day 5 after the first STZ injection, islets from mice treated with MLDS displayed significantly increased *HGF* and *c-Met* mRNA expression (Fig. 1*A*). Mouse islets treated with 1 mmol/L STZ for 24 h in vitro display increased *HGF*, but not *c-Met*, mRNA expression (Fig. 1*B*). Mouse islets and βTC-3 insulinoma cells treated in vitro with a combination of cytokines for 16–24 h showed increased *c-Met*, but not *HGF* mRNA expression (Fig. 1*C* and *D*). This suggests that in the MLDS-treated mouse islets, perhaps both STZ and inflammation are upregulating *HGF* and *c-Met* mRNA. Both HGF and c-Met proteins are upregulated in MLDS-treated mouse islets in vivo and in mouse islets treated with cytokines in vitro (Fig. 1*E* and *F*). This latter result suggests that posttranscriptional alterations might be responsible for HGF accumulation in mouse islets treated with cytokines. Collectively, these data suggest that islet and β-cell damaging agents, such as islet inflammation and STZ, induce the expression of both c-Met and its ligand HGF.

Generation and characterization of PancMet KO mice. We generated conditional KO mice (PancMet KO mice) with selective elimination of c-Met expression in pancreas and islets by combining Pdx-Cre with c-Met$^{lox/lox}$ mice (Fig. 2*A*). Compared with WT (c-Met$^{lox/lox}$ or c-Met$^{lox/+}$) mice, PancMet KO mice exhibit efficient Cre-mediated exon 16 deletion, and decreased c-Met levels, as assessed by PCR analysis of pancreas genomic DNA and Western blot of pancreas and islet protein extracts (Fig. 2*B–D*). The detection of c-Met expression in pancreas extracts from PancMet KO mice could be due to the presence of c-Met in nonendocrine and nonexocrine cell types, such as vascular cells, fibroblasts, immune cells, and cells in lymph nodes, all of which are present in the pancreas. PancMet KO mice display marked downregulation of c-Met in islets and ducts as assessed by immunofluorescent staining (Fig. 2*E–H*). Furthermore, HGF-mediated signaling via ERK1/2 was markedly attenuated in PancMet KO mouse islets (Fig. 2*I*). Taken together, these results indicate that PancMet KO mice display effective and efficient recombination of c-Met in pancreas and islets. Notably, c-Met deficiency in the pancreas and β-cells of adult mice did not significantly

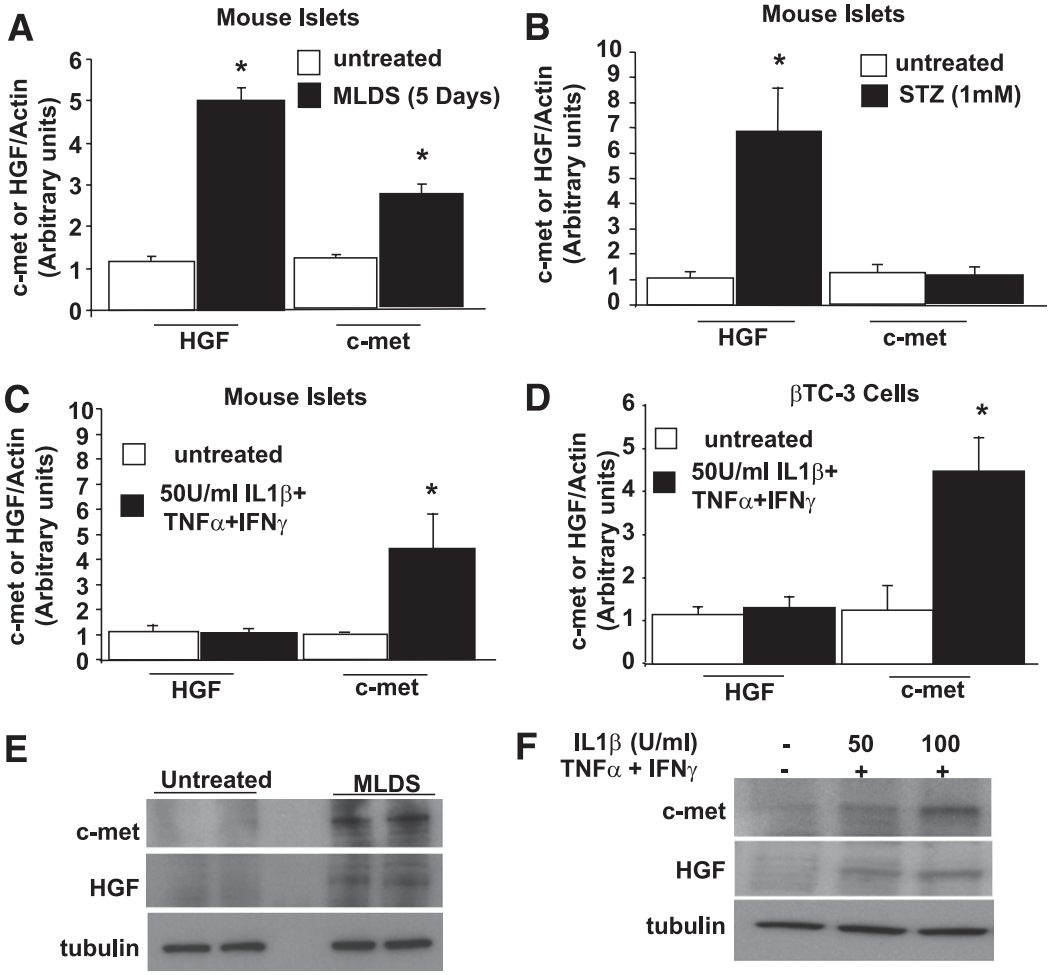

FIG. 1. ***A*: Expression of *HGF* and *c-Met* mRNA in islets obtained from mice after 5 days of MLDS (40 mg/kg) administration (*n* = three mice/group). *HGF* and *c-Met* mRNA expression in islets treated in vitro (*B*) with 1 mmol/L STZ for 24 h or (*C*) with 50 units/mL of IL-1β, 1,000 units/mL TNF-α, 1,000 units/mL IFN-γ for 24 h, and (*D*) in βTC-3 insulinoma cells treated with the same concentration of cytokines for 16 h. Real-time PCR results are mean ± SEM of three experiments in triplicate. **P* < 0.05 vs. untreated. HGF and c-Met protein expression (*E*) in islets obtained from mice after 5 days of MLDS administration and (*F*) in islets treated in vitro with 1,000 units/mL TNF-α, 1,000 units/mL IFN-γ, and 50 or 100 units/mL of IL-1β for 24 h. Western blot results are representative of three different experiments.**

alter glucose or β-cell homeostasis, although a trend to display lower nonfasting blood glucose was observed in PancMet KO mice (Supplementary Figs. 1 and 2). In addition to being expressed in insulin-positive cells, c-Met is also present in glucagon- and somatostatin-positive cells in mouse islets (Supplementary Fig. 3), and its absence could lead to alterations in the proportion of these endocrine cells in PancMet KO mice. Analysis of α-cell/β-cell and δ-cell/β-cell ratios per islet reveals normal values in PancMet KO mice (Supplementary Fig. 2*I* and *J*). These results show that HGF actions in the pancreas are dispensable for α-, δ-, and β-cell growth, and β-cell maintenance and function under basal conditions.

