Disruption of the Jnk2 (Mapk9) gene reduces destructive insulitis and diabetes in a mouse model of type 1 diabetes

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The c-Jun NH2-terminal kinase isoform (JNK) 1 is implicated in type 2 diabetes. However, a potential role for the JNK2 protein kinase in diabetes has not been established. Here, we demonstrate that JNK2 may play an important role in type 1 (insulin-dependent) diabetes that is caused by autoimmune destruction of β cells. Studies of nonobese diabetic (NOD) mice demonstrated that disruption of the Mapk9 gene (which encodes the JNK2 protein kinase) decreased destructive insulitis and reduced disease progression to diabetes. CD4+ T cells from JNK2-deficient nonobese diabetic mice produced less IFN-γ but significantly increased amounts of IL-4 and IL-5, indicating polarization toward the Th2 phenotype. This role of JNK2 in control of the Th1/Th2 balance of the immune response represents a mechanism of protection against autoimmune diabetes. We conclude that JNK protein kinases may have important roles in diabetes, including functions of JNK1 in type 2 diabetes and JNK2 in type 1 diabetes.

Failure of the insulin-producing β cells in the pancreatic islets of Langerhans is the common characteristic of type 1 (insulin-dependent) and type 2 (insulin-independent) diabetes mellitus. Inflammatory cytokines and oxidative stress produced under hyperglycemic conditions are involved in the progression of β cell dysfunction in both types of diabetes. The (c-Jun N-terminal kinase) JNK group of mitogen-activated protein kinase (MAPK) is activated by cytokines and reactive oxygen species and may therefore contribute to this process. Indeed, the JNK group of MAPKs is implicated in type 2 diabetes because these protein kinases can phosphorylate the insulin receptor substrate IRS1, thereby suppressing insulin signal transduction (1, 2). Genetic evidence that supports this conclusion has been obtained from studies of knockout mice. Thus, disruption of the Mapk8 or Mapk8ip1 genes (which encode the JNK1 protein kinase and the JNK scaffold protein JIP1, respectively) prevents peripheral insulin resistance and obesity caused by feeding a high fat diet (3, 4). Interestingly, signaling by JNK1, but not by JNK2, is implicated in obesity and insulin resistance associated with type 2 diabetes (4). The role of JNK2 in diabetes is therefore unclear. One possibility is that JNK2 may play a role in type 1 diabetes.

In type 1 diabetes, the cooperation of CD4+ and CD8+ T cells is required for islet infiltration and destruction of β cells (5, 6). Proinflammatory cytokines produced by islet-infiltrating immune cells have been implicated as effector molecules. Indeed, IL-1β in combination with IFN-γ and TNF-α triggers apoptosis of β cells (7). The cytokine environment enables the differentiation of CD4+ T cells to two different phenotypes: Th1 cells produce IL-2 and IFN-γ, which induce a cellular immune response. In contrast, Th2 cells secrete IL-4, IL-5, and IL-10, which support humoral immunity and down-regulate the inflammatory actions of Th1 cells. The Th1/Th2 balance is critical for the development and resolution of immune responses. Importantly, benign insulitis is associated with Th2 cells, whereas destructive insulitis appears to be associated with Th1 cells (8). One signaling pathway that has been implicated in the regulation of CD4+ T cell differentiation is the JNK group of MAPKs (9, 10).

Here, we tested whether JNK2 may play a role in type 1 diabetes by using the nonobese diabetic (NOD) mouse model of autoimmune diabetes (11). Disruption of the Mapk9 gene (which encodes the JNK2 protein kinase) decreased destructive insulitis and reduced disease progression to diabetes. CD4+ T cells from JNK2-deficient NOD mice produced less IFN-γ but significantly increased amounts of IL-4 and IL-5. These findings suggest a role for JNK2 in controlling the Th1/Th2 balance of the immune response, thereby providing protection against autoimmune diabetes.

Methods

Mice. NOD/Lt (NOD) and NOD/LtSz-Prkdcscid/Prkdcscid (NOD/Scid) mice were obtained from The Jackson Laboratory. C57BL/6.129-Mapk9tm1 Flv/J (Mapk9−/−) mice (12) were back-crossed with NOD mice for six generations and Mapk9−/− mice that are homozygous for the 15 Idd NOD alleles were identified by PCR genotyping, as described in ref. 13. These NOD/Mapk9−/− mice were back-crossed for an additional four generations onto the NOD background. NOD/Mapk9−/− mice were obtained by crossing NOD/Mapk9−/− mice. The Mapk9 genotype was examined by PCR analysis (12). Blood glucose levels were monitored weekly with a Dex-Glucometer (Bayer). Animals with a blood glucose level >200 mg/dl for two consecutive weeks were considered diabetic. The mice were housed in a facility accredited by the American Association for Laboratory Animal Care, and the animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Adaptive Cell Transfer Studies. NOD/Scid mice were injected intravenously with 2 × 106 total splenocytes and were monitored (14 weeks) for diabetes. NOD mice (7-week-old males) were irradiated (725 rad) one day before adoptive transfer of 2 × 107 total splenocytes from recently diagnosed diabetic donors by i.v. injection; the mice were monitored (8 weeks) for diabetes.

