Signaling between pancreatic β cells and macrophages via S100 calcium-binding protein A8 exacerbates β-cell apoptosis and islet inflammation

Hideaki Inoue[‡], **Jun Shirakawa**[‡1], **Yu Togashi**[‡2], **Kazuki Tajima**[‡2], **Tomoko Okuyama**[‡2], **Mayu Kyohara**[‡], **Yui Tanaka**[‡], **Kazuki Orime**[‡§], **Yoshifumi Saisho**[¶], **Taketo Yamada**[∥], **Kimitaka Shibue**[§], **Rohit N. Kulkarni**[§3], and **Yasuo Terauchi**[‡4]

From the [‡]*Department of Endocrinology and Metabolism, Graduate School of Medicine, Yokohama-City University, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan, the Departments of* [¶]*Internal Medicine and* [∥]*Pathology, School of Medicine, Keio University, Tokyo 108-8345, Japan, and the* [§]*Section of Islet Cell and Regenerative Biology, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital, Harvard Stem Cell Institute, Harvard Medical School, Boston, Massachusetts 02138*

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Chronic low-grade inflammation in the pancreatic islets is observed in individuals with type 2 diabetes, and macrophage levels are elevated in the islets of these individuals. However, the molecular mechanisms underlying the interactions between the pancreatic β cells and macrophages and their involvement in inflammation are not fully understood. Here, we investigated the role of S100 calcium-binding protein A8 (S100A8), a member of the damage-associated molecular pattern molecules (DAMPs), in β-cell inflammation. Co-cultivation of pancreatic islets with unstimulated peritoneal macrophages in the presence of palmitate (to induce lipotoxicity) and high glucose (to induce glucotoxicity) synergistically increased the expression and release of islet-produced S100A8 in a Toll-like receptor 4 (TLR4)-independent manner. Consistently, a significant increase in the expression of the *S100a8* gene was observed in the islets of diabetic db/db mice. Furthermore, the islet-derived S100A8 induced TLR4-mediated inflammatory cytokine production by migrating macrophages. When human islet cells were co-cultured with U937 human monocyte cells, the palmitate treatment up-regulated S100A8 expression. This S100A8-mediated interaction between islets and macrophages evoked β-cell apoptosis, which was ameliorated by TLR4 inhibition in the macrophages or S100A8 neutralization in the pancreatic islets. Of note, both glucotoxicity and lipotoxicity triggered S100A8 secretion from the pancreatic islets, which in turn promoted macrophage infiltration of the islets. Taken together, a positive feedback loop between islet-derived S100A8 and macrophages drives β-cell apoptosis and pancreatic islet inflammation. We conclude that developing therapeutic approaches to inhibit S100A8 may serve to prevent β-cell loss in patients with diabetes.

Activation of the innate immune system and circulating levels of acute-phase inflammatory proteins play important roles in the onset and development of type 2 diabetes (1–3). Evidence of chronic inflammation has been demonstrated in the adipose tissue, liver, vascular endothelial cells, circulating leukocytes, and pancreatic islets in obese and/or diabetic humans (4–8). Chronic islet inflammation evokes a decline in the β-cell mass by promoting β-cell apoptosis, which is a hallmark of type 2 diabetes (9, 10).

It has been reported that macrophages are elevated in the pancreatic islets in patients with type 2 diabetes (11). Chronic hyperglycemia promotes amyloid formation in the islets by inducing the secretion of islet amyloid polypeptide (12), production of reactive oxygen species in β cells (13), and formation of advanced glycation end products (14, 15). These conditions lead to activation of the NLRP3 inflammasomes, IL-1β secretion, macrophage infiltration of the β cells, and pro-apoptotic processes (12, 16). Thus, islet inflammation is closely related to β-cell failure and apoptosis in diabetes. A previous study showed that the co-culture of MIN6 insulinoma cells with RAW264.7 macrophage cells in the presence of palmitate increased the expression of inflammatory genes in the MIN6 cells and decreased insulin secretion (17). However, the precise mechanisms involved in the mutual interaction between the pancreatic β cells and macrophages in diabetes remain unclear.

In this study, we identified *S100a8* as an up-regulated gene after chronic glucose stimulation, which reflects a state of sustained hyperglycemia, in the pancreatic islets. S100A8 is a small calcium-binding protein that is found at high levels in the extracellular milieu under inflammatory conditions. Furthermore, the S100A8 protein is known to be associated with various chronic inflammatory diseases and both type 1 and type 2 diabetes (18, 19). S100A8 is thought to be a member of the damage-associated molecular pattern molecules and stimulates macrophages (20–23). Consequently, to test the

Figure 1. Glucose stimulation up-regulated S100A8/A9 expression in the islets. *A*, mRNA expression levels in the islets stimulated with GKA Cpd A (glucokinase activator; 30 μmol/liter) for 2, 6, or 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$ *versus* other groups ($n = 4$/group). *B*, mRNA expression levels in islets cultured in the presence of 2.8, 5.6, 11.1, or 22.2 mmol/liter glucose for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 4$/group).

hypothesis that S100A8 contributes to islet inflammation, we established a co-culture system with freshly isolated primary pancreatic islets and resident peritoneal macrophages to investigate the role(s) of S100A8 in the sustenance of islet inflammation.