PancMet KO mice are more susceptible than WT mice to MLDS-induced diabetes. Because c-Met and HGF are upregulated in islets after exposure to cytokines in vitro or after MLDS treatment in vivo (Fig. 1), we sought to address the functional importance of c-Met in the adaptive responses of the β-cell to the injury induced by MLDS administration in vivo. We measured blood glucose levels in PancMet KO and WT mice during 20 days after the first STZ injection. MLDS-treated PancMet KO mice displayed significantly increased blood glucose levels compared with WT mice from day 4 to day 20 (Fig. 3*A*). In addition, MLDS-treated PancMet KO mice displayed a nonsignificant trend toward faster and higher frequency of hyperglycemia compared with WT mice (Fig. 3*B*).These results correlated with significant hypoinsulinemia in PancMet KO mice at day 20 after the first STZ injection compared with the reduced insulin levels in WT mice treated with MLDS (Fig. 3*C*).

Together with a more pronounced deterioration in glucose homeostasis after MLDS administration, PancMet KO mice also displayed significantly decreased β-cell mass (Fig. 4*A–C*). This decrease was not due to diminished number of islets or decreased β-cell neogenesis, measured as the number of singlet and doublet insulin-positive cells in the pancreas (Supplementary Fig. 4), but to a reduction of insulin-positive area per islet (Fig. 4*D–F*). The number of islets with >80% insulin-positive area was markedly and significantly decreased in PancMet KO mice compared with WT littermates (Fig. 4*F*). Conversely, the number of islets with <20% insulin-positive area was significantly increased in PancMet KO mice, suggesting a decrease in the number of insulin-positive cells per islet in these mice. An increase in β-cell death would likely explain the decrease in insulin-positive cells per islet and the diminished β-cell mass in PancMet KO mice compared with WT littermates. Indeed, the percentage of TUNEL-positive β-cells at day 8 after the

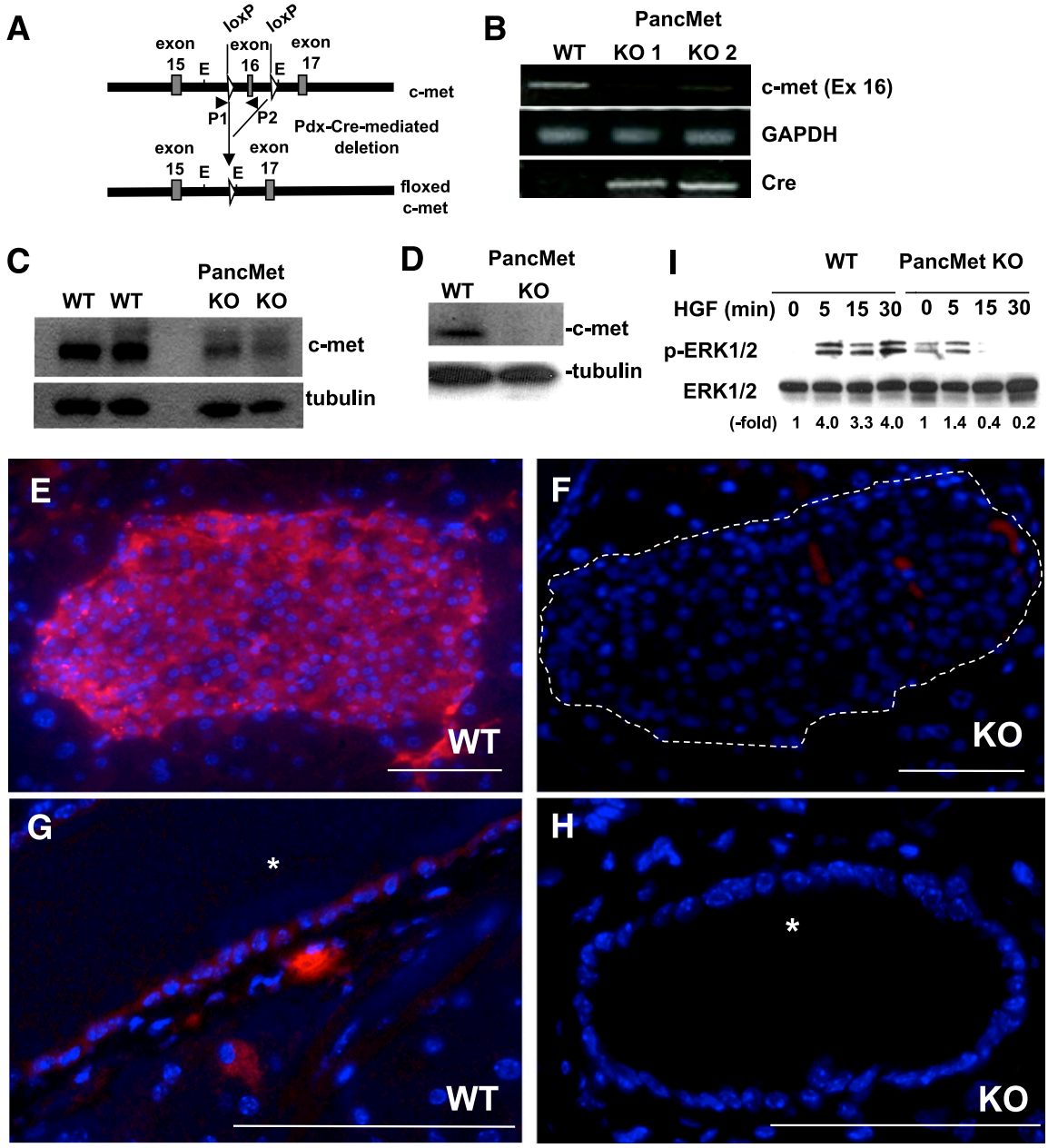

FIG. 2. Assessment of exon 16 deletion and c-Met downregulation in pancreas and islets of PancMet KO mice. *A*: Schematic illustration of Cre-mediated deletion of exon 16 from the *c-Met* allele. Cre action removes exon 16 and intron sequences between the two LoxP sites (white arrowheads). *E*: *Eco*RI restriction sites; P1 (LoxP) and P2 (exon 16) primers used for genotyping and assessment of *c-Met* deletion from pancreas genomic DNA. *B*: PCR analysis of pancreas genomic DNA from WT and PancMet KO mice. PCR fragments corresponding to floxed *c-Met* alleles (600 bp), *GADPH* (259 bp), and *Cre* (332 bp) are shown. Western blot of c-Met expression in (*C*) pancreas and (*D*) islet protein extracts from WT and PancMet KO mice. *E–H*: Immunofluorescent staining for c-Met on pancreatic sections from WT and PancMet KO mice. A profound decrease in c-Met staining was observed in (*F*) islets and (*H*) cells lining the duct (duct lumen labeled with *) of PancMet KO mice compared with WT mice (*E* and *G*). Scale bar = 50 μm. *I*: Western blot of ERK1/2 activation in isolated islets from WT and PancMet KO mice treated with 25 ng/mL HGF. (A high-quality digital representation of this figure is available in the online issue.)

first STZ injection was strikingly and significantly increased in PancMet KO mice, even when compared with the expected cell death in WT mice treated with MLDS (Fig. 4*G–I*).