Abbreviations: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NOD, nonobese diabetic.

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The presence of hyperglycemia (blood glucose >200 mg/dl) was examined in a cohort of 32 female NOD mice and 28 female NOD/Mapk9<sup>−/−</sup> mice. The data are presented as the percent of mice with hyperglycemia. The time to diabetes onset estimated by Kaplan-Meier analysis demonstrated that the NOD/Mapk9<sup>−/−</sup> mice and the NOD mice were significantly different (log-rank test, \( P = 0.0011 \)).

**Immunohistochemical Analysis.** Pancreata were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Immunohistochemistry was performed by using tissue embedded in Tissue-Tek OCT and frozen in 2-methylbutane. Five-micrometer sections were stained with fluorescein isothiocyanate-conjugated antibodies to CD4 and CD8 (Pharmingen) and mounted in Vectashield with DAPI (Vector Laboratories). Infiltration was assessed by examining the pancreas of 6 mice per group and analysis of 10 to 20 islets per mouse.

**Cell Culture and Cytokine Secretion.** Total CD4<sup>+</sup> cells were isolated from spleen and lymph nodes by negative selection with anti-NK1.1 (Pharmingen), anti-CD8 (TB105), anti-Mac1 (Pharmingen), and anti-MHC class II (m5/115) mAbs after depletion with magnetic beads as described in ref. 13. CD4<sup>+</sup> T cells were activated (10<sup>6</sup> cells per ml) with immobilized anti-CD3 mAb (2C11) (5 \( \mu \)g/ml) and anti-CD28 mAb (Pharmingen) (1 \( \mu \)g/ml) in the presence of medium (for Th0 cells), IL-4 (for Th2 cells) (R & D Systems) (10<sup>3</sup> units/ml) or IL-12 (for Th1 cells) (Genetics Institute, Cambridge, MA) (3.5 ng/ml) for 4 days. Cells were then extensively washed, counted, and equal numbers of cells were restimulated with immobilized anti-CD3 mAb for 24 h when the supernatant was harvested. Cytokine production was determined by ELISA with anti-IL4, anti-IL5, or anti-IFN-\( \gamma \) mAb (2 \( \mu \)g/ml), biotinylated anti-IL4, anti-IL5, or anti-IFN-\( \gamma \) mAb (1 \( \mu \)g/ml) (Pharmingen), horseradish peroxidase-conjugated avidin D (Sigma), and peroxidase substrate and reaction stop solutions (Kirkegaard & Perry Laboratories) according to the manufacturer’s recommended protocol (Pharmingen).

**Results**

**Spontaneous Diabetes and Insulitis Is Decreased in JNK2-Deficient Mice.** Comparison of the spontaneous incidence of disease in female NOD and NOD/Mapk9<sup>−/−</sup> mice indicated that JNK2 deficiency decreased the cumulative incidence of diabetes (log-rank test, \( P = 0.0011 \); Fig. 1). To investigate the mechanisms underlying the effect of JNK2 on diabetes in NOD mice, we performed histological analysis of the pancreas. At 13 weeks of age, control NOD mice exhibited severe islet infiltration with <20% of the islets showing normal appearance and >50% of the islets showing invasive and destructive insulitis. In contrast, >70% of the islets in JNK2-deficient mice were not infiltrated, and the residual 30% showed mostly peri-insulitis (Fig. 2A and B). At 30 weeks of age, all of the islets of NOD mice showed severe destructive insulitis, but normal uninfiltreated islets were detected in JNK2-deficient NOD mice (data not shown).

**JNK2-Deficiency Impairs the Generation of Diabetogenic T Cells.** Autoimmune diabetes can be adoptively transferred to euglycemic recipients by injection of splenic T cells. To examine the diabetogenic potential of T cells from JNK2-deficient mice, T cells from young (13 week old) non-diabetic female NOD and female NOD/Mapk9<sup>−/−</sup> mice were transferred into NOD/Scid recipients. The incidence of diabetes was significantly reduced if recipient mice received splenic T cells from JNK2-deficient donors compared with control NOD donors (\( P = 0.035 \); Fig. 3A). This observation indicated that the generation of \( \beta \)-cell-specific diabetogenic T cells may be impaired in JNK2-deficient mice.

