Laboratory Investigation (2008) 88, 398–407

MKK3 signalling plays an essential role in leukocyte-mediated pancreatic injury in the multiple low-dose streptozotocin model

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In vitro studies have implicated activation of the p38 mitogen-activated protein kinase (MAPK) signalling pathway in cytokine-mediated pancreatic β-cell injury. Activation of the p38 MAPK occurs through two different upstream kinases, mitogen-activated protein kinase kinase 3 (MKK3) and MKK6. This study examined the role of MKK3 signalling in an *in vivo* model of cytokine-dependent pancreatic injury induced by multiple low doses of streptozotocin (MLD-STZ). Groups of wild-type (WT) or *Mkk3−/−* C57BL/6J mice received 5 daily injections of STZ (40 mg/kg) and were killed on day 5, week 2 or week 4. MLD-STZ in WT mice exhibited two distinct phases of pancreatic damage: islet cell apoptosis (immunostaining for cleaved caspase-3) on day 5 in the absence of leukocyte infiltration, and this was followed by islet inflammation (leukocyte infiltration and cytokine production) and further islet cell apoptosis on day 14 resulting in a loss of insulin-producing β-cells and an 80% incidence of hyperglycaemia. *Mkk3−/−* mice were not protected from the initial phase of STZ-induced islet cell apoptosis day 5. However, *Mkk3−/−* mice were completely protected from the induction of hyperglycaemia. This was attributed to inhibition of leukocyte infiltration, production of pro-inflammatory cytokines and islet cell apoptosis at day 14 of MLD-STZ. *In vitro* studies showed that cultured islets from *Mkk3−/−* and WT mice are equally susceptible to STZ and cytokine-induced apoptosis. In conclusion, MKK3 signalling plays an essential role in the development of islet inflammation leading to destruction of β-cells and hyperglycaemia in MLD-STZ-induced pancreatic injury.

Laboratory Investigation (2008) **88,** 398–407; doi:10.1038/labinvest.2008.10; published online 18 February 2008

KEYWORDS: apoptosis; β-cell; cytokine; hyperglycaemia; macrophage; p38 MAPK

The destruction of pancreatic β-cells results in hypoinsulinaema and hyperglycaemia. Immunity and inflammation play an important role in β-cell destruction through the recruitment and activation of T cells and macrophages in pancreatic islets and the local production of inflammatory cytokines such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).[1,2] The pro-inflammatory cytokine IL-1β induces cell death in cultured islets.[3] TNF-α can augment IL-1β-induced cytotoxicity of cultured β cells,[4] whereas a combination of the cytokines IL-1β, TNF-α and IFN-γ has additive cytotoxicity for cultured islets.[5] Evidence that this mechanism contributes to β-cell death *in vivo* comes from IL-1 and TNF-α blockade studies in the multiple low-dose streptozotocin (MLD-STZ) model of hyperglycaemia,[6,7] and blocking IL-1 action prolongs survival of mouse islets in an allograft model.[8] In addition, a recent clinical trial showed that treatment with the IL-1 receptor antagonist improved blood glucose levels and β-cell secretory function and reduced markers of systemic inflammation in patients with type 2 diabetes.[9]

IL-1β and TNF-α are known to activate the three different mitogen-activated protein kinase (MAPK) pathways in pancreatic islets: extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK).[10–12] Several studies

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Received 14 June 2007; revised 15 December 2007; accepted 23 December 2007

have shown that JNK signalling is involved in IL-1β-induced β-cell death *in vitro*,[13,14] whereas most studies suggest that activation of ERK does not participate in cytokine-induced β-cell death.[12,15] However, the role of the p38 MAPK pathway is less clear with some studies finding that blockade of the p38 MAPK pathway can suppress β-cell death in the setting of cytokine or amylin-induced cytotoxicity or in islet grafts,[15–17] whereas other studies have reported that p38 MAPK blockade can exacerbate cytokine-induced β-cell death or has no effect upon cytokine-induced β-cell death.[13,18]

The p38 MAPK is activated by a wide variety of stresses such as pro-inflammatory cytokines (IL-1 and TNF-α), Toll-like receptor activation (innate immunity), reactive oxygen species, osmotic stress and UV irradiation.[10] The p38 kinase is activated by dual phosphorylation of its activation loop through the action of the upstream kinases mitogen-activated protein kinase kinase 3 (MKK3) and MKK6, although other mechanisms of p38 activation can operate in response to specific stimuli.[19] Once activated, the p38 kinase can phosphorylate a variety of transcription factors resulting in cellular responses such as apoptosis, inflammation and fibrosis.[10] However, activation of this pathway can lead to different outcomes depending upon the individual stimulus and cell type involved. For example, p38 signalling promotes reactive oxygen species induced apoptosis of cultured tubular epithelial cells, whereas p38 signalling can suppress apoptosis in this cell type induced by the toxin 1,2-(dichlorovinyl)-L-cysteine.[20,21]

Most of our knowledge on the contribution of p38 MAPK signalling to pancreatic islet damage comes from *in vitro* studies. However, one study has reported the use of systemic administration of a p38 inhibitor to prevent the onset of hyperglycaemia in non-obese diabetic mice.[22] This is a very promising finding, and consistent with studies showing beneficial effects of systemic p38 inhibitor drugs in a range of disease models.[23–27] However, there are concerns that systemic p38 blockade could potentially have deleterious effects.[28] This raises the question of whether it may be possible to dissect the p38 pathway at the level of the upstream kinases, MKK3 and MKK6. A requirement for MKK3–p38 signalling has been demonstrated in mouse models of inflammatory arthritis.[29] MKK3 is expressed in islets;[30] however, the potential contribution of MKK3–p38 signalling in the development of pancreatic islet damage is unknown. To examine this question, we used mice deficient for the *Mkk3* gene to examine the contribution of MKK3–p38 signalling in the MLD-STZ model in which both pro-inflammatory cytokines and the T-cell immune response play a pathogenic role in β-cell destruction leading to hyperglycaemia.[6,7,31–33]

MATERIALS AND METHODS

Multiple Low-Dose Streptozotocin (MLD-STZ) Model

Mkk3−/− gene deficient mice on the C57BL/6J background were bred in house at Monash Animal Services, Clayton, Australia.[34] Male mice (22–26 g) were given daily intraperitoneal injections of 40 mg/kg streptozotocin (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.1 M sodium citrate pH 4.5 for 5 consecutive days. Blood glucose was measured once weekly following a 16 h fast using the glucose oxidase method. Hyperglycaemia was defined as >7 mmol/l fasting blood glucose. Animals were killed at different times (5 days, 2 or 4 weeks) after the start of STZ injections for analysis of the following parameters (group sizes are provided in the figure legends). Day 5 is the peak of STZ-induced islet cell apoptosis, which occurs before leukocyte infiltration. Week 2 is the peak of islet leukocyte infiltration and the peak of leukocyte-induced islet cell apoptosis. Week 4 is used for analysis of pancreatic insulin content and to allow for serial measurements of blood glucose. All animal experimentation was approved by the Monash Medical Centre Animal Ethics Committee.

Histochemistry

Gomori's aldehyde fuchsin staining was performed on 4 μm paraffin sections of formalin-fixed pancreatic tissue as described previously.[35] The percent islet area stained was determined by image analysis using Image Pro Plus 4.0 software (Media Cybernetics, CA, USA). The perimeter of the islet was traced to measure the islet area and then the area of aldehyde fuchsin staining within the islet was assessed using a pre-set threshold and expressed as a percentage.