PancMet KO mice display increased lymphocyte infiltration in response to MLDS. To determine whether the increased sensitivity of PancMet KO mice to the diabetogenic effects of MLDS was associated with exaggerated insulitis, hematoxylin–eosin-stained pancreatic sections from MLDS-treated mice were examined histologically for the degree of insulitis based on the scale described by Flodström et al. (28): 0, no infiltration; 1, mild infiltration; 2, minor peri-insular infiltration; 3, clear peri-insular infiltration; 4, clear intraislet infiltration. PancMet KO mouse islets displayed clear intraislet infiltration that also strongly stained with an anti-CD3 antibody, a general marker for lymphocytes (Fig. 5*A–D*). Determination of insulitis degree showed that the number of islets without infiltration was significantly decreased, and the number of islets with clear infiltration was significantly increased, in PancMet KO compared with WT mice (Fig. 5*E*).

Chemokines and cytokines are mediators of the immune response by attracting and activating leukocytes. Because

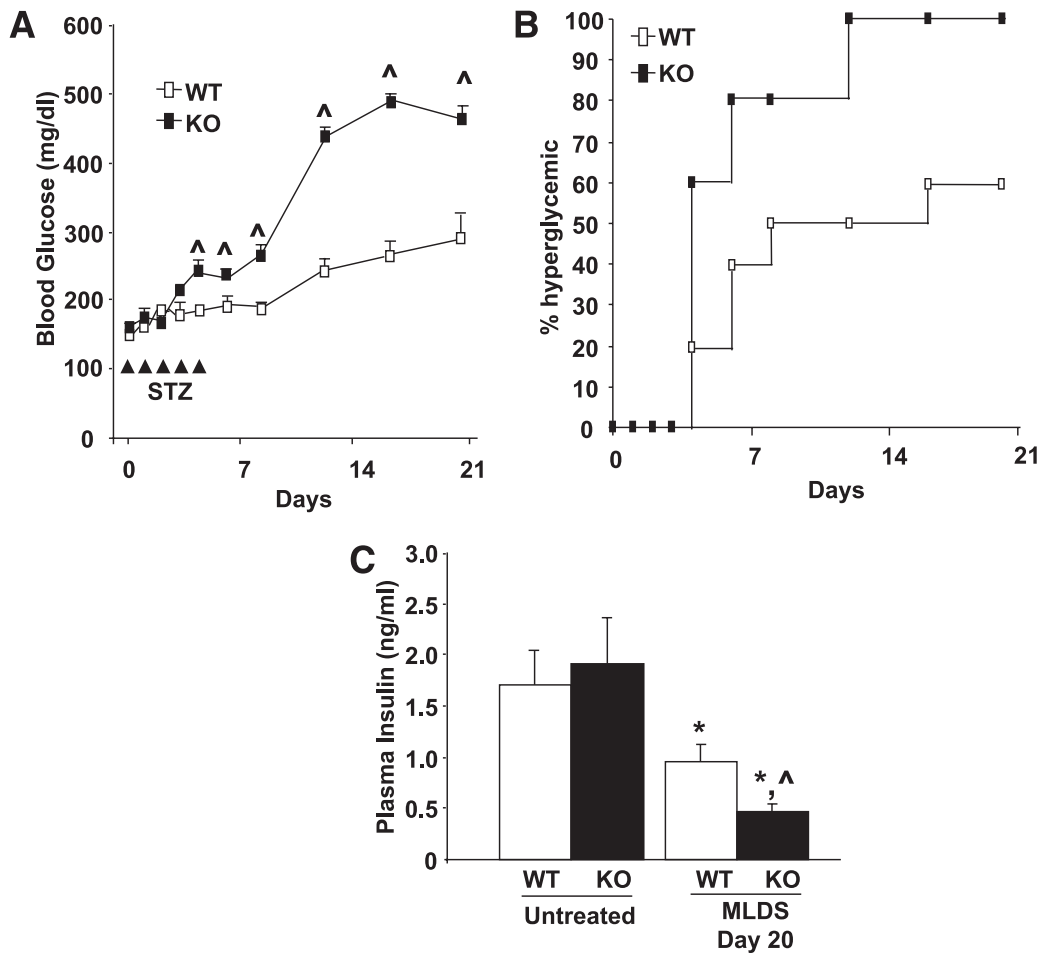

FIG. 3. PancMet KO mice treated with MLDS display (*A*) significantly increased blood glucose levels (^*P* < 0.05 vs. WT) and (*B*) a trend toward faster and higher frequency of hyperglycemia (>200 mg/dL on at least two consecutive measurements), although not significant (*P* = 0.084 at day 12 and *P* = 0.154 at days 16 and 20 determined by Fisher exact test). On the other hand, PancMet KO mice display a significantly faster and higher frequency of severe hyperglycemia (>300 mg/dL on at least two consecutive measurements) compared with WT mice (day 16, PancMet KO 100% vs. WT 10%, *P* = 0.002; day 20, PancMet KO 100% vs. WT 20%, *P* = 0.007). *C*: PancMet KO mice treated with MLDS display profound hypoinsulinemia at day 20 (P* < 0.05 vs. untreated and ^*P* < 0.05 vs. WT). WT (*n* = 10) and PancMet KO mice (*n* = 5) were injected with 40 mg/kg STZ for 5 days.**

PancMet KO mice display increased lymphocyte infiltration, we measured the level of the secreted chemokines MCP-1 and MIG from PancMet KO and WT mouse islets exposed to cytokines. As shown in Fig. 5*F* and *G*, cytokine-induced chemokine secretion is significantly increased in PancMet KO compared with WT mouse islets.

PancMet KO β-cells are more sensitive to STZ- and cytokine-mediated cell death. The results presented thus far indicate that β-cells deficient in c-Met are more sensitive to cell death in vivo after MLDS administration, but they do not address whether they are more sensitive to the initial cytotoxic effects of STZ, the concomitant inflammatory insult generated in this model, or both. To directly address this issue, we performed TUNEL and insulin staining of primary islet cell cultures from WT and PancMet KO mice treated with STZ or cytokines in vitro. β-Cell death was significantly increased in PancMet KO islet cell cultures treated with STZ or cytokines compared with WT cells (Fig. 6*A*–*D*).

Inhibition of NF-κB activation eliminates the increased sensitivity of PancMet KO β-cells to cytokine-mediated cytotoxicity. Accumulating evidence suggests that the transcription factor NF-κB is an important intracellular mediator initiating the cascade of events that lead to β-cell death in the presence of cytokines (31–33). Therefore, we examined activation of NF-κB as measured by phosphorylated p65/RelA in cytokine-treated islets and found enhanced phospho-p65 levels in PancMet KO mouse islets compared with WT islets (Fig. 6*E* and *F*). *iNOS* is a well-known NF-κB target gene induced by cytokines (31,34,35). To determine whether *iNOS* induction was greater in c-Met-null islets, we measured *iNOS* mRNA and protein expression, and NO formation as nitrite accumulation in the culture media of cytokine-treated PancMet KO and WT islets. PancMet KO mouse islets displayed significantly increased *iNOS* expression levels and NO production compared with WT islets (Fig. 6*G*–*J*). In addition, another NF-κB target gene *A20*, a pro-survival gene in β-cells (36), was also further induced in PancMet KO islets compared with WT islets (Fig. 6*G*). Collectively, these data confirm the increased cytokine-mediated activation of NF-κB in PancMet KO islets. The addition of the NOS inhibitor L-N^G-monomethyl Arginine (L-NMMA) (Fig. 6*K*) or two different NF-κB inhibitors, sodium salicylate, which binds to and inhibits NF-κB activator IκB kinase (IKK) β (37), or the cell-permeable peptide SN-50, which inhibits the nuclear translocation of the NF-κB active complex (38), completely blocked the increased sensitivity of PancMet KO β-cells to the cytotoxic effects of cytokines (Fig. 6*L*). However, SN-50 did not alter STZ-mediated cytotoxicity in PancMet KO β-cells (Supplementary Fig. 5*A*).