Results

S100A8/A9 expression in the islets was up-regulated by chronic glucose stimulation

Chronic hyperglycemia induces β-cell apoptosis, in part, through continuous glucokinase activation (24). We previously identified the target genes of glucokinase by examining the gene expression profiles of glucokinase activator (GKA)[5]-treated isolated islets (NCBI GEO database GSE41248) (25). Among them, *S100a8* and *S100a9* (*S100a8/a9*) expression showed the greatest increase following chronic glucokinase activation of the islets (90- and 254-fold increase, respectively). We validated the gene expression changes in isolated islets stimulated with a glucokinase activator (Fig. 1A) and confirmed that glucose stimulation increased the expression of *S100a8/a9* in the islets in a concentration-dependent manner (Fig. 1B). Thus, S100A8 expression was induced by high glucose in the islets without macrophages.

S100A8/A9 expression in the islets was enhanced by co-culture with macrophages in the presence of palmitate

We co-cultured islets with macrophages using co-culture inserts (Fig. 2A) and observed that the islet expression of

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[5] The abbreviations used are: GKA, glucokinase activator; TLR, Toll-like receptor; TUNEL, TdT-mediated dUTP nick-end labeling; ER, endoplasmic reticulum.

S100a8, *S100a9*, *Il-1b*, *Tnf-a*, *Il-6*, and *Ccl2* was increased in the presence of macrophages (Fig. 2B). The absence of elevated expression of the macrophage markers, *Cd11b* and *F4/80*, suggested that it was unlikely that there was contamination of the co-cultured islet samples with macrophages (Fig. 2C). The expression of *S100a8* and *S100a9*, but not of *Il-1b*, *Tnf-a*, *Il-6*, or *Ccl2*, was enhanced in the islets co-cultured with macrophages in the presence of the saturated fatty acid palmitate (16:0) (Fig. 2B). We confirmed the secretion of S100A8, but not of S100A9, by ELISAs in the supernatant of islets co-cultured with macrophages in the presence of palmitate (Fig. 2D). Notably, secretion of S100A8 from the macrophages was not affected by the concentration of glucose or palmitate (Fig. 2E). S100A8 proteins were predominantly expressed in the mouse pancreatic islets, but not in acinar cells (Fig. 2F).

To test the possibility that the adipocyte-derived fatty acids contributed to the macrophage-mediated islet inflammation *in vivo*, we added isolated white adipocytes from the epididymal fat to the co-culture of islets with macrophages and examined islet gene expression. As expected, the adipocytes and macrophages synergistically increased the expression of *S100a8* and *S100a9* in the islets, and this was not associated with elevation of the expression of macrophage or adipocyte markers (Fig. 3A). Palmitate has been reported to induce islet inflammation through the TLR4/MyD88 pathway (17). Islets obtained from TLR4-knockout mice and co-cultured with WT macrophages in the presence of palmitate showed a significant increase in the expression of *S100a8/a9* (Fig. 3B). These results suggest that TLR4-mediated signaling was not required for the S100A8 production induced by macrophage-derived factors and palmitate in the co-cultured islets.

Glucotoxicity further enhanced the induction of S100A8/A9 in co-cultured islets

Chronic high ambient glucose concentration has been shown to accelerate inflammation in various tissues in diabetes (26, 27). We undertook experiments under normal glucose (5.6 mmol/liter) conditions and under high glucose (11.1 mmol/liter) conditions to mimic the environment in diabetes. The protein expression of S100A8/A9 in the co-cultured islets was enhanced following culture in the presence of 11.1 mmol/liter glucose (high concentration) (Fig. 4A). S100A8 secretion from islets co-cultured with macrophages in the presence of palmitate was also enhanced by glucose stimulation (Fig. 4B). High glucose enhanced palmitate-induced *S100a8* and *S100a9* gene expression, whereas expression of other inflammatory or macrophage markers in the co-cultured islets was not influenced by the glucose concentration (Fig. 4C). In addition, inflammatory gene expression in the macrophages was up-regulated by the ambient glucose level (Fig. 4D).