PAS staining of 4 μm formalin-fixed sections was used to score the degree of insulitis using a semiquantitative scoring system as follows: 0, normal; 1+, minor peri-islet mononuclear cell infiltration; 2+, moderate intra-islet mononuclear cell infiltration ($<50\%$ of islet area); 3+, severe intra-islet cell infiltration ($>50\%$ of islet area) with damage to islet architecture.

Pancreas Insulin Content

Groups of 4 wild-type (WT) and *Mkk3*−/− mice were killed 4 weeks after MLD-STZ. The pancreas was removed and weighed, and then insulin was extracted by homogenization in 5 ml acid ethanol (150 mM HCl in 75% ethanol). After overnight incubation at 4°C, insulin was quantified in the sample supernatant by ELISA (Linco Research, St Charles, MO, USA).

Immunohistochemistry Staining

Immunoperoxidase staining using an avidin–biotin complex (ABC) based system was performed on 4 μm paraffin sections. The following primary antibodies were used: rabbit antibody against cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA); guinea-pig anti-insulin antibody (Dako); F4/80, which detects mouse macrophages (Serotec, Oxford, UK); GK1.5, which detects mouse CD4 and; YTS169.4, which detects mouse CD8.

To quantify apoptotic islet cells, serial sections were stained for cleaved caspase-3 and insulin. For each islet cross-section,

the number of cleaved caspase-3 positive cells was counted within the islet. In the serial section, the area of insulin staining in each islet was analysed by image analysis to normalize the number of apoptotic cells relative to the area of insulin staining and record the number of apoptotic cells per mm^2. This method was also used to confirm that apoptotic cells were insulin positive and to determine the proportion of apoptotic β-cells in the islets.

Isolation of Mouse Pancreatic Islets

Normal mice, or mice after 2 weeks of MLD-STZ, were killed and the common bile duct ligated at the distal end with nylon suture and then cannulated with a 30-gauge needle and syringe to infuse 3 ml of 1 mg/ml ice-cold collagenase P (Roche Biochemicals, Mannheim, Germany) in Hank's balanced salt solution (HBSS). The inflated pancreas was excised and incubated in 2 ml of collagenase P solution at 37°C for 12 min. After a brief vigorous shake to disperse the digested pancreas, islets were washed three times in HBSS with centrifugation for 30 s at $200 \times g$. The islet pellet was either resuspended in RNAlater (Ambion) for RNA extraction, or in culture media for apoptosis studies. The material was then put in petri dishes under a dissecting microscope with reflected lighting and a dark base. Individual islets were recovered using sterile forceps and then used for RNA extraction or cell culture studies.

Glucose-Induced Insulin Secretion by Isolated Islets

Islets were isolated from groups of four normal WT and *Mkk3−/−* mice. Triplicate samples of 8–15 hand-picked islets were incubated overnight at 37°C in RPMI-1640 media with 10% fetal calf serum. The islets were washed and incubated in 0.5 ml Krebs-Ringer Hepes-buffered saline (KRHS) in 3 mmol/l glucose at 37°C for 30 min and then incubated in 0.5 ml KRHS in 20 mmol/l glucose for another 30 min. Supernatants were assessed for insulin content by ELISA. Islets were extracted and analysed for total insulin. The percentage of insulin secreted by islets within the 30 min period of high glucose stimulation was calculated.

Induction of Apoptosis in Cultured Mouse Islets

Isolated pancreatic islets from normal WT or *Mkk3−/−* mice were isolated as described above, cultured for 72 h in DMEM with 10% FCS, and then incubated for 24 h with either 0.5 mM STZ or with a recombinant cytokine mix (10 ng/ml IL-1β, 10 ng/ml TNF-α, 10 ng/ml IFN-γ) (R&D Systems). Apoptosis was quantified by Cell Death Detection ELISA kit (Roche) and normalized against cellular DNA content.

Real Time RT-PCR

Total cellular RNA was extracted from isolated pancreatic islets using the RNAeasy Micro kit (Qiagen, Doncaster, Victoria, Australia) and reverse transcribed using the Superscript First-Strand Synthesis kit (Invitrogen) with oligo-dT primers. Real-time PCR was performed with the primers and probes listed in Table 1 using Rotor-Gene 3000 system (Corbett Research, Sydney, Australia) using the QuantiTect Probe PCR kit (Qiagen) with thermal cycling conditions of 37°C for 10 min to activate uracil–DNA glycosylase, 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. Primers and probes used are listed in Table 1. Standard curves were established for target PCR products including cyclophilin, using serial dilutions of each purified PCR product. Samples were run and the relative abundance of each mRNA was calculated using the $\Delta\Delta C_t$ method and normalized against the cyclophilin mRNA level. All samples were analysed twice and the average result taken.

Table 1 Sequences of primers and probes for real-time RT-PCR

Molecule	Sequences of primers and minor groove binder (mgb) probes
Glut2	ATGAAGAGGAGACTGAAGGA AGCCACCCACCAAAGAATGA TGGTCTCTGTCTGTGTC mgb
IL-1β	CAAGATAGAAGTCAAGAGCAAA TAGAAACAGTCCAGCCCATAC CACAAGCAGAGCACAAG mgb
TNFα	TCTACTCCCAGGTTCTCTTC GCAGAGAGGAGGTTGACTTT TCACCCACACCGTCAG mgb
IFN-γ	CAGCAACAACATAAGCGTCA ACCTCAAACTTGGCAATACTC CAACAGCAAGGCGAAA mgb
IL-4	TGAACGAGGTCACAGGAGAA ACCTTGGAAGCCCTACAGA CACAGCAACGAAGAACAC mgb
iNOS	ACTACTAAATCTCTCTCCTCTCC TCTCTGCTCTCAGCTCCAA TCCCTCCCCTCTCTCC mgb
MCP-1	GACCCGTAAATCTGAAGCTAA CACACTGGTCACTCCTACAGAA ACAACCACCTCAAGCAC mgb
Cyclophilin	GAAGGTGAAAGAAGGCATGAA GCCCGCAAGTCAAAAGAAA CAAGACCAGCAAGAAGA mgb

Statistical Analysis

Data are presented as mean ± s.e.m. Data were analysed by one-way ANOVA with Newman–Keuls multiple comparison *post-test* or by Mann–Whitney *U*-test.

RESULTS

Characterization of Pancreatic Islet Function in Mkk3−/− Mice

Adult Mkk3−/− and WT mice had equivalent levels of fasting blood glucose (5.3 ± 0.6 and 4.8 ± 0.5 mmol/l in

Mkk3−/− *vs* WT, respectively) and fasting plasma insulin (213 ± 23 and 218 ± 39 nmol/l in Mkk3−/− *vs* WT). In addition, glucose-induced insulin released by isolated pancreatic islets was equivalent in Mkk3−/− and WT mice (Figure 1a). Furthermore, basolateral glucose transporter (Glut2) mRNA levels were equivalent in isolated islets from Mkk3−/− and WT mice, which is important since STZ is taken up into β-cells via this transporter.[36,37] We attempted to analyse MKK3 and MKK6 expression and phosphorylation in islets from WT and Mkk3−/− mice; however, immunostaining of tissue sections was unsuccessful despite trying several commercial antibodies and a wide range of antigen retrieval conditions and western blotting was not sufficiently sensitive to detect MKK3 or MKK6 in lysates of isolated islets.