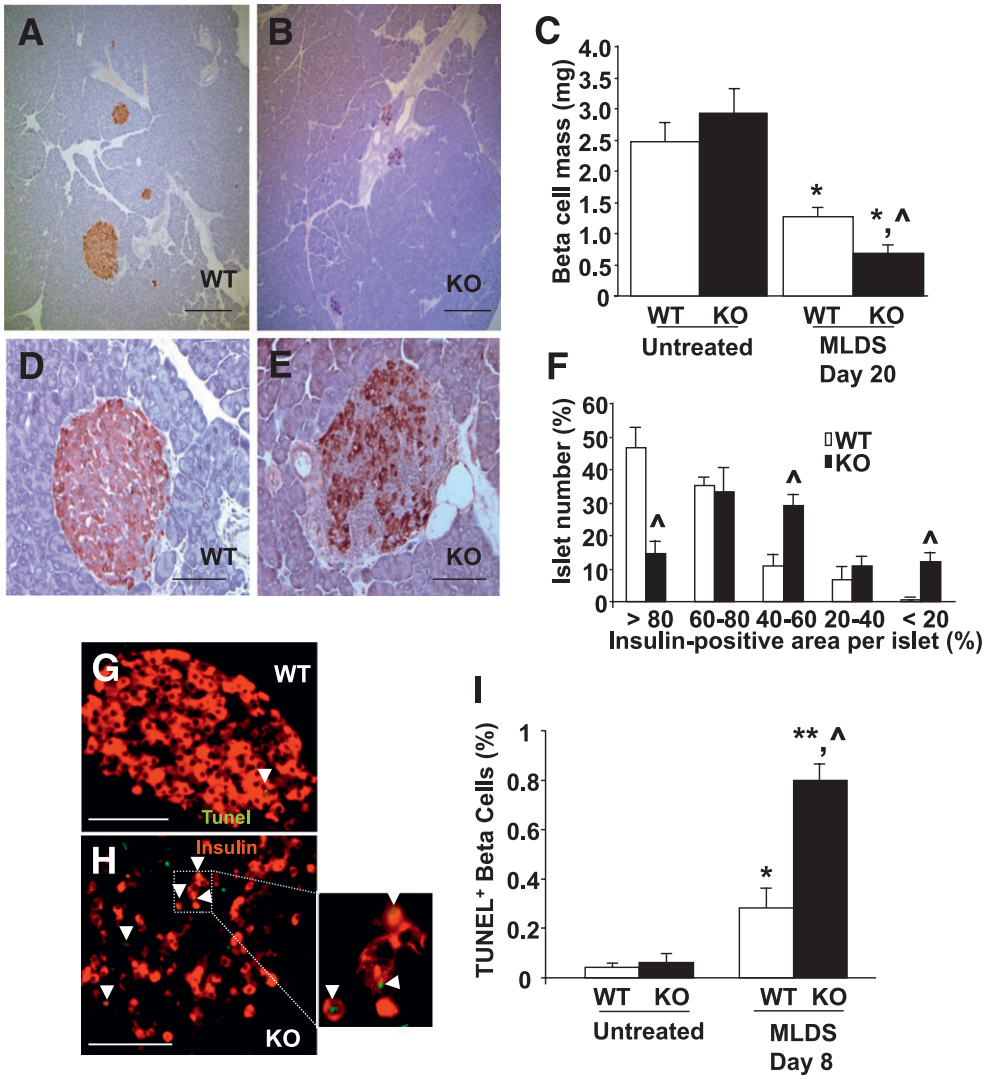

FIG. 4. Decreased β-cell mass and increased β-cell death in PancMet KO mice treated with MLDS. *A* and *B*: Representative photomicrographs of pancreas sections obtained from MLDS-treated mice (day 20) stained for insulin (brown) and counterstained with hematoxylin. Scale bar = 200 μm. *C*: Histomorphometric quantitation of β-cell mass in three pancreatic sections per mouse separated by 50 μm and stained for insulin. Pancreata were harvested at day 20 after MLDS from PancMet KO (n = 5) and WT (n = 10) mice. *D* and *E*: Photomicrographs of representative islets from PancMet KO and WT littermates treated with MLDS and stained for insulin. Scale bar = 50 μm. *F*: Percentage of islets with insulin-positive areas ranging from >80 to <20% assessed in three pancreatic sections from WT (n = 10) and PancMet KO mice (n = 5) 20 days after MLDS treatment. *G* and *H*: Representative photomicrographs of islets from PancMet KO and WT littermates at day 8 after treatment with MLDS and stained for insulin (red) and TUNEL (green). Scale bar = 50 μm. A higher magnification photo of an area in the pancreas of PancMet KO mice is displayed on the right side of *H*. Arrows indicate TUNEL-positive β-cell nuclei. *I*: Quantitation of TUNEL-positive β-cell nuclei in pancreatic sections from PancMet KO (n = 5) and WT (n = 5) mice treated with MLDS and harvested at day 8. *P < 0.05 and **P < 0.01 vs. untreated and ^P < 0.05 vs. WT. (A high-quality digital representation of this figure is available in the online issue.)

Furthermore, PancMet KO and WT mouse β-cells were equally sensitive to cytokines + FasL (Jo-2) cell death stimulus (Supplementary Fig. 5*B*). These results suggest that increased NF-κB activation and NO production in PancMet KO islets affect cytokine-induced but not Fas/FasL- or STZ-mediated β-cell death, and that proapoptotic genes induced by NF-κB counteract the potential prosurvival effects of *A20* in c-Met-null β-cells.

HGF decreases NF-κB activation and protects rodent and human β-cells against cytokines. To ascertain whether activation of the HGF/c-Met signaling pathway protects β-cells from cytokines, we added HGF to normal mouse primary islet cell cultures treated with increasing doses of cytokines and analyzed the percentage of TUNEL-positive β-cells. HGF completely protected normal mouse β-cells against cytokines (Fig. 7*A*), but not PancMet KO β-cells, suggesting that HGF-induced protective effects are mediated through c-Met (Supplementary Fig. 6). Opposite to what was observed in PancMet KO islets, normal cytokine-treated islets incubated with HGF displayed significantly decreased NF-κB activation (Fig. 7*B* and *C*), iNOS expression (Fig. 7*D* and *E*), and NO production (Fig. 7*F*). Collectively, these results in PancMet KO β-cells and in islets treated with HGF indicate that HGF may protect mouse β-cells against cytokine-induced cell death by inactivation of NF-κB and decreased NO production.