We next examined the expression of *S100a8/a9* in the islets of the db/db mouse, an established model of diabetes. Six- and 12-week-old db/db mice exhibited morbid obesity, severe hyperglycemia, and irregular α/β-cell distribution within the islets; however, the ratio of β to α cells and the proportion of apoptotic β cells were not altered in the db/db mice compared with control db/+ mice (Table S1 and Fig. 5 (*A* and *B*)). Isolated pancreatic islets from db/db mice, at both 6 and 12 weeks of age,
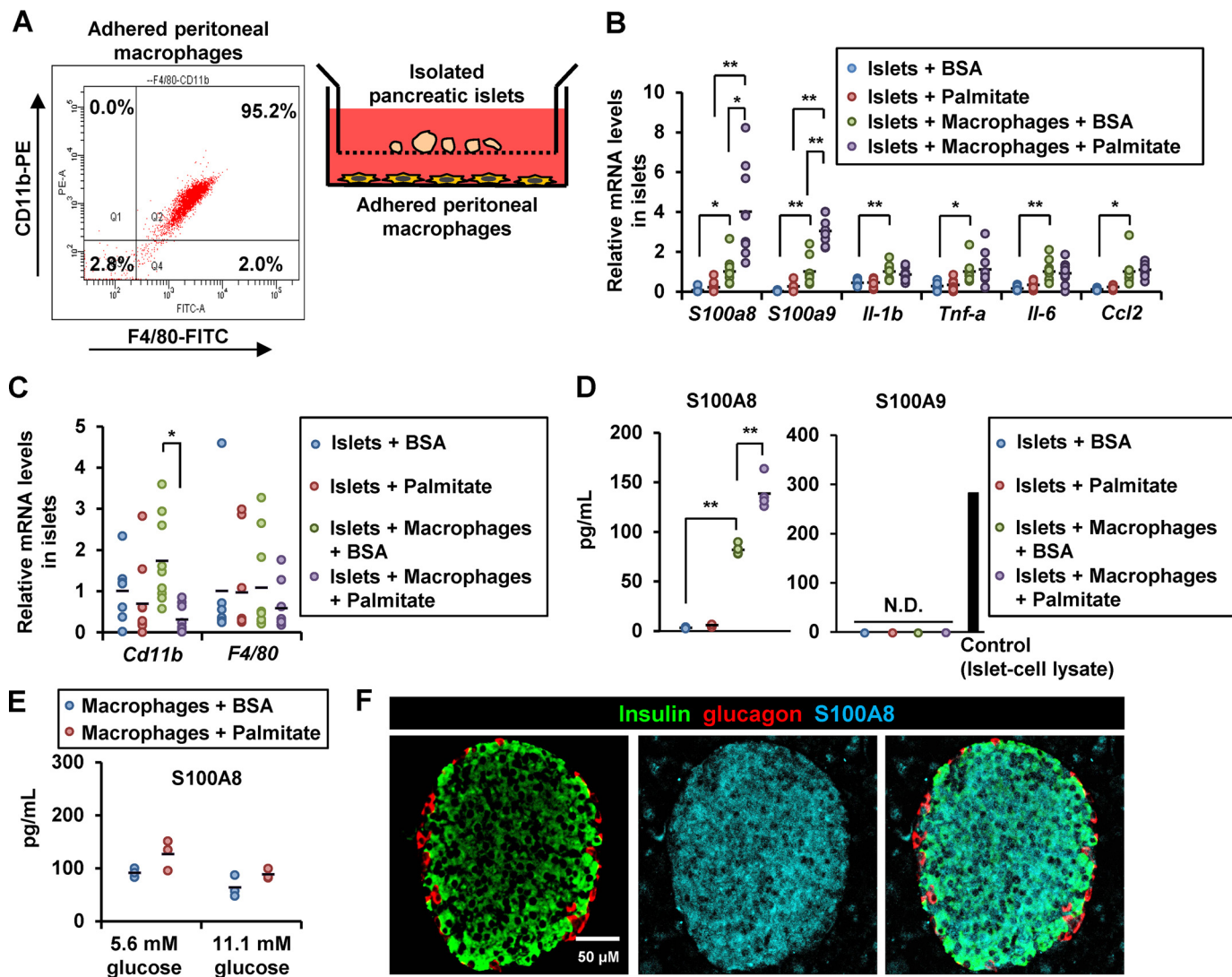
Figure 2. Chronic stimulation with high concentrations of glucose, palmitate, and macrophages induced S100A8/A9 expression in the pancreatic islets. *A*, illustration of co-cultured islets and macrophages (*right*). Flow cytometry of the adherent peritoneal macrophages (*left*). *B–D*, isolated mouse pancreatic islets (50 islets) were co-cultured with peritoneal macrophages (1×10^5 cells) in the presence of BSA (0.5%) or palmitate (500 μmol/liter). *B* and *C*, mRNA expression levels in islets co-cultured with macrophages in the presence/absence of palmitate for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 9$). *D*, secreted S100A8 protein (*left*) and S100A9 protein (*right*) levels in the supernatant obtained from co-culture of islets with macrophages in the presence/absence of palmitate for 48 h. *Horizontal bars*, mean values. *N.D.*, not detected; **, $p \leq 0.01$ ($n = 4$). *E*, secreted S100A8 protein levels in the supernatant of macrophages cultured in the presence of 5.6 or 11.1 mmol/liter glucose with/without palmitate for 48 h. *Horizontal bars*, mean values ($n = 3$/group). *F*, pancreatic sections from 8-week-old male WT C57BL/6J mice were stained with antibodies to insulin (*green*), glucagon (*red*), and S100A8 (*blue*). Scale bar, 50 μm.

showed higher levels of expression of *S100a8/a9*, of the inflammatory cytokine gene *Il-6*, and of the macrophage marker *F4/80* compared with db/+ islets (Fig. 5C).

Factors from the islets, but not from macrophages, activated the macrophages in co-culture via TLR4

In macrophages co-cultured with islets in the presence of palmitate, expression of *Tnf-a*, *Ccl2*, *Il-1b*, *Il-6*, *Il-12*, *Il-22*, *Il-23*, and *Il-24* genes were elevated (Fig. 6A). IL-23 and IL-24 are potent inducers of oxidative and ER stress in β cells (28). No increase in the expression of *S100a8/a9* genes was observed in the macrophages co-cultured with the islets in the presence of palmitate or in the presence of high ambient glucose (Fig. 6B). This implies that the production of S100A8 induced by co-culture with macrophages was predominantly derived from the

islets. The aforementioned increase in the cytokine gene expression was blunted by the TLR4-inhibitory peptide VIPER (Fig. 6C). These results suggest that a humoral factor derived from the co-cultured islets stimulated the macrophages via TLR4.