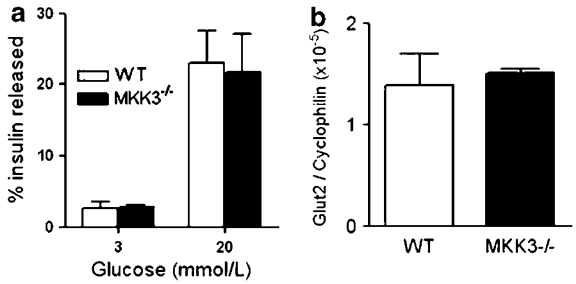

Figure 1 Pancreatic islet function in *Mkk3*−/− mice. (**a**) Islets isolated from normal WT (open bars) or *Mkk3*−/− (closed bars) mice showed a very similar ability to secrete insulin in response to stimulation with high glucose. (**b**) RT-PCR analysis showed no difference in Glut2 mRNA levels between islets isolated from normal WT (open bars) and *Mkk3*−/− (closed bars) mice.

MLD-STZ-Induced Hyperglycaemia

Control WT mice developed hyperglycaemia within 2 weeks of MLD-STZ injections (Figure 2a), with an accumulative 80% incidence (33/41 mice with hyperglycaemia at week 4). Consistent with the development of hyperglycaemia, STZ-WT mice showed a marked reduction in pancreatic insulin levels (Figure 2b), and a substantial loss of Fuchsin stained insulin granules in islet β-cells at the 4-week time point (Figure 2c–e). In contrast, *Mkk3*−/− mice were completely protected from MLD-STZ-induced hyperglycaemia (0/17 mice with hyperglycaemia at week 4) (Figure 2a). Indeed, Mkk3−/− mice were still protected from hyperglycaemia at 8 weeks after MLD-STZ administration (data not shown). There was a partial reduction in pancreatic insulin levels and in the area of islet Fuchsin staining in STZ-*Mkk3*−/− mice, but this was mild compared to the changes seen in STZ-WT mice, and did not affect blood glucose levels (Figure 2b–f).

MLD-STZ-Induced Islet Cell Apoptosis

Loss of β-cells through apoptosis is an important mechanism in the induction of hyperglycaemia in the MLD-STZ model.[38] There are two peaks of β-cell apoptosis—an early induction of apoptosis before islet inflammation on day 5 and a second peak during the development of islet inflammation.[38] We examined islet cell apoptosis by immunostaining for cleaved caspase-3. Examination of MLD-STZ WT mice demonstrated significant islet cell apoptosis on day 5 (in the absence of detectable islet inflammation) and on day 14 (Figure 3). MLD-STZ in *Mkk3*−/− mice induced significant islet cell apoptosis on day 5; however, *Mkk3*−/− mice showed a marked reduction in the second phase of islet cell apoptosis on day 14 (Figure 3). To examine whether MKK3 signalling is directly involved in islet cell apoptosis, we performed *in vitro* studies using islets isolated from normal WT and *Mkk3*−/− mice. The addition of STZ or a cytokine mix induced significant apoptosis in WT islets, with no protection from STZ- or cytokine-induced apoptosis evident in *Mkk3*−/− islets (Figure 4).

MLD-STZ-Induced Islet Inflammation

The second phase of MLD-STZ-induced pancreatic damage results in the development of hyperglycaemia at 2 weeks. Previous studies in this model have shown that insulitis leading to a loss of insulin producing β-cells and the development of hyperglycaemia is dependent on T cells and macrophages,[31,39–43] with roles for individual cytokines (IL-1, TNF-α and IFN-γ) in β-cell destruction identified.[6,7,32,44] In the current study, MLD-STZ WT mice developed significant insulitis at 2 weeks (Figure 5). Immunohistochemistry staining showed peri-islet and intra-islet infiltration of F4/80+ macrophages, CD4+ T cells and CD8+ T cells in islets at the 2-week time point (Figure 6). Furthermore, MLD-STZ WT mice showed a significant increase in islet mRNA levels for IFN-γ, IL-4, IL-1β, TNF-α, inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein-1 (MCP-1) as determined by real-time RT-PCR analysis of isolated islets (Figure 7). Western blotting was attempted to examine the islet content of these proteins, but was unsuccessful due to insufficient material in these samples.

Mkk3−/− mice were largely protected from the second phase of MLD-STZ-induced pancreatic damage at the 2-week time point. This was shown by a reduction in the severity of insulitis (Figure 5). Consistent with this analysis, immunostaining found no significant peri- or intra-islet infiltration by F4/80+ macrophages, CD4+ T cells or CD8+ T cells (Figure 6). This reduction in leukocyte infiltration in STZ *Mkk3*−/− mice was associated with a lack of upregulation of the islet mRNA levels for IFN-γ, IL-1β, TNF-α and MCP-1 (Figure 7). However, there was an increase in islet IL-4 and iNOS mRNA levels in MLD-STZ *Mkk3*−/− mice that is comparable to that seen in MLD-STZ in WT mice (Figure 7), which is likely due to expression of these molecules by intrinsic islet cells.

DISCUSSION

Mice deficient in the *Mkk3* gene were found to have normal islet function in terms the ability to secrete insulin in re-