More important, HGF completely protected human β-cells from cytokine-induced cell death (Fig. 8*A*–*C*) and significantly decreased p65/RelA phosphorylation in human islets (Fig. 8*D* and *E*). Activation of p65/NF-κB and binding to an NF-κB consensus sequence were also inhibited by HGF in human islets (Fig. 8*F*). Furthermore,

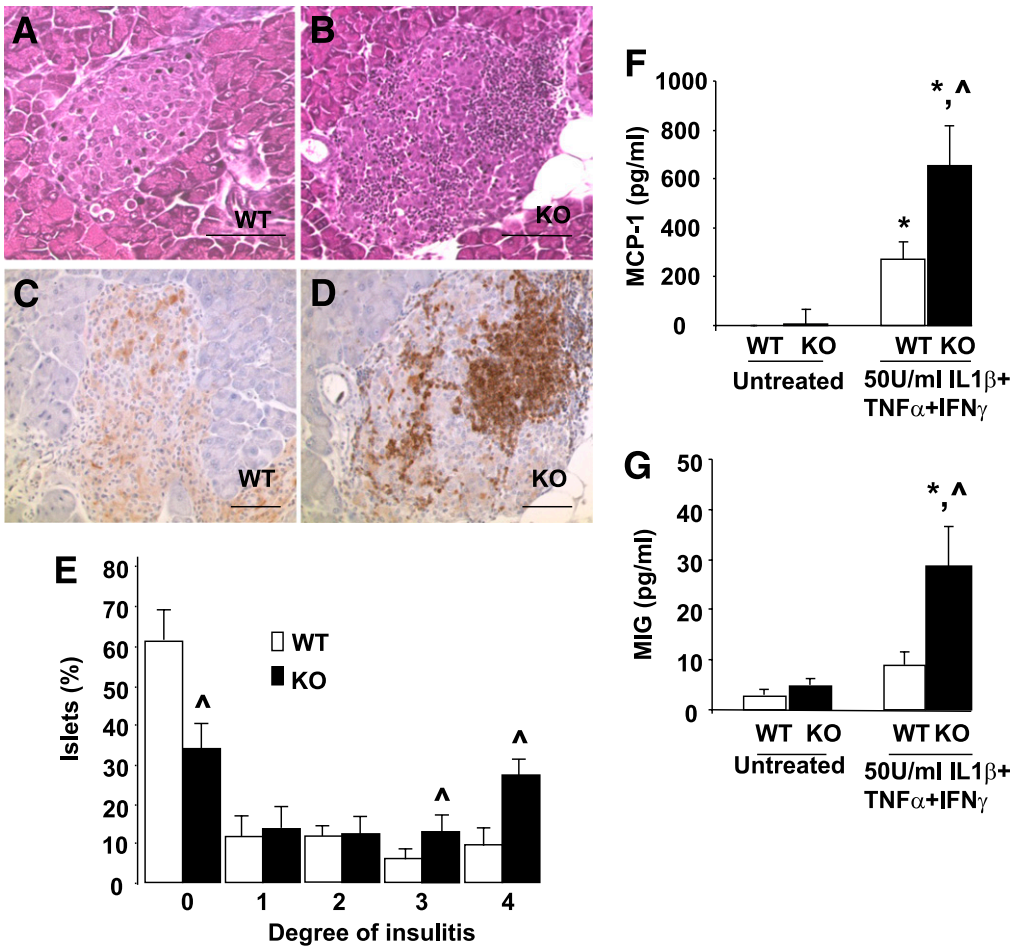

FIG. 5. Increased degree of intraislet infiltrate in PancMet KO mice treated with MLDS compared with WT littermates. Hematoxylin–eosin (*A* and *B*) and anti-CD3 (*C* and *D*) staining of pancreas sections from MLDS-treated mice. A representative islet from a PancMet KO mouse showing severe lymphocyte intraislet infiltration compared with an islet from WT mice displaying mild lymphocyte infiltration. Scale bar = 50 μm. *E*: Degree of insulitis at day 20 after MLDS administration in WT (n = 10) and PancMet KO (n = 5) mice. Insulitis was calculated as percent of islets per mouse in each stage of insulitis (0–4 as described in RESULTS). Secreted chemokines MCP-1 (*F*) and MIG (*G*) accumulated in the media of islets exposed to cytokines 50 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 24 h. Data pooled from five different experiments are shown (n = 10 WT and n = 10 PancMet KO mice). *P < 0.05 vs. untreated and ^P < 0.05 vs. WT. (A high-quality color representation of this figure is available in the online issue.)

HGF was found to modulate specific upstream regulators of NF-κB activation that are involved in cytokine-mediated β-cell death, significantly decreasing the phosphorylation of inhibitor of κ-B (IκB) α and increasing the phosphorylation of AKT and GSK-3β in cytokine-treated human islets (Fig. 8*D* and *E*) (39,40). HGF-mediated inhibition of NF-κB activation in islets was significantly decreased by the PI3K inhibitor Wortmannin (Fig. 8*F*). Taken together, these results suggest that HGF may protect human β-cells against cytokine-induced cell death by inactivation of the NF-κB and activation of the PI3K/Akt signaling pathways.

DISCUSSION

The current study provides the first direct evidence that endogenous pancreatic HGF/c-Met signaling is important for β-cell survival in diabetogenic conditions. On one hand, the absence of c-Met in the mouse pancreas enhances β-cell death, islet chemokine and NO production, insulitis, and β-cell mass depletion, leading to further pronounced hypoinsulinemia, further increased blood glucose levels, and a nonsignificant trend toward faster and higher frequency of hyperglycemia in response to MLDS treatment. On the other hand, HGF protects rodent and, more important, human β-cells from cytokine-induced cell death. Therefore, these observations indicate that activation of the HGF/c-Met signaling pathway attenuates β-cell death and identifies this pathway as a therapeutic target for the treatment of the disease.

PancMet KO mice display normal glucose and β-cell homeostasis, suggesting that HGF actions in the pancreas are dispensable for β-cell growth, maintenance, and function under basal conditions. This is in contrast with our previous results indicating that elimination of c-Met from β-cells in RIP-Cre-lox-Met mice leads to mildly impaired glucose tolerance and decreased glucose-stimulated insulin secretion (12). Because heterozygote RIP-Cre mice (CD-1 background) used in our studies display normal glucose homeostasis (not shown), there are two possible reasons for the difference in the metabolic phenotype between RIP-Cre-lox-Met mice and PancMet KO mice: *1*) the differential elimination of c-Met from β-cells in one case and from pancreatic precursors that give rise to endocrine, exocrine, and ductal cells in the other; or *2*) because the RIP-Cre transgene is also expressed in the hypothalamus (41), the metabolic defects observed in RIP-Cre-lox-c-Met