Because S100A8 is reported as a ligand of TLR4, we examined whether S100A8 induces inflammation in the co-cultured macrophages. Treatment with the recombinant S100A8-GST peptide increased the expression of the *Tnf-a*, *Ccl2*, *Il-1b*, *Il-6*, *Il-12*, *Il-22*, and *Il-23* genes in the macrophages and prompted macrophage migration (Fig. 7, *A and B*). Neutralization of S100A8 using an antibody significantly reduced the migration of the macrophages induced by co-culture with islets (Fig. 7C). Neutralization of S100A8 with an antibody also reduced the cytokine expression of the macrophages induced by co-culture
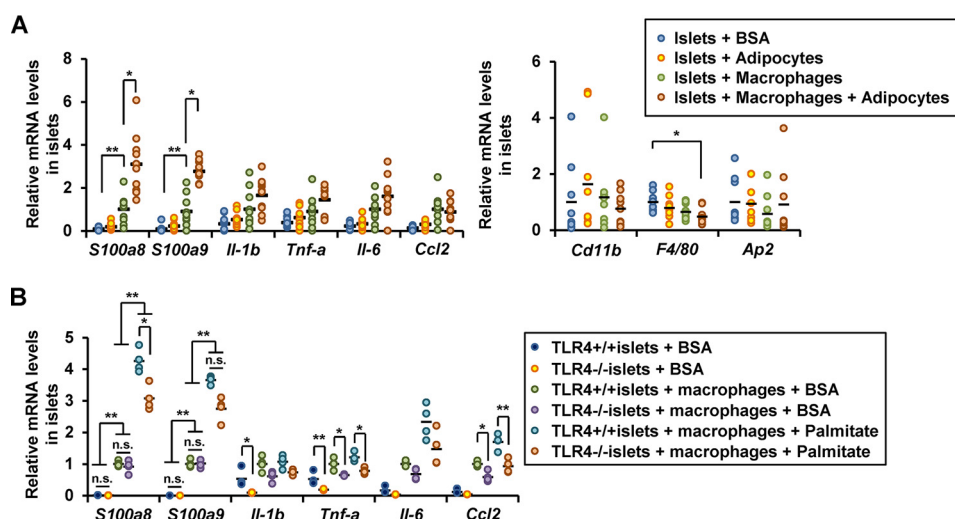
Figure 3. TLR4-independent S100A8 production induced by macrophage-derived factors and palmitate in the co-cultured islets. *A*, isolated pancreatic islets (50 islets) were co-cultured with white adipocytes in the presence of BSA (0.5%) or palmitate (500 μmol/liter) for 24 h. mRNA expression levels in co-cultured islets. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 9$). *B*, mRNA expression levels in TLR4$^{+/+}$ or TLR4$^{-/-}$ islets co-cultured with TLR4$^{+/+}$ macrophages in the presence of BSA (0.5%) or palmitate (500 μmol/liter) for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 6$).

of the macrophages with islets in the presence of palmitate (Fig. 7D). Inhibition of TLR4 with VIPER or TAK-242 attenuated the migration of the macrophages and the cytokine expression in these cells induced by purified S100A8-GST peptide (Fig. 7, *E* and *F*). TLR4$^{-/-}$ macrophages exhibited a significant decrease of cytokine expression following co-culture with islets in the presence of palmitate as compared with TLR4$^{+/+}$ macrophages (Fig. 7G). Taken together, S100A8 leads to the up-regulation of inflammation mediators in macrophages via TLR4.

Co-cultivation of islets with macrophages in the presence of palmitate coordinately promoted β-cell apoptosis via islet-derived S100A8 and macrophages

Exogenous *S100a8/a9* expression induced by adenoviral transduction was also influenced by the ambient glucose levels (Fig. 8A), suggesting that glucose stimulation possibly enhanced translation or stabilized the S100A8/A9 mRNA or protein. Overexpression of S100A8/S100A9 exerted no effect on glucose-induced insulin secretion from the islets (Fig. 8B) or on the degree of apoptosis in the islets (Fig. 8C). S100A8 overexpression in the MIN6K8 β cells slightly decreased insulin secretion from the cells, and conversely, *S100a8* knockdown was capable of restoring the insulin secretion, even in the absence of macrophages (Fig. 8D). In the presence of macrophages, however, S100A8 overexpression increased insulin secretion, whereas *S100a8* knockdown tended to decrease insulin secretion in S100A8-overexpressing MIN6K8 β cells (Fig. 8E).

To assess the effects of the co-cultivation on the islets, β-cell apoptosis was evaluated. Co-culturing with macrophages increased the number of apoptotic β cells, and palmitate enhanced macrophage-induced β-cell apoptosis (Fig. 9A). The combination of glucose stimulation further induced apoptosis of β cells in the presence of macrophages (Fig. 9A). The apoptosis-associated Bax protein expression level, but not that of the necrosis-associated HMGB1 protein, increased in the islets co-cultured with macrophages in the presence of palmitate and

ambient high glucose levels (Fig. 9B). The TLR4-inhibitory peptides VIPER and TAK-242 showed a tendency to reduce β-cell apoptosis caused by co-culturing of the islets with macrophages (Fig. 9, *C* and *D*). Furthermore, neutralization of S100A8 with an antibody specific to S100A8 significantly reduced the degree of β-cell apoptosis in the islets co-cultured with macrophages (Fig. 9, *C* and *D*).