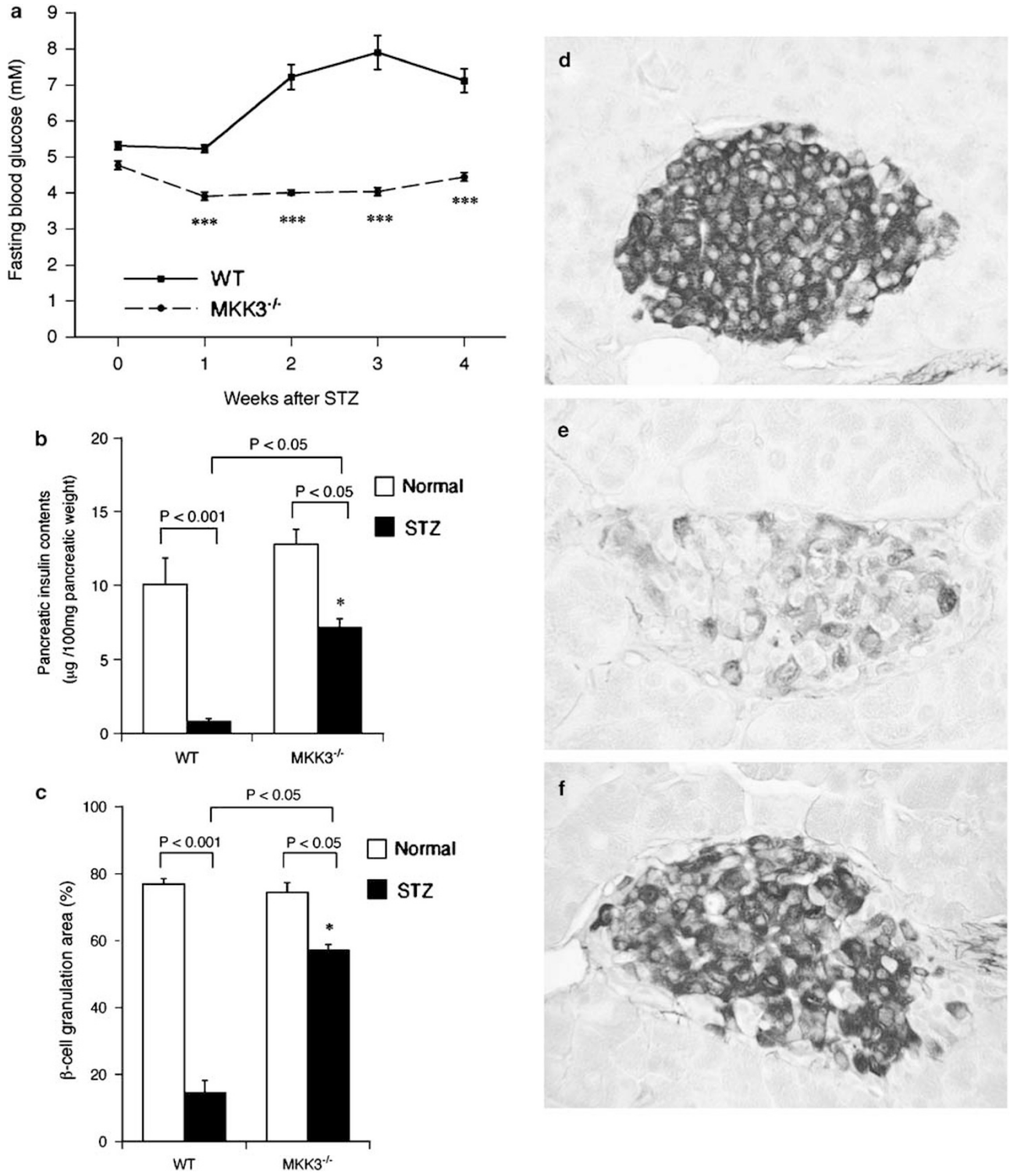

Figure 2 *Mkk3−/−* mice are resistant to hyperglycaemia in MLD-STZ. (**a**) Fasting blood glucose was measured on a weekly basis after MLD-STZ administration. Most WT mice developed hyperglycaemia (>7 mmol/l) by week 3. In contrast, fasting blood glucose remained normal in *Mkk3−/−* mice after MLD-STZ administration (***$P<0.0001$ *vs* WT). (**b**) The insulin content of whole pancreas tissue was measured in normal mice (open bars) and mice at week 4 of MLD-STZ (closed bar) ($n=4$). (**c**) The degree of β-cell granulation in pancreatic islets was assessed by Gomori's aldehyde fuchsin staining in normal mice (open bars) and at week 4 of MLD-STZ (closed bars) in WT and *Mkk3−/−* mice ($n=7$–8). Examples of Fuchsin staining in: (**d**) normal WT mice; (**e**) MLD-STZ in WT mice showing a dramatic reduction in β-cell granulation, and; (**f**) MLD-STZ in *Mkk3−/−* mice which are largely protected from loss of β-cell granulation.

sponse to glucose stimulation. The normal levels of blood glucose and plasma insulin in fasted animals also indicates normal islet function in *Mkk3−/−* mice. Thus, the remarkable resistance of *Mkk3−/−* mice to MLD-STZ induced hyperglycaemia is likely to be due to a modified immune response. The potential mechanisms by which *Mkk3−/−* mice are protected from hyperglycaemia in this model are considered below.

The MLD-STZ model has two distinct phases of pancreatic injury.[38] STZ induces acute toxic effects upon islet β-cells which is followed by leukocytic infiltration and the production of pro-inflammatory cytokines which completes β-cell destruction leading to hyperglycaemia. *Mkk3−/−* mice were not protected from the acute toxic effects of MLD-STZ administration on the basis that levels of islet cell apoptosis were comparable to than seen in MLD-STZ WT mice on day 5. In addition, cultured *Mkk3−/−* islets were equally susceptible to STZ induced apoptosis as those from WT mice. This is consistent with the equivalent expression of Glut2, the transport molecule that takes STZ into β-cells,[36,37] in WT and *Mkk3−/−* islets.

The protective effect of *Mkk3* gene deletion in the MLD-STZ model was attributed to suppression of islet inflammation on day 14 following the initial STZ-mediated pancreatic insult. It is known that both T cells and macrophages play an important role in islet destruction leading to hyperglycaemia in the MLD-STZ model,[31,39–43] and thus the lack of T cell and macrophage infiltration seen in *Mkk3−/−* mice is likely to be a major reason for their protection from MLD-STZ induced hyperglycaemia. Furthermore, infiltrating macrophages and T cells are a major source of the pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ that promote islet destruction in this model.[6,7,17,32,44] However, it should be noted that *Mkk3−/−* islets were not protected from cytokine-induced apoptosis *in vitro*, suggesting that protection of *Mkk3−/−* mice from islet destruction in the MLD-STZ model is due to a failure to upregulate islet mRNA levels for these pro-inflammatory cytokines rather than any intrinsic resistance of *Mkk3−/−* β-cells to cytokine-induced cytotoxicity. In addition, this finding is consistent with a study in which treatment with a p38 inhibitor drug prevented upregulation of IL-1β and TNF-α mRNA levels in human islets and improved their function after transplantation into diabetic athymic mice.[17]

The lack of islet monocyte/macrophage infiltration in *Mkk3−/−* mice in the MLD-STZ model may be due to a