**JNK2 Deficiency Causes Altered T Cell Function in NOD Mice.** Analysis of the distribution of T cell populations (CD4 and CD8) and their activation markers (CD44 and CD25) in lymph nodes and spleen showed no difference between nondiabetic NOD and NOD/Mapk9<sup>−/−</sup> mice (data not shown). We also examined the presence of T cells in infiltrated islets by immunohistochemistry on frozen pancreatic sections. Although the number of infiltrated islets in NOD mice was greater than the number of infiltrated islets in NOD/Mapk9<sup>−/−</sup> mice (Fig. 2), the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the infiltrated islets appeared similar in NOD and NOD/Mapk9<sup>−/−</sup> mice (Fig. 4A). It is known that a Th1...
by Th2 cells and Th1 cells was substantially increased in these mice compared with wild-type mice (Fig. 4B). Similarly, the secretion of IL-5, another Th2 cytokine, was also augmented in Th2 and Th1 cells from JNK2-deficient mice (Fig. 4D).

Selective Th2 Polarization of JNK2-Deficient CD4+ T Cells from NOD Mice. To test whether JNK2 deficiency promoted the production of Th2 cytokines, we examined the cytokine profile of nonpolarized effector (Th0) cells differentiated with anti-CD3 and anti-CD28 mAbs in the absence of exogenous cytokines. Although IL-4 secretion by wild-type NOD Th0 cells was not detected, high levels of IL-4 were produced by JNK2-deficient NOD Th0 cells (Fig. 4B). Furthermore, the amount of IL-5 secreted by JNK2-deficient NOD Th0 cells was similar to the high level produced by Th2 cells, but no IL-5 production by NOD Th0 cells was detected (Fig. 4D). In addition, the expression of IFN-γ by Th0 cells from JNK2-deficient mice was greatly reduced (Fig. 4C). To test whether this phenotype was due to an impaired differentiation or to an impaired activation of effector cells, we examined cytokine production during the differentiation (days 3 and 4) of CD4+ T cells in the absence of exogenous cytokines. IL-4 was detected in cultures of differentiating JNK2-deficient CD4+ T cells (Fig. 4E). In contrast, the JNK2-deficient CD4+ T cells produced lower levels of IFN-γ compared with NOD CD4+ T cells (Fig. 4F). Together, these data indicate that JNK2 deficiency caused an intrinsic polarization of NOD CD4+ T cells toward Th2 effectors independently of the cytokine environment. Control studies demonstrated that the Th1/Th2 polarization of CD4+ T cells from JNK1-deficient (Mapk8−/−) NOD mice was similar to wild-type NOD mice (data not shown), indicating a selective role for JNK2 in the regulation of CD4+ T differentiation in NOD mice.

JNK2-Deficiency Increases the Resistance of β Cells to Apoptosis. We tested whether the resistance of JNK2-deficient NOD mice to insulitis was exclusively due to the Th2 phenotype of CD4+ T cells or whether JNK2 could also contribute to islet β cell death. Adoptive transfer of T cells isolated from diabetic NOD donor mice causes rapid development of diabetes in sublethally irradiated NOD host mice within 2 to 4 weeks. We therefore transferred T cells from recently diagnosed diabetic female NOD mice into irradiated male NOD and NOD/Mapk9−/− mice. The frequency of diabetes caused by the T cells isolated from diabetic NOD mice was significantly reduced when these cells were introduced into JNK2-deficient NOD mice compared with control NOD mice (P = 0.002; Fig. 3B). The JNK2-deficient NOD group of recipient mice exhibited reduced diabetes compared with the control group of NOD mice after 8 weeks. These data suggest that JNK2-deficiency may increase the resistance of β cells to apoptosis. Although NOD/Mapk9−/− β cells and NOD β cells were found to be equally sensitive to apoptosis induced by IL-1β, TNF-α, and IFN-γ in vitro (data not shown), it is possible that JNK2 deficiency might enhance resistance to death induced by other T cell mediators, including perforin, granzyme, or Fas ligand.