Expression of S100A8 in human islets

Next, investigation of human islets revealed that expression of S100A8 was significantly enhanced after stimulation with GKA in both nondiabetes and type 2 diabetes donors (Fig. 10A). Co-existence of human monocyte U937 cell line and palmitate significantly up-regulated the expression of *S100A8* in human islets (Fig. 10B). We also explored the localization of S100A8 in human islets. Immunohistochemical staining for S100A8 was predominantly detected in β cells in the islets (Fig. 10C). The degree of β-cell apoptosis in the human islets co-cultured with U937 cells and palmitate tended to be decreased by the S100A8-specific neutralizing antibody (Fig. 10D). Further study is warranted to clarify the pathological significance of S100A8 in human islet inflammation.

Discussion

The results of the present study identified S100A8 as an endogenous islet-derived secretory peptide that is induced by a combination of infiltrating macrophages, palmitate (lipotoxicity), and high glucose (glucotoxicity), resulting in the activation of macrophages and potentiation of islet inflammation and β-cell death through a positive feedback loop (Fig. 11). The current results are consistent with a recent report suggesting that the serum level of the S100A8/A9 complex is a sensitive marker of acute inflammation associated with islet transplant rejection (29).

Several studies have shown that TLR4 signaling and MyD88 signaling in β cells play important roles in the development of islet inflammation (17, 30, 31). However, TLR4 stimulation did not induce S100A8/A9 production in the islets, whereas
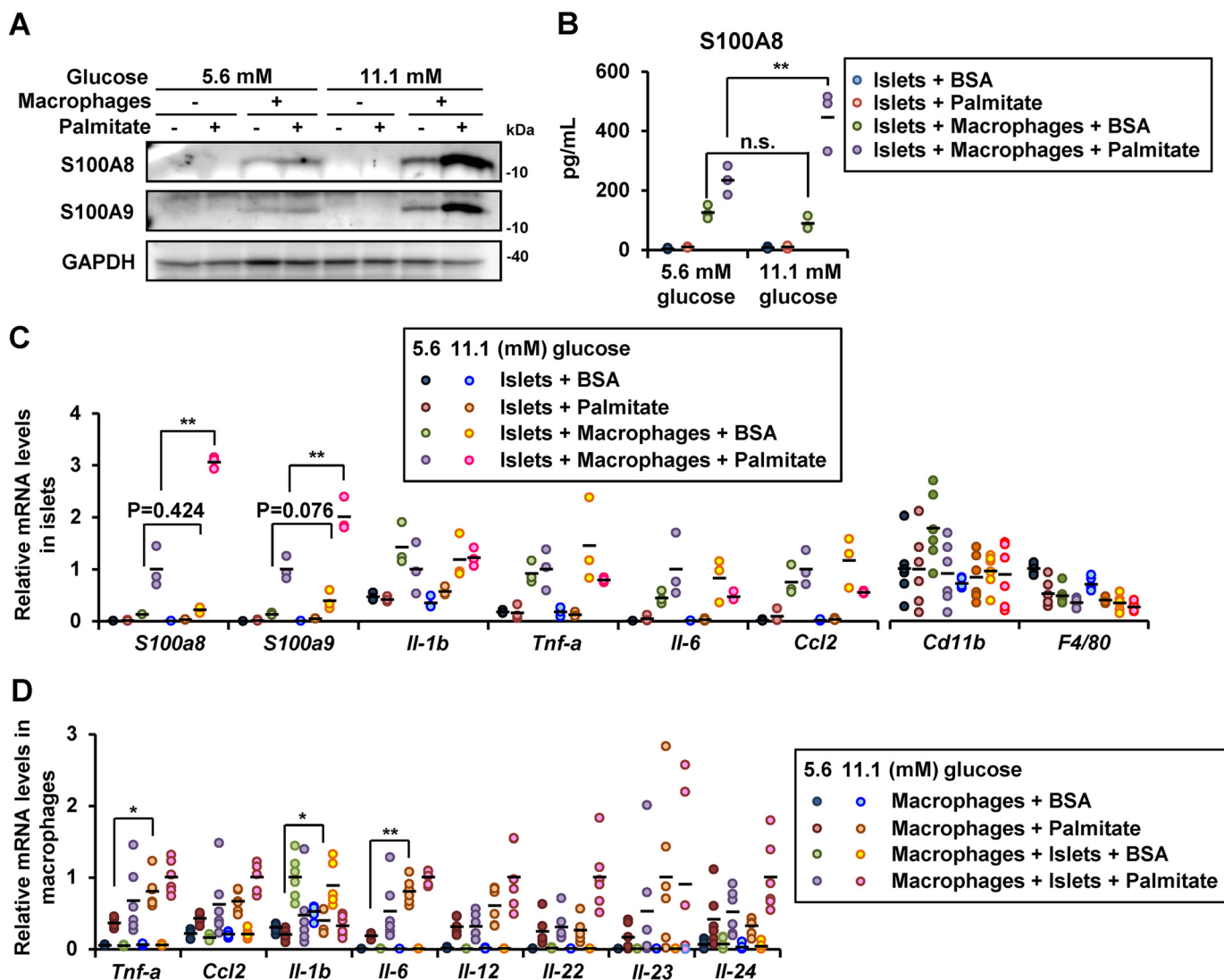
Figure 4. Increased ambient glucose concentrations enhanced the expressions of S100A8/A9 in co-cultured islets with macrophages. Isolated pancreatic islets (200 islets for *A* and *B*, 50 islets for *C* and *D*) were co-cultured with peritoneal macrophages (5×10^5 cells for *A* and *B*, 1×10^5 cells for *C* and *D*) in medium containing BSA in the presence/absence of palmitate. *A*, isolated islets were co-cultured with macrophages in the presence of 5.6 or 11.1 mmol/liter of glucose with/without palmitate for 48 h. Total cell extracts from the islets were subjected to immunoblotting as indicated. *B*, secreted S100A8 protein levels in the supernatant from a co-culture of islets with macrophages in the presence/absence of palmitate and 5.6 or 11.1 mmol/liter glucose for 48 h. *Horizontal bars*, mean values. *n.s.*, not significant; **, $p \leq 0.01$ ($n = 3$). *C*, mRNA expression levels in islets co-cultured with macrophages in the presence of 5.6 or 11.1 mmol/liter glucose with/without palmitate for 24 h. *Horizontal bars*, mean values. **, $p \leq 0.01$ ($n = 6$). *D*, mRNA expression levels in macrophages co-cultured with islets in the presence of 5.6 or 11.1 mmol/liter glucose with/without palmitate for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 6$).