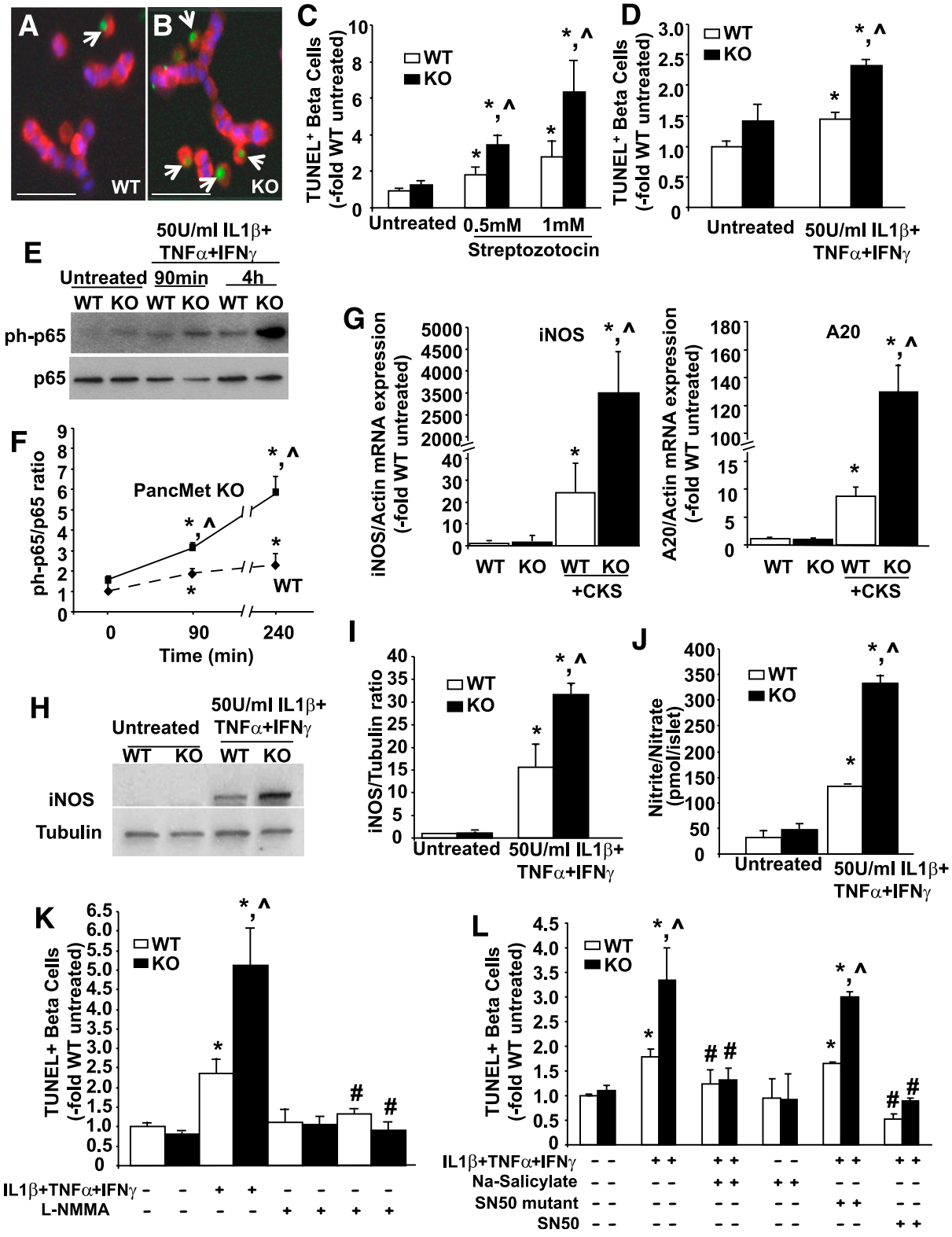

FIG. 6. Increased sensitivity of PancMet KO β-cells to STZ- or cytokine-induced cell death is mediated by NF-κB activation. *A* and *B*: Representative photomicrographs of islet cultures treated with STZ for 24 h and stained for TUNEL (green), insulin (red), and DAPI (blue). Arrows indicate TUNEL-positive β-cell nuclei. Scale bar = 25 μm. Quantitation of TUNEL-positive β-cell nuclei in five experiments performed in duplicate of islet cell cultures of PancMet KO and WT mice treated with (*C*) STZ or (*D*) 50 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 24 h. *E*: Representative Western blot displaying phospho-p65 and p65 expression in protein extracts from PancMet KO and WT islets treated with or without 50 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ. *F*: Densitometric quantitation of phospho-p65 and p65 in five Western blots performed with five different islet extract samples per time point. *G*: mRNA expression of *iNOS* and *A20*, two NF-κB target genes, in islets from WT (n = 4), and PancMet KO (n = 5) mice treated with or without cytokines (5 units/mL IL-1β, 100 units/mL TNF-α, and 100 units/mL IFN-γ) for 6 h. *H*: Representative Western blot displaying iNOS and tubulin expression in protein extracts from PancMet KO and WT islets treated with or without 50 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 24 h. *I*: Densitometric quantitation of iNOS expression in three Western blots performed with three different islet extract samples per condition. *J*: Medium nitrite levels secreted from islets exposed in vitro to