Discussion

Type 1 diabetes is characterized by failure of the insulin-producing β cells in pancreatic islets. Proinflammatory cytokines produced by islet infiltrating immune cells have been implicated as effector molecules. Here, we demonstrate that the protein kinase JNK2 plays an important role in a mouse model of type 1 diabetes (NOD) and that the role of JNK2, in part, is to regulate cytokine secretion by CD4+ T cells. Two different effector CD4+ T cell subtypes can be distinguished based on the spectrum of cytokines that they are programmed to secrete. Th1 cells produce IL-1 and IFN-γ, which induce a cellular immune response. In contrast, Th2 cells secrete (IFN-γ) environment accelerates the recruitment of islet-specific CD4+ T cells and also accelerates the onset of diabetes, whereas a Th2 environment protects against autoimmune diabetes (14). We therefore examined whether JNK2-deficiency in NOD CD4+ T cells could promote the differentiation of these cells into Th2 effector cells. CD4+ T cells were isolated from nondiabetic 8-week-old NOD and JNK2-deficient NOD mice and were activated with immobilized anti-CD3 mAb and soluble anti-CD28 mAb in the presence of IL-4 (to promote Th2 differentiation) or IL-12 (to promote Th1 differentiation). After 4 days of differentiation, the cells were washed and restimulated with immobilized anti-CD3 mAb. Culture supernatants were then harvested 24 h later for analysis of cytokine production. Although IFN-γ secretion by Th1 cells was not significantly affected in JNK2-deficient mice (Fig. 4B), the production of IL-4
IL-4, IL-5, and IL-10, which support humoral immunity and down-regulate the inflammatory actions of Th1 cells. The Th1/Th2 balance is critical for the development and resolution of immune responses. Importantly, benign insulitis is associated with Th2 cells, whereas destructive insulitis appears to be associated with Th1 cells (8). One signaling pathway that has been implicated in the regulation of CD4$^+$ T cell differentiation is the JNK group of MAPKs (9, 10). In this study, we demonstrate that the JNK2 protein kinase plays a key role in the differentiation of CD4$^+$ T cells in NOD mice.

It is known that the transition from nonpathogenic insulitis to diabetes correlates with the change from a predominance of Th2 to Th1 cytokines in the islets (15). A Th1 (IFN-γ) environment accelerates the recruitment of islet-specific CD4$^+$ T cells and also accelerates the onset of diabetes (14). In contrast, a Th2 pancreatic environment appears to protect against autoimmune diabetes. Thus, administration of IL-4 systemically or expression of IL-4 in NOD mice in vivo interferes with the islet infiltration by T cells and prevents Th1-mediated destructive insulitis and diabetes (16–20). The observation that JNK2 deficiency causes selective polarization of CD4$^+$ T cells to the Th2 phenotype with increased IL-4 expression (Fig. 4) may lead to creation of a Th2 pancreatic environment that protects against autoimmune diabetes. This finding may account for the reduced insulitis (Fig. 2) and reduced progression to diabetes (Figs. 1 and 3) in NOD/Mapk9$^{-/-}$ mice.

Inappropriate activation of T cells initiates many autoimmune diseases. JNK may play several roles in this process. JNK may play a suppressive role in autoimmune disease because it is implicated in the negative selection of autoreactive T cells in the thymus (21, 22). JNK may also play an enabling role in autoimmune disease. In type 1 diabetes, JNK may contribute to β-cell apoptosis caused by cytokines (23, 24) and oxidative stress caused by reactive oxygen species and nitric oxide (25). Furthermore, JNK may regulate the production of macrophage-derived cytokines, including TNF-α (26), that may contribute to the development of destructive insulitis and diabetes (27, 28). It is possible that, in addition to the regulation of the Th1/Th2 balance (Fig. 4), one or more of these additional potential mechanisms may also contribute to the effects of JNK2 deficiency to suppress insulitis and diabetes in NOD/Mapk9$^{-/-}$ mice.

We conclude that JNK may play multiple roles in diabetes. The JNK1 protein kinase (encoded by the Mapk8 gene) and the JNK scaffold protein JIP1 (encoded by the Mapk8ip1 gene) are implicated in type 2 diabetes (3, 4). Thus, Mapk8$^{-/-}$ and
Mapk8ip1−/− mice exhibit profound defects in peripheral insulin resistance and obesity caused by eating a high fat diet (3, 4). In contrast, the JNK2 protein kinase (encoded by the Mapk9 gene) appears to play no role in type 2 diabetes, and studies of Mapk9−/− mice confirm this conclusion. However, JNK2 does play an important role in the development of type 1 (insulin-dependent) diabetes, an autoimmune disease. JNK2 deficiency reduces insulitis and hyperglycemia in the NOD mouse model of type 1 diabetes (Fig. 1). The mechanism of JNK2 deficiency is mediated, in part, by an increased polarization of CD4+ T cells to the Th2 phenotype and increased IL-4 production. Previous studies have identified a Th2 pancreatic environment (with increased IL-4) as protective against type 1 diabetes in NOD mice (13). Indeed, IL-4 administration has been proposed as a potential therapy for type I diabetes (16–20). The results of this study suggest that JNK2 represents an alternative pharmacological target for the design of a small molecule inhibitor that could increase Th2 responses and, therefore, protect against type 1 diabetes. Potential candidate drugs that could be used have recently been described in ref. 29.

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