S100A8 protein from the islets acted as a ligand for the TLR4 expressed in macrophages. Because TLR4 and RAGE (receptor for advanced glycation end products) receptors are also expressed in the pancreatic β cells (17), it was suggested that S100A8 also exerts direct effects on the β cells. TLR4 stimulation by S100A8 triggered the release of the inflammatory cytokines *Il-12*, *Il-23*, and *Il-24* from the macrophages, which resulted in β-cell apoptosis. Further production of S100A8 may evoke inflammation of the surrounding islets, neighboring tissues, or feeding vessels. TNFα, CCL2, and IL-1β are known to induce islet inflammation through the NF-κB pathway (3), and IL-23 and IL-24 have been shown to be potent inducers of oxidative and ER stress in β cells (28). The expression of these cytokines was also induced in the macrophages following stimulation with S100A8 in the present study.

Overexpression of S100A8/A9 had no inhibitory effect on insulin secretion from isolated islets (Fig. 8B). We also report that S100A8 *per se* impaired insulin secretion from MIN6K8 β cells in the absence of macrophages (Fig. 8D). However, overexpression of S100A8 enhanced insulin secretion from MIN6K8 β cells in the presence of macrophages (Fig. 8E). Overexpression of S100A8 has been reported in ductal adenocarcinoma of the pancreas, and it has been suggested that a peptide metabolite of S100A8 released from pancreatic cancer possibly suppresses insulin secretion to induce diabetes (32). Further research is required to clarify the effects of S100A8 on insulin secretion under similar pathophysiological conditions.