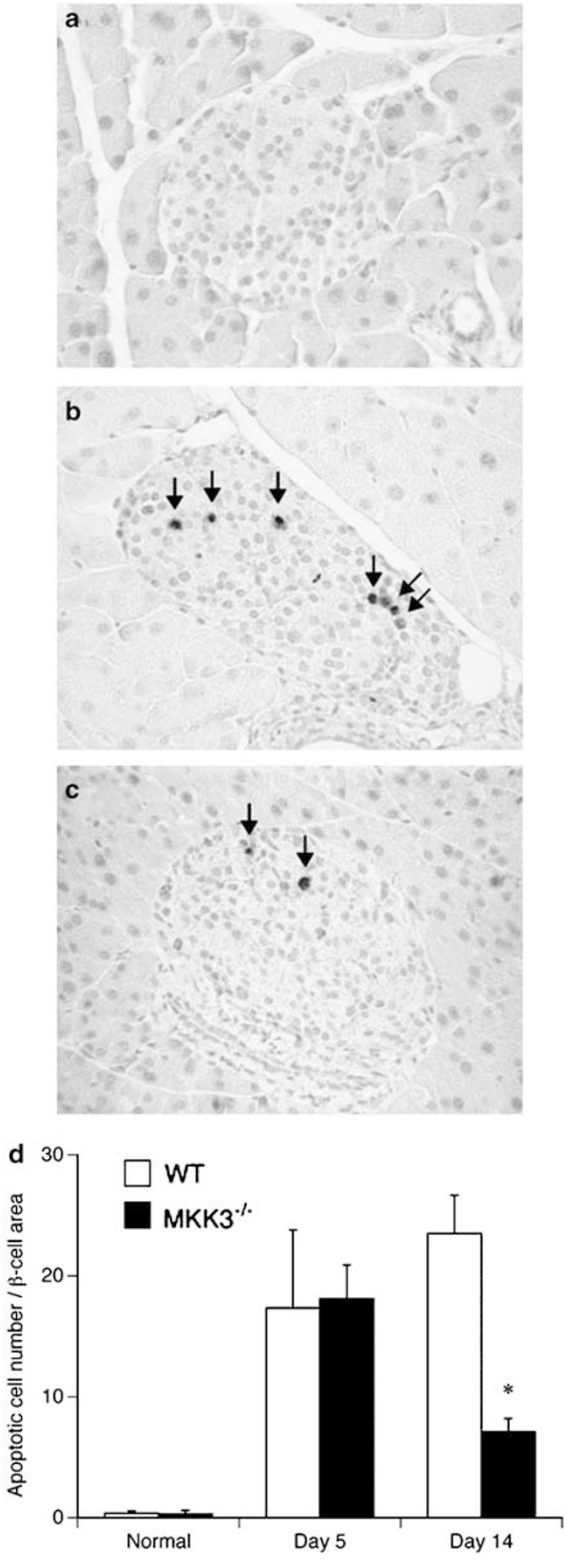

Figure 3 Islet cell apoptosis in MLD-STZ. Immunostaining for cleaved caspase-3 in pancreas tissue sections. (**a**) Normal WT mouse pancreas with no staining. (**b**) Day 14 of MLD-STZ in WT mice showing several apoptotic cells with nuclear staining for cleaved caspase-3 (arrows). (**c**) Fewer apoptotic cells (arrows) are seen in islets of *Mkk3−/−* mice on day 14 of MLD-STZ. (**d**) Quantification of apoptotic cells based upon immunostaining for cleaved caspase-3 in normal mice ($n=3$), on day 5 ($n=4$–5), or on day 14 ($n=8$–10) after STZ administration in WT (open bars) and *Mkk3−/−* (closed bars) mice. $^*P<0.05$ *vs* WT by ANOVA with Newman–Keuls multiple comparison post-test.

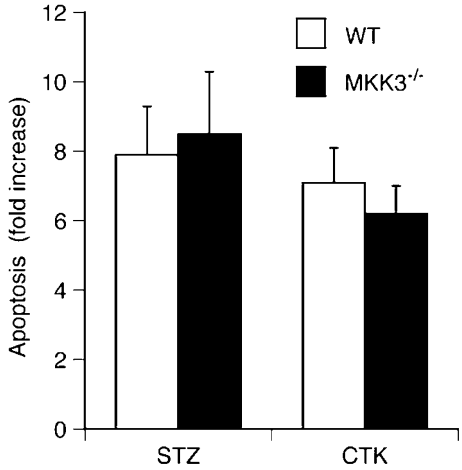

Figure 4 STZ- and cytokine-induced apoptosis of cultured islets. Islets were isolated from normal WT or *Mkk3−/−* mice, cultured and then stimulated for 24 h with either 0.5 mM STZ or a cytokine mix (CTK = 10 ng/ml IL-1β, 10 ng/ml TNF-α, 10 ng/ml IFN-γ). Apoptosis was measured using the cell death detection ELISA kit. Values are presented as an OD ratio of sample to untreated negative control after normalizing by DNA content. There are no significant differences between WT and *Mkk3−/−* islets.

failure to upregulate islet MCP-1 mRNA levels as shown by real-time RT-PCR analysis of isolated islets. This, in turn, may be due to the failure to upregulate islet IL-1β mRNA levels since IL-1 has been shown to induce MCP-1 production by cultured human islets and rat β-cells.[45,46] Furthermore, IL-1 induced upregulation of MCP-1 mRNA levels in cultured rat β-cells is, in part, dependent upon p38 MAPK signalling.[45]

It is interesting that the lack of MKK3–p38 signalling did not prevent upregulation of IL-4 mRNA in islets in the MLD-STZ model. Previous studies have shown that IL-4 is produced by intrinsic islet cells,[32] indicating that STZ-induced pancreatic injury was able to upregulate IL-4 mRNA levels in the absence of leukocyte infiltration and MKK3–p38 signalling. However, IL-4 is not protective in the MLD-STZ model.[32]

The role of nitric oxide in islet damage, including cytokine-induced islet cytotoxicity, is a controversial topic.[47] A number of studies have argued against a role for nitric oxide in the MLD-STZ model,[48,49] whereas iNOS−/− mice are partially protected from MLD-STZ-induced hyperglycaemia with a reduction in islet leukocytic infiltration.[50] Consistent with previous studies, we found a significant increase in islet iNOS mRNA in WT mice in the MLD-STZ model. Of interest, normal *Mkk3−/−* mice showed higher basal levels of iNOS mRNA in islets compared to normal WT mice, suggesting an inhibitory role for MKK3–p38 signalling in regulation of iNOS gene expression in intrinsic islet cells. MLD-STZ in *Mkk3−/−* mice induced an increase in islet iNOS mRNA levels even in the absence of an increase in the expression of well-known inducers of iNOS expression,

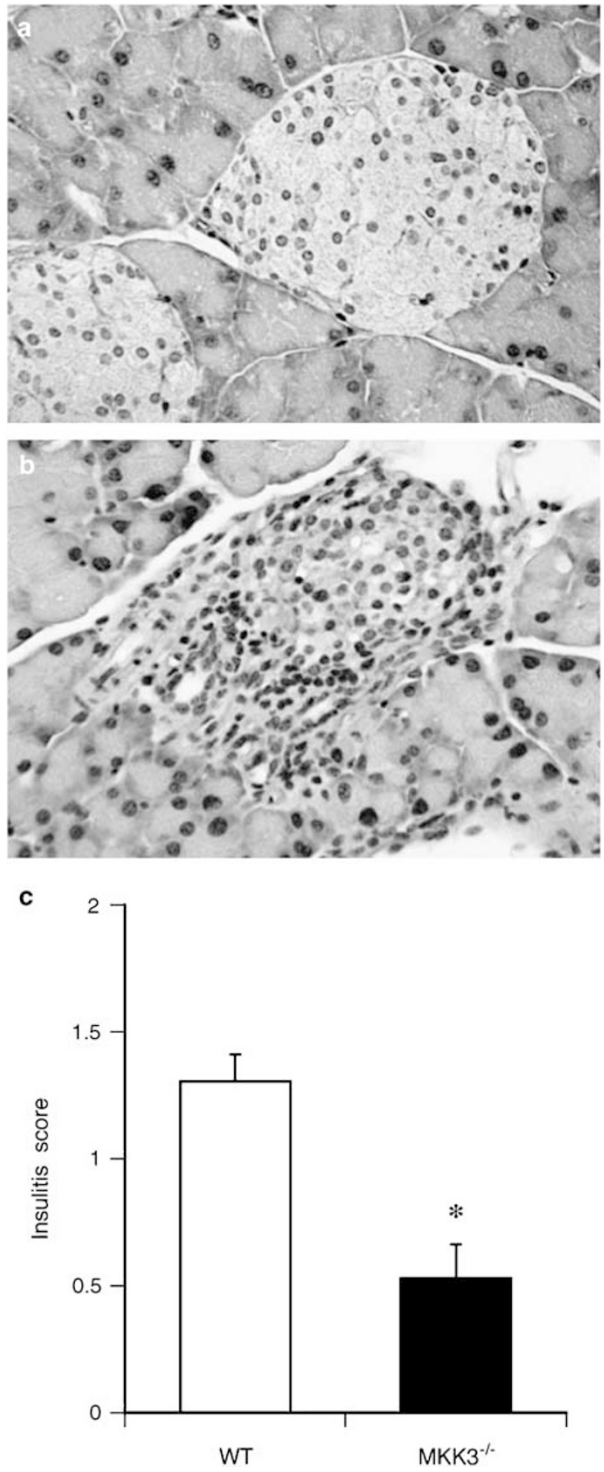

Figure 5 Insulitis in MLD-STZ. The degree of insulitis was assessed on PAS-stained sections. (**a**) An untreated WT mouse shows normal islet structure. (**b**) An example of peri-islet and intra-islet mononuclear cell infiltration with a marked loss of architecture in a WT mouse 2 weeks after MLD-STZ (3+ score). (**c**) Graph showing the degree of insulitis as assessed using a semi-quantitative scoring system. *$P<0.001$ compared to WT by Mann–Whitney *U*-test.