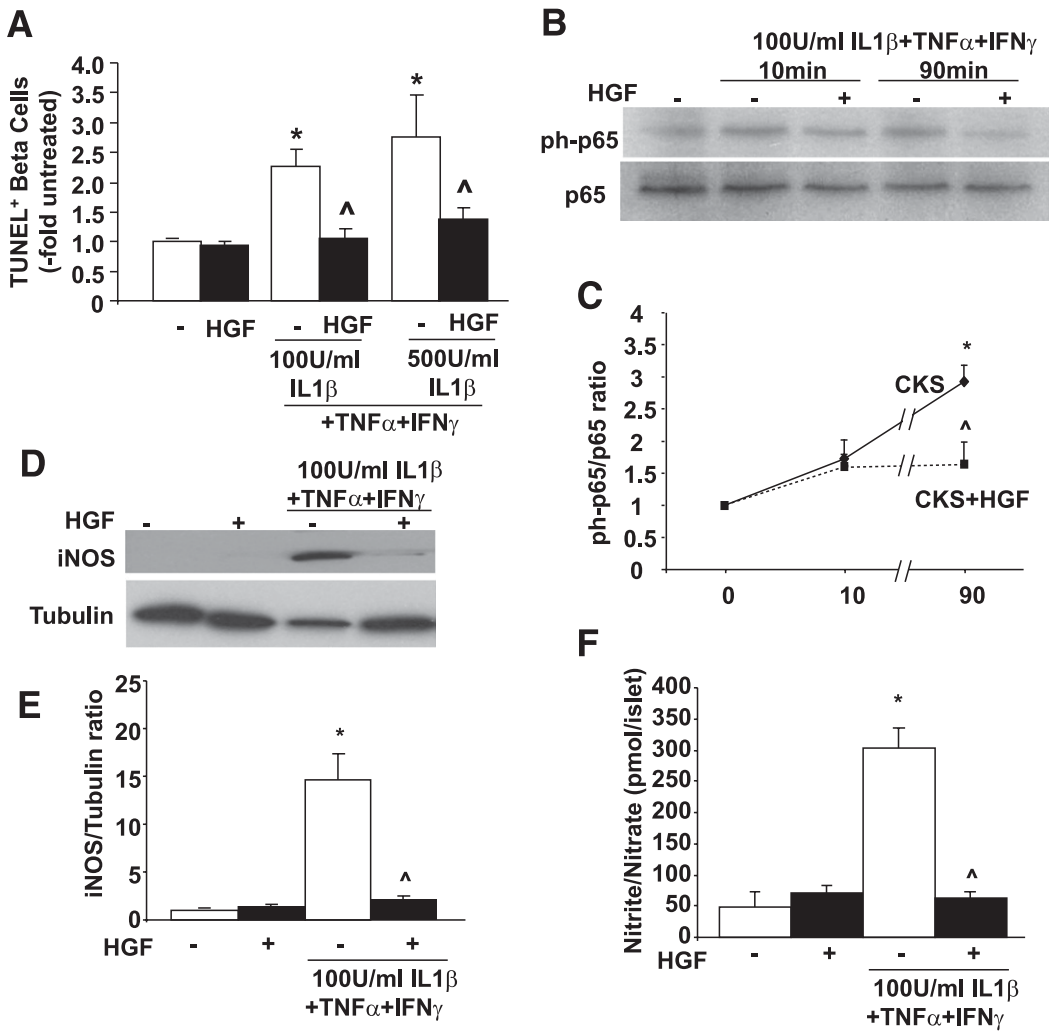

FIG. 7. Protective effect of HGF on primary mouse β-cells treated with cytokines. *A*: Mouse islet cell cultures were treated with or without 25 ng/mL HGF and 100 or 500 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 24 h. Results are means ± SE of five experiments in duplicate. *B*: Representative Western blot displaying phospho- and total p65 levels in protein extracts from mouse islets treated with or without 25 ng/mL HGF and 100 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for different time periods. *C*: Densitometric quantitation of phospho- and total p65 in four Western blots performed with four different protein extracts. *D*: Representative Western blot displaying iNOS and tubulin levels in protein extracts from mouse islets treated with or without the same doses of cytokines and HGF for 24 h. *E*: Densitometric quantitation of iNOS expression in three Western blots performed with three different protein extracts. *F*: Medium nitrite levels secreted from islets treated with or without the same doses of cytokines and HGF for 24 h. *$P < 0.05$ vs. untreated and ^$P < 0.05$ vs. cytokine-treated but HGF-untreated cells. CKS, cytokines.

mice might be caused by the loss of c-Met not only from β-cells but also from the hypothalamus.

HGF is a prosurvival agent in multiple cell types, including the β-cell (7,8,15–19). HGF increases β-cell survival in vivo after administration of high doses of STZ, as well as in an islet transplant setting in diabetic mice in which hypoxia- and nutrient deprivation-mediated β-cell damage are present (15–19). In vitro, exogenously added HGF protects β-cells against STZ (17). The current study found that HGF also protects both mouse and human β-cells against high doses of cytokines. HGF and c-Met are both upregulated in islets at early stages (day 5) in the MLDS mouse model and in vitro after cytokine and STZ treatment. This suggests that STZ and islet inflammation activate the HGF/c-Met pathway in islet cells, and potentially in islet infiltrating cells (20–24,42), perhaps in an attempt to counteract the damage induced by these cytotoxic agents. Indeed, removal of HGF/c-Met signaling from islets renders β-cells more sensitive to STZ and cytokines in vitro and, more important, leads to exacerbated β-cell death, further increased blood glucose levels, and a nonsignificant trend toward faster and higher frequency of hyperglycemia in the MLDS mouse model. This indicates that the autocrine action of the upregulated HGF/c-Met system, or the paracrine or endocrine HGF from other sources (43,44), might participate in delaying β-cell death

50 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 24 h. Effect of the NOS inhibitor L-NMMA citrate (2 mmol/L) (*K*) and the NF-κB inhibitors Na-salicylate (5 mmol/L) and SN-50 (50 μg/mL) (*L*) in β-cell death induced by cytokines assessed as in *A*–*D*. SN-50 mutant was used as control for SN-50 treatment. Four to five experiments were performed in duplicate with islet cell cultures of PancMet KO and WT mice treated as in *D*. Results are means ± SE. *$P < 0.05$ vs. untreated ^$P < 0.05$ vs. WT and #$P < 0.05$ vs. WT or KO treated with cytokines alone or with SN-50 mutant; assessed by Student *t* test or one-way ANOVA and Tukey's honestly significant difference post hoc test where appropriate. CKS, cytokines. (A high-quality digital representation of this figure is available in the online issue.)