Our results suggested that S100A8 did not act directly to induce β-cell apoptosis, but via a mutual interaction with
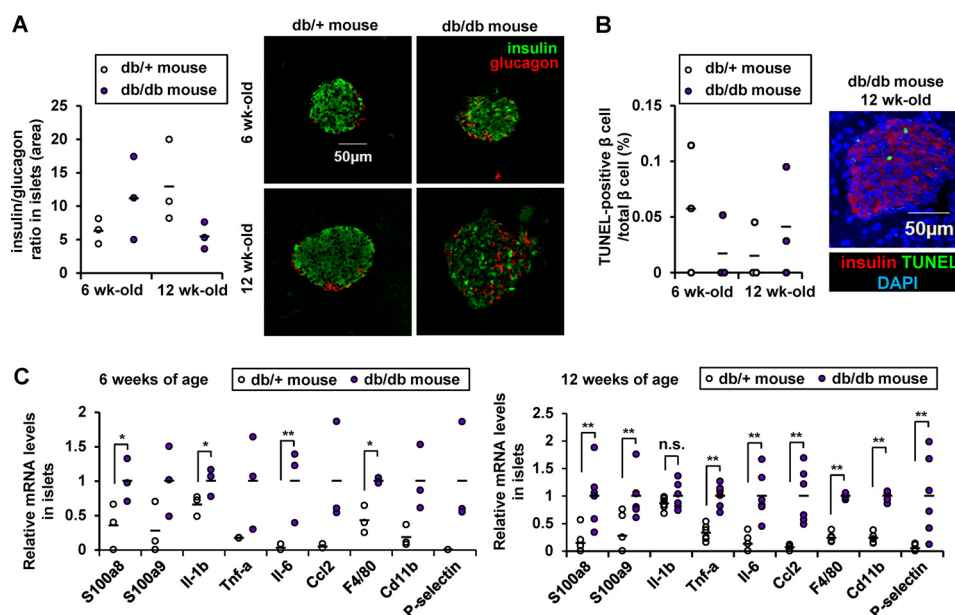
Figure 5. Increased S100A8/A9 expressions in diabetic islets. *A*, quantification of β-cell mass as a proportion of the total α-cell mass in the islet. *Horizontal bars*, mean values (*n* = 6). *B*, the proportion of TUNEL-positive cells is shown as a percentage of the total number of insulin-positive cells in the sections. *Horizontal bars*, mean values (*n* = 5). *Scale bar*, 50 μm. *C*, mRNA expression levels in islets from 6- or 12-week-old db/db or db/+ mice. *Horizontal bars*, mean values. *n.s.*, not significant; *, *p* ≤ 0.05; **, *p* ≤ 0.01 (*n* = 6).
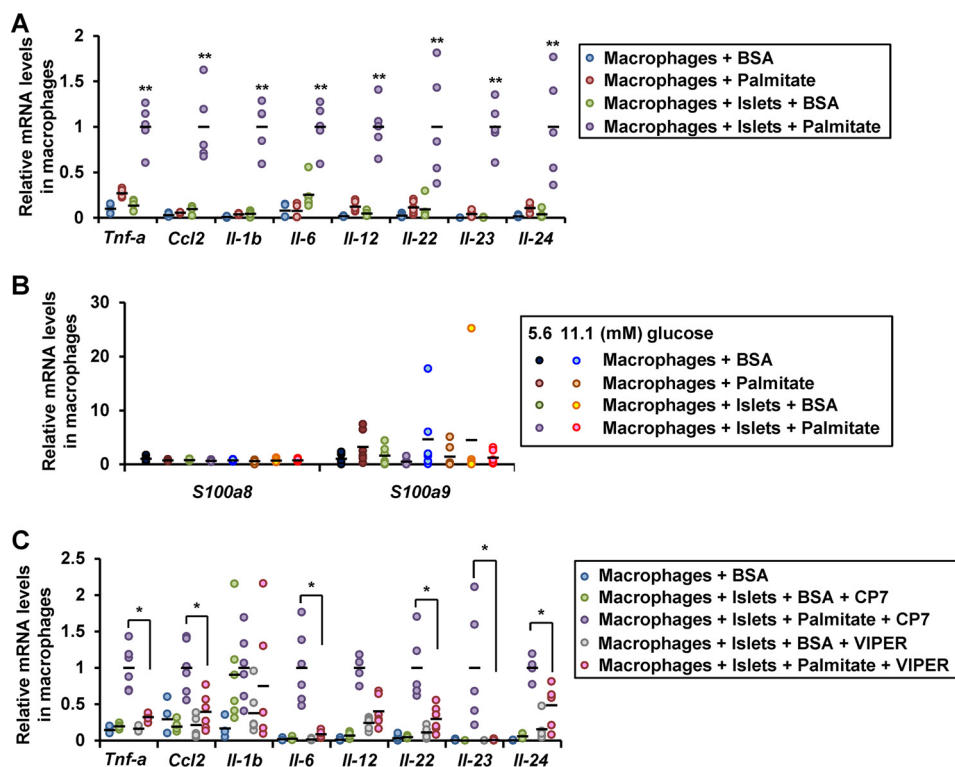

Figure 6. Activation of the macrophages in co-culture with islets via TLR4. Peritoneal macrophages (1 × 10⁵ cells) were co-cultured with isolated pancreatic islets (50 islets) in a medium containing BSA (0.5%) in the presence/absence of palmitate. *A* and *B*, mRNA expression levels in macrophages co-cultured with islets for 24 h. *Horizontal bars*, mean values. **, *p* ≤ 0.01 (*n* = 5). *C*, mRNA expression levels in macrophages co-cultured with islets in the presence of the TLR4 inhibitory peptide VIPER or the control peptide CP7 for 24 h. *Horizontal bars*, mean values. *, *p* ≤ 0.05 (*n* = 5).

macrophages. S100A8 in β cells probably triggers destruction of the β-cell membrane, because S100A8 and S100A9 have the ability to form oligomers and induce amyloid deposition (33). Because S100A9 protein was detected in the islets but not in the culture medium, the contribution of S100A9 in our experimental conditions remains unclear. S100A9 protein, a hetero-

dimerization partner of S100A8, possibly plays an essential role in the protein expression of S100A8 protein, as indicated by previous studies that found that S100A8 was not detectable in S100A9-KO peripheral tissues (20, 34).