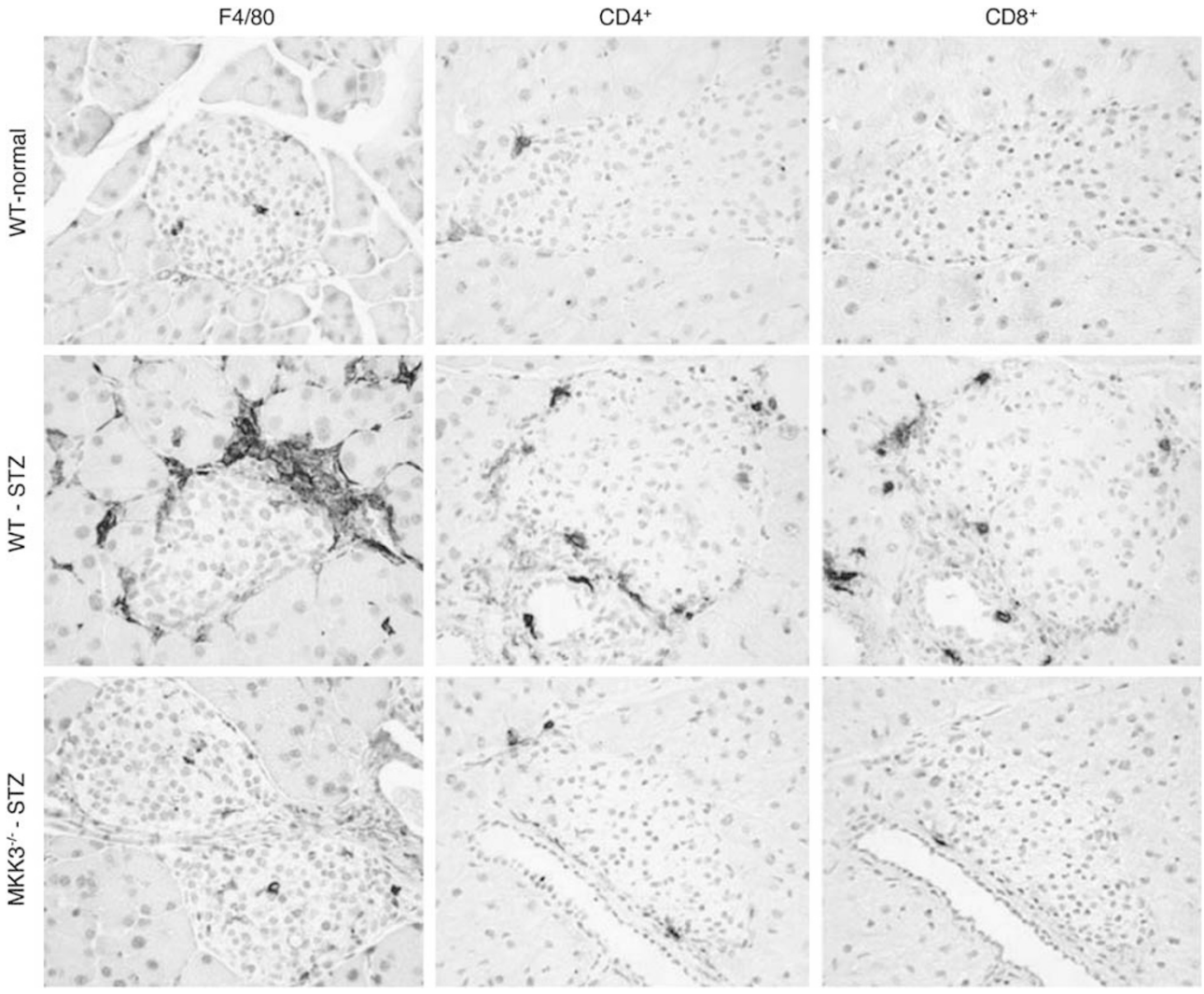

Figure 6 Macrophage and T-cell infiltration in MLD-STZ. Immunostaining of normal WT mice shows a small number of F4/80+ macrophages in pancreatic islets and only occasional CD4+ or CD8+ T cells in normal islets. At 2 weeks after MLD-STZ in WT mice there is a marked peri-islet and intra-islet F4/80+ macrophage infiltrate and a marked islet infiltrate of CD4+ and CD8+ T cells. In contrast, no leukocytic infiltrate is seen in *Mkk3*−/− mice at 2 weeks after MLD-STZ. Photomicrographs of immunostaining are representative of leukocyte infiltration as analysed in groups of 4–6 mice.

such as IFN-γ, IL-1β and TNF-α. Indeed, *Mkk3*−/− mice were protected from apoptosis at day 14 of MLD-STZ despite augmented islet iNOS mRNA levels, suggesting that nitric oxide may only be a minor player in islet apoptosis *in vivo*, although further studies are needed to validate this conclusion.

The results of the current study are consistent with a recent report in which administration of a p38 inhibitor, FR167653, was shown to reduce insulitis and development of hyperglycaemia in non-obese diabetic mice.[22] However, there are concerns that p38 blockade could have deleterious effects based upon the fetal lethal phenotype of p38α gene knockout mice,[51] impaired clearance of pneumococcal pneumonia and tuberculosis infections in mice treated with a p38 inhibitor,[52] exacerbation of renal injury in some models of kidney disease[53,54] and clinical trials in which p38 inhibitors have caused hepatotoxicity.[28] Our study has identified that signalling via MKK3 is sufficient for leukocyte-mediated islet cell destruction leading to hyperglycaemia. This opens up the possibility for the use of selective MKK3 inhibitors to prevent the onset of hyperglycaemia, or to prevent its reoccurrence after transplantation.

In conclusion, MKK3 signalling plays an essential role in the development of hyperglycaemia in the MLD-STZ model. This is postulated to operate via MKK3 signalling in damaged islets leading to MCP-1 production that, in turn, induces macrophage infiltration with the leukocyte-derived cytokines causing destruction of the remaining islet cells resulting in insulin deficiency and hyperglycaemia.