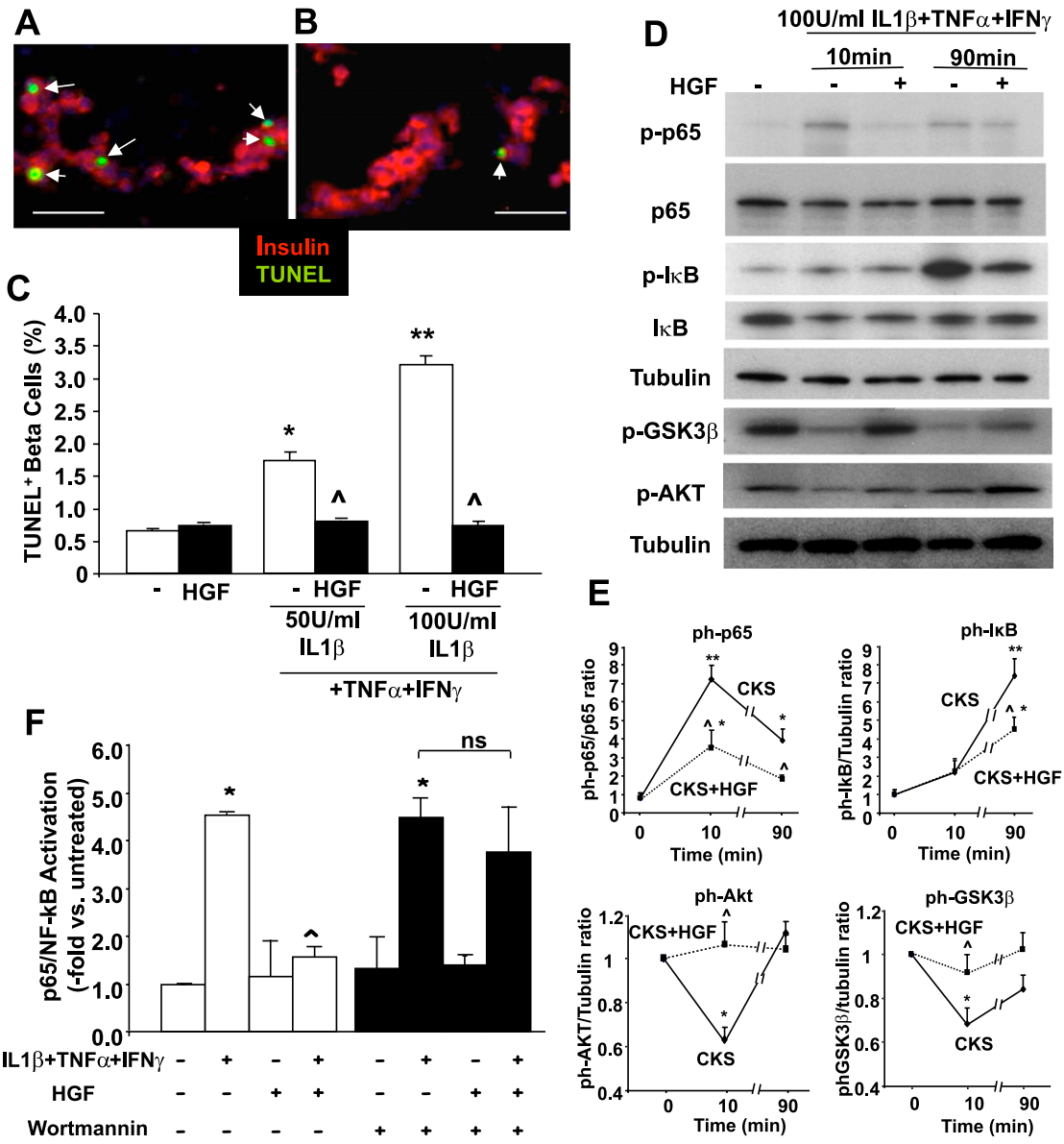

FIG. 8. Protective effect of HGF on primary human β-cells treated with cytokines. *A*: Representative images of human islet cell cultures treated with 100 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 24 h in the absence or (*B*) presence of 25 ng/mL HGF and stained for TUNEL (green), insulin (red), and DAPI (blue). Arrows indicate TUNEL-positive β-cell nuclei. Scale bar = 25 μm. *C*: Quantitation of TUNEL-positive β-cell nuclei in five experiments per duplicate performed with human islet cell cultures from five different donors treated with 50 or 100 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 24 h. *D*: Representative Western blots displaying the expression of phospho- and total p65, phospho- and total IκB, phospho-GSK-3β, phospho-AKT, and tubulin in protein extracts from human islets treated with or without HGF and 100 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ. *E*: Densitometric quantitation of these proteins in four Western blots performed with four different human islet extract samples per time point obtained from four different donors. *F*: Activation of p65/NF-κB in human islet extracts treated with 50 units/mL IL-1β, 1,000 units/mL TNF-α, and 1,000 units/mL IFN-γ for 10 min and with or without 25 ng/mL HGF and assessed by an ELISA-based TransAM assay measuring p65/NF-κB binding activity (see RESEARCH DESIGN AND METHODS). In some cases, human islets were pretreated for 30 min with 10 nM Wortmannin. Results are means ± SEM of three experiments in triplicate. *$P < 0.05$ and **$P < 0.01$ vs. untreated and ^$P < 0.05$ vs. cytokine treated. CKS, cytokines; ns, not significant. (A high-quality digital representation of this figure is available in the online issue.)

in diabetogenic situations. Collectively, the results included in this study establish the possibility that alterations in the expression or activation of HGF/c-Met signaling might further predispose individuals toward the development of diabetes.

This study found that mice deficient in c-Met in the pancreas display extensive intraislet lymphocyte infiltration after treatment with MLDS. Recent studies indicate that HGF has potent anti-inflammatory effects in multiple organ systems, including inflammatory bowel disease, airway and kidney inflammation, autoimmune myocarditis, and autoimmune arthritis (20–24). In the kidney, HGF decreases the expression of chemokines such as Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) and MCP-1 in mouse models of subtotal nephrectomy and obstructive nephropathy (24,45). We found that c-Met-null islets exposed to cytokines display enhanced secretion of MCP-1 and MIG, which are known to recruit macrophages and T cells to sites of tissue injury and infection (46,47). This suggests that *1*) the increased chemokine production in c-Met-null islets might be responsible for the enhanced insulitis observed in PancMet

KO mice after MLDS administration and *2*) HGF/c-Met signaling is an endogenous regulator of islet inflammation. However, it is also possible that the increased sensitivity to β-cell death in PancMet KO mice is an important contributor to enhanced islet inflammation.

NF-κB regulates the expression of genes involved in cellular stress responses, cell growth, inflammation, survival, and apoptosis (48). The predominant species in NF-κB pathway in most cell types is the p65:p50 heterodimer, which associates with the inhibitors of NF-κB (IκBs) in the cytoplasm of resting cells. Activation of NF-κB mainly occurs via IKK-mediated phosphorylation of inhibitory molecules, including IκBα. However, optimal induction of NF-κB target genes also requires phosphorylation of NF-κB proteins, such as p65, within their transactivation domain by a variety of kinases, including protein kinase A, protein kinase Cζ, and glycogen synthase kinase-3 (GSK-3). NF-κB activation is a key event for β-cell destruction in vitro after cytokine treatment (31,32). However, the role of NF-κB in the β-cell in vivo during islet inflammation and autoimmunity remains uncertain. Mice in which signaling of the entire family of NF-κB/Rel transcription factors is specifically and conditionally inhibited in adult β-cells by expressing a dominant-negative form of IκBα in the β-cell under the control of the tetracycline (on/off) system display nearly complete protection against MLDS-induced diabetes (33). Our studies found that c-Met-null islets display increased p65 phosphorylation compared with WT islets after treatment with cytokines. This increase in NF-κB activation could be responsible for the enhanced NO and chemokine production and intraislet infiltration, and the increased β-cell sensitivity to cytokines in PancMet KO mouse islets. Conversely, HGF treatment downregulated the NF-κB-iNOS-NO pathway in normal mouse islets. Inhibiting NOS with L-NMMA or blocking the degradation of the NF-κB inhibitor, IκB, with salicylate or inhibition of NF-κB nuclear translocation with SN-50 clearly eliminated cytokine-induced β-cell death in WT islets and in c-Met-null islets. These results suggest that HGF/c-Met signaling might act as a regulator of NF-κB-iNOS-NO pathway in β-cells in the presence of cytokines. These results could also suggest that c-Met deficiency in β-cells of NOD mice could accelerate diabetes onset in NOD-PancMet KO mice. However, NOD–RIP–mIκBα mice expressing a nondegradable form of IκBα in pancreatic β-cells display accelerated diabetes onset, indicating that NF-κB may play an antiapoptotic role in NOD mouse β-cells and protects from developing diabetes (49). Future studies describing whether c-Met absence from β-cells affects diabetes onset in NOD mice are warranted.

Recent evidence indicates that HGF disrupts NF-κB signaling in endothelial and renal tubule cells by IκB and GSK-3–dependent mechanisms (24,45,50). HGF decreased p65/NF-κB activation, diminished IκBα phosphorylation, and increased Akt and GSK-3 phosphorylation in cytokine-treated human islets. HGF-mediated inhibition of cytokine-induced p65/NF-κB activation was reduced by the PI3K inhibitor Wortmannin, indicating that both aspects of NF-κB inactivation—sequestration of NF-κB and decreased kinase-induced activation—might be involved in the effect of HGF in human islets. Taken together, these results suggest that HGF-mediated protection of β-cells is likely through downregulation of NF-κB signaling pathway.

In conclusion, although HGF/c-Met signaling in the pancreas is dispensable for normal β-cell growth, function, and maintenance, its absence renders β-cells highly susceptible to cell death against diabetogenic agents. These observations also highlight a novel role for HGF as a protector of mouse and, more important, human β-cells against cytokines. Collectively, these results point out the physiologic and therapeutic importance of the entire HGF/c-Met pathway for the survival of the β-cell in diabetes.

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