Glucokinase-mediated induction of S100A8 production seems specific to pancreatic β cells, as deduced from the results
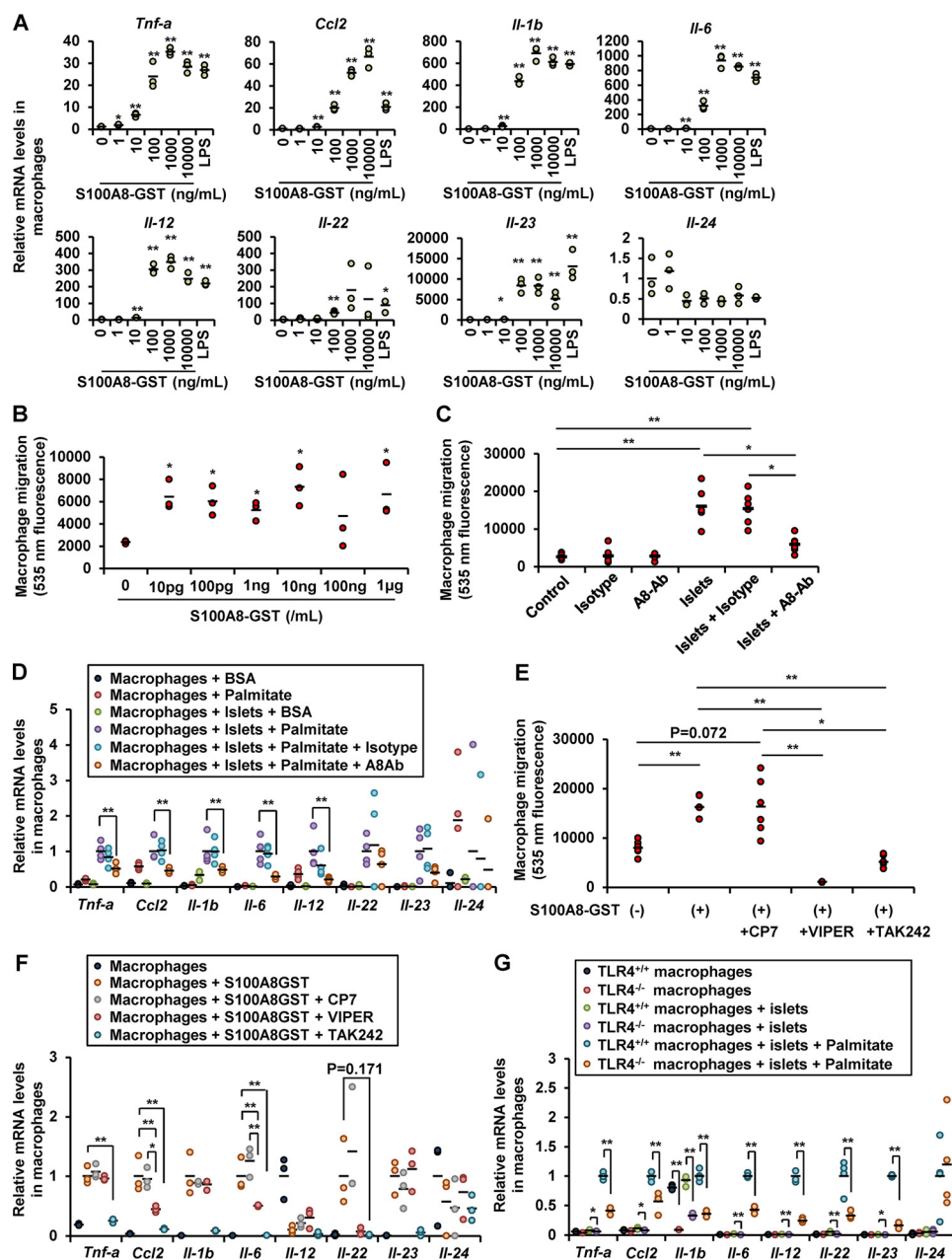
Figure 7. Islet-derived S100A8 activated macrophage migration and inflammation. *A*, mRNA expression levels in peritoneal macrophages (1×10^5 cells) stimulated with S100A8-GST peptide for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ *versus* 0 ng/ml control ($n = 3$). *B*, fluorescence intensity of Calcein-AM–labeled macrophages migrating in response to S100A8-GST peptide stimulation for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$ *versus* 0 ng/ml control ($n = 3$). *C*, fluorescence intensity of migrated Calcein-AM–labeled macrophages migrating in response to co-culture with islets in the presence of anti-S100A8 neutralizing antibody (10 µg/ml) or IgG$_{2B}$ isotype control for 48 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 6$). *D*, mRNA expression levels in macrophages co-cultured with the islets with/without palmitate in the presence of anti-S100A8 neutralizing antibody or IgG$_{2B}$ isotype control. *Horizontal bars*, mean values. **, $p \leq 0.01$ ($n = 4$). *E*, fluorescence intensity of migrated Calcein-AM–labeled macrophages in response to co-culture with islets in the presence of recombinant S100A8 peptide and the TLR4-inhibitory peptide VIPER or TAK-242 (100 nmol/liter) for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 6$). *F*, mRNA expression levels in S100A8-stimulated macrophages cultured in the presence of the TLR4 inhibitory peptide VIPER or TAK-242 for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 3$). *G*, mRNA expression levels in TLR4$^{+/+}$ or TLR4$^{-/-}$ macrophages co-cultured with TLR4$^{+/+}$ islets in the presence of palmitate for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 4$).

of previous studies carried out in other organs (35–37). Glucokinase activation enhances adaptive β-cell proliferation and prevents β-cell apoptosis induced by glucotoxicity or ER stress (25, 38, 39). However, chronic glucokinase activation and high glucose act to trigger β-cell apoptosis (24, 39). There is considerable debate about the role of glucokinase in the protection of β cells from the S100A8-mediated positive feedback loop of

islet inflammation under glucolipotoxicity. Additional studies are necessary to confirm the production of S100A8 in human β cells from obese or diabetic subjects.

In summary, our studies support the identification of S100A8 as a secretory protein to promote β-cell apoptosis and constitute an important step in the development of approaches to protect β cells in patients with diabetes.