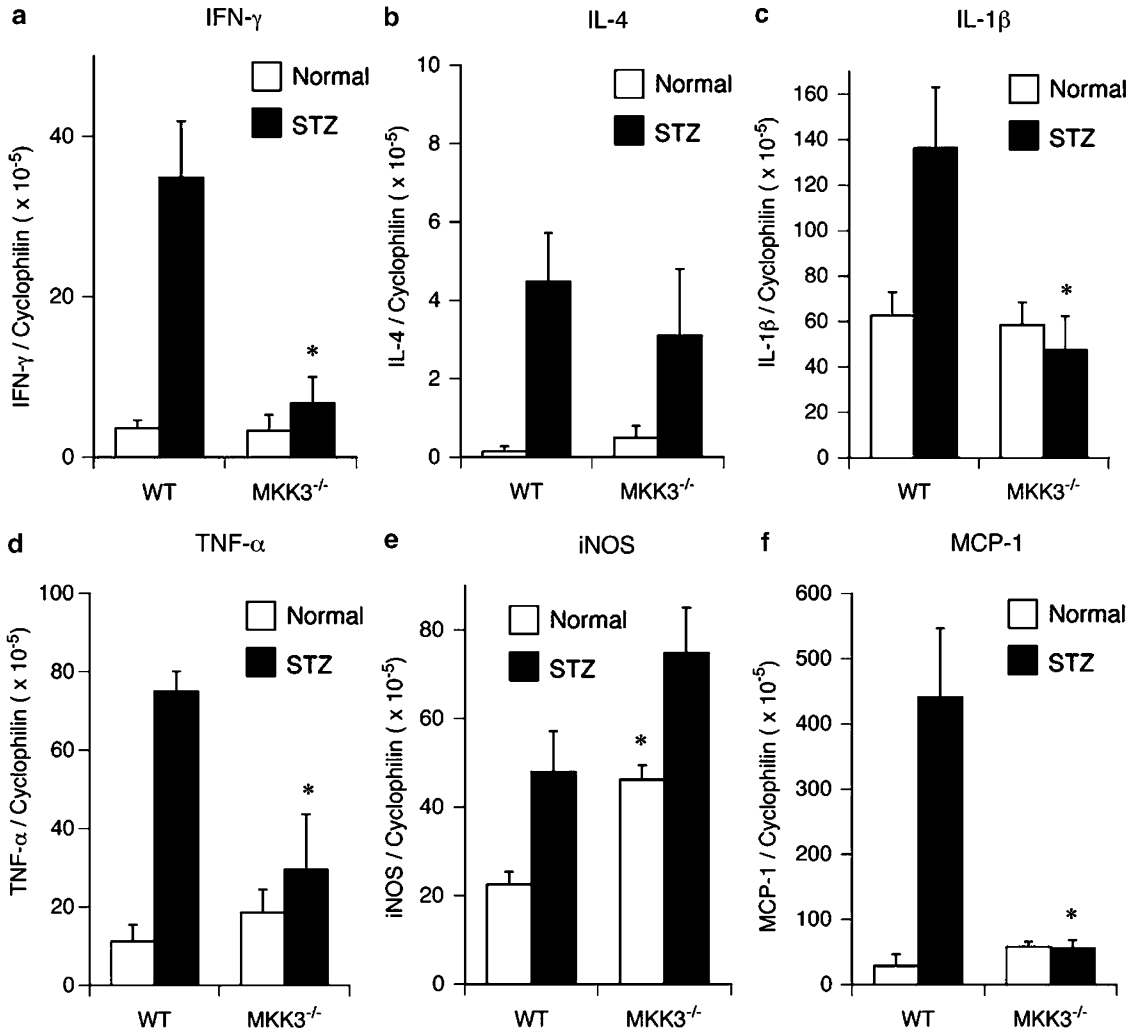

Figure 7 Analysis of pro-inflammatory molecules in islets in MLD-STZ. Pancreatic islets were isolated from normal WT and *Mkk3−/−* mice ($n = 4–5$), and from WT and *Mkk3−/−* mice 2 weeks after MLD-STZ ($n = 8–10$). Islet mRNA levels were analysed by real-time RT-PCR for: (**a**) IFN-γ, (**b**) IL-4, (**c**) IL-1β, (**d**) TNF-α, (**e**) iNOS and (**f**) MCP-1. Normal mice (day 0, open bars) and MLD-STZ mice (day 14 MLD-STZ, closed bars). $^*P < 0.05$ compared to WT by ANOVA.

ACKNOWLEDGEMENT

This study was supported by the National Health and Medical Research Council of Australia.

1. Hohmeier HE, Tran VV, Chen G, *et al.* Inflammatory mechanisms in diabetes: lessons from the beta-cell. Int J Obes Relat Metab Disord 2003;27(Suppl 3):S12–S16.
2. Kawasaki E, Abiru N, Eguchi K. Prevention of type 1 diabetes: from the view point of beta cell damage. Diabetes Res Clin Pract 2004;66(Suppl 1):S27–S32.
3. Bendtzen K, Mandrup-Poulsen T, Nerup J, *et al.* Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. Science 1986;232:1545–1547.
4. Mandrup-Poulsen T, Bendtzen K, Dinarello CA, *et al.* Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic beta-cell cytotoxicity. J Immunol 1987;139: 4077–4082.
5. Eizirik DL, Sandler S, Welsh N, *et al.* Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. J Clin Invest 1994;93:1968–1974.
6. Sandberg JO, Andersson A, Eizirik DL, *et al.* Interleukin-1 receptor antagonist prevents low dose streptozotocin induced diabetes in mice. Biochem Biophys Res Commun 1994;202: 543–548.
7. Holstad M, Sandler S. A transcriptional inhibitor of TNF-alpha prevents diabetes induced by multiple low-dose streptozotocin injections in mice. J Autoimmunity 2001;16:441–447.
8. Sandberg JO, Eizirik DL, Sandler S, *et al.* Treatment with an interleukin-1 receptor antagonist protein prolongs mouse islet allograft survival. Diabetes 1993;42:1845–1851.
9. Larsen CM, Faulenbach M, Vaag A, *et al.* Interleukin-1-receptor antagonist in type 2 diabetes mellitus. N Engl J Med 2007;356: 1517–1526.
10. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81:807–869.
11. Welsh N. Interleukin-1 beta-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F. J Biol Chem 1996;271:8307–8312.
12. Larsen CM, Wadt KA, Juhl LF, *et al.* Interleukin-1beta-induced rat pancreatic islet nitric oxide synthesis requires both the p38 and

extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases. J Biol Chem 1998;273:15294–15300.
13. Ammendrup A, Maillard A, Nielsen K, *et al.* The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. Diabetes 2000;49:1468–1476.
14. Bonny C, Oberson A, Negri S, *et al.* Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. Diabetes 2001;50:77–82.
15. Saldeen J, Lee JC, Welsh N. Role of p38 mitogen-activated protein kinase (p38 MAPK) in cytokine-induced rat islet cell apoptosis. Biochem Pharmacol 2001;61:1561–1569.
16. Zhang S, Liu H, Liu J, *et al.* Activation of activating transcription factor 2 by p38 MAP kinase during apoptosis induced by human amylin in cultured pancreatic beta-cells. FEBS J 2006;273:3779–3791.
17. Matsuda T, Omori K, Vuong T, *et al.* Inhibition of p38 pathway suppresses human islet production of pro-inflammatory cytokines and improves islet graft function. Am J Transplant 2005;5:484–493.
18. Pavlovic D, Andersen NA, Mandrup-Poulsen T, *et al.* Activation of extracellular signal-regulated kinase (ERK)1/2 contributes to cytokine-induced apoptosis in purified rat pancreatic beta-cells. Eur Cytokine Netw 2000;11:267–274.
19. Kang YJ, Seit-Nebi A, Davis RJ, *et al.* Multiple activation mechanisms of p38alpha mitogen-activated protein kinase. J Biol Chem 2006;281:26225–26234.
20. Dong J, Ramachandiran S, Tikoo K, *et al.* EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death. Am J Physiol Renal Physiol 2004;287:F1049–F1058.
21. de Graauw M, Tijdens I, Cramer R, *et al.* Heat shock protein 27 is the major differentially phosphorylated protein involved in renal epithelial cellular stress response and controls focal adhesion organization and apoptosis. J Biol Chem 2005;280:29885–29898.
22. Ando H, Kurita S, Takamura T. The specific p38 mitogen-activated protein kinase pathway inhibitor FR167653 keeps insulitis benign in nonobese diabetic mice. Life Sci 2004;74:1817–1827.
23. Jackson JR, Bolognese B, Hillegass L, *et al.* Pharmacological effects of SB 220025, a selective inhibitor of P38 mitogen-activated protein kinase, in angiogenesis and chronic inflammatory disease models. J Pharmacol Exp Ther 1998;284:687–692.
24. Underwood DC, Osborn RR, Bochnowicz S, *et al.* SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. Am J Physiol Lung Cell Mol Physiol 2000;279: L895–L902.
25. Behr TM, Nerurkar SS, Nelson AH, *et al.* Hypertensive end-organ damage and premature mortality are p38 mitogen-activated protein kinase-dependent in a rat model of cardiac hypertrophy and dysfunction. Circulation 2001;104:1292–1298.
26. Stambe C, Atkins RC, Tesch GH, *et al.* The role of p38alpha mitogen-activated protein kinase activation in renal fibrosis. J Am Soc Nephrol 2004;15:370–379.
27. Stambe C, Atkins RC, Tesch GH, *et al.* Blockade of p38alpha MAPK ameliorates acute inflammatory renal injury in rat anti-GBM glomerulonephritis. J Am Soc Nephrol 2003;14:338–351.
28. Kumar S, Boehm J, Lee JC. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov 2003;2:717–726.
29. Inoue T, Boyle DL, Corr M, *et al.* Mitogen-activated protein kinase kinase 3 is a pivotal pathway regulating p38 activation in inflammatory arthritis. Proc Natl Acad Sci USA 2006;103:5484–5489.
30. Abdelli S, Ansite J, Roduit R, *et al.* Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes 2004;53:2815–2823.
31. Kim YT, Steinberg C. Immunologic studies on the induction of diabetes in experimental animals. Cellular basis for the induction of diabetes by streptozotocin. Diabetes 1984;33:771–777.
32. Herold KC, Vezys V, Sun Q, *et al.* Regulation of cytokine production during development of autoimmune diabetes induced with multiple low doses of streptozotocin. J Immunol 1996;156:3521–3527.
33. Muller A, Schott-Ohly P, Dohle C, *et al.* Differential regulation of Th1-type and Th2-type cytokine profiles in pancreatic islets of C57BL/6 and BALB/c mice by multiple low doses of streptozotocin. Immunobiology 2002;205:35–50.
34. Wysk M, Yang DD, Lu HT, *et al.* Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. Proc Natl Acad Sci USA 1999;96:3763–3768.
35. Peterson JD, Pike B, McDuffie M, *et al.* Islet-specific T cell clones transfer diabetes to nonobese diabetic (NOD) F1 mice. J Immunol (Baltimore, MD, USA) 1994;153:2800–2806.
36. Schnedl WJ, Ferber S, Johnson JH, *et al.* STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes 1994;43:1326–1333.
37. Wang Z, Gleichmann H. GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice. Diabetes 1998;47:50–56.
38. O'Brien BA, Harmon BV, Cameron DP, *et al.* Beta-cell apoptosis is responsible for the development of IDDM in the multiple low-dose streptozotocin model. J Pathol 1996;178:176–181.
39. Herold KC, Montag AG, Fitch FW. Treatment with anti-T-lymphocyte antibodies prevents induction of insulitis in mice given multiple doses of streptozocin. Diabetes 1987;36:796–801.
40. Herold KC, Montag AG, Buckingham F. Induction of tolerance to autoimmune diabetes with islet antigens. J Exp Med 1992;176: 1107–1114.
41. Herold KC, Bloch TN, Vezys V, *et al.* Diabetes induced with low doses of streptozotocin is mediated by V beta 8.2+ T-cells. Diabetes 1995;44:354–359.
42. Ihm SH, Lee KU, Rhee BD, *et al.* Initial role of macrophage in the development of anti-beta-cell cellular autoimmunity in multiple low-dose streptozotocin-induced diabetes in mice. Diabetes Res Clin Pract 1990;10:123–126.
43. Mensah-Brown E, Shahin A, Parekh K, *et al.* Functional capacity of macrophages determines the induction of type 1 diabetes. Ann N Y Acad Sci 2006;1084:49–57.
44. Cockfield SM, Ramassar V, Urmson J, *et al.* Multiple low dose streptozotocin induces systemic MHC expression in mice by triggering T cells to release IFN-gamma. J Immunol 1989;142: 1120–1128.
45. Chen MC, Proost P, Gysemans C, *et al.* Monocyte chemoattractant protein-1 is expressed in pancreatic islets from prediabetic NOD mice and in interleukin-1 beta-exposed human and rat islet cells. Diabetologia 2001;44:325–332.
46. Welsh N, Cnop M, Kharroubi I, *et al.* Is there a role for locally produced interleukin-1 in the deleterious effects of high glucose or the type 2 diabetes milieu to human pancreatic islets? Diabetes 2005;54: 3238–3244.
47. Eizirik DL, Pavlovic D. Is there a role for nitric oxide in beta-cell dysfunction and damage in IDDM? Diabetes Metab Rev 1997;13: 293–307.
48. Holstad M, Sandler S. Aminoguanidine, an inhibitor of nitric oxide formation, fails to protect against insulitis and hyperglycemia induced by multiple low dose streptozotocin injections in mice. Autoimmunity 1993;15:311–314.
49. Sternesjo J, Welsh N, Sandler S. S-methyl-L-thiocitrulline counteracts interleukin 1 beta induced suppression of pancreatic islet function *in vitro*, but does not protect against multiple low-dose streptozotocin-induced diabetes *in vivo*. Cytokine 1997;9:352–359.
50. Flodstrom M, Tyrberg B, Eizirik DL, *et al.* Reduced sensitivity of inducible nitric oxide synthase-deficient mice to multiple low-dose streptozotocin-induced diabetes. Diabetes 1999;48: 706–713.
51. Tamura K, Sudo T, Senftleben U, *et al.* Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. Cell 2000;102:221–231.
52. van den Blink B, Juffermans NP, ten Hove T, *et al.* p38 mitogen-activated protein kinase inhibition increases cytokine release by macrophages *in vitro* and during infection *in vivo*. J Immunol 2001;166:582–587.
53. Ohashi R, Nakagawa T, Watanabe S, *et al.* Inhibition of p38 mitogen-activated protein kinase augments progression of remnant kidney model by activating the ERK pathway. Am J Pathol 2004;164: 477–485.
54. Aoudjit L, Stanciu M, Li H, *et al.* p38 mitogen-activated protein kinase protects glomerular epithelial cells from complement-mediated cell injury. Am J Physiol Renal Physiol 2003;285:F765–F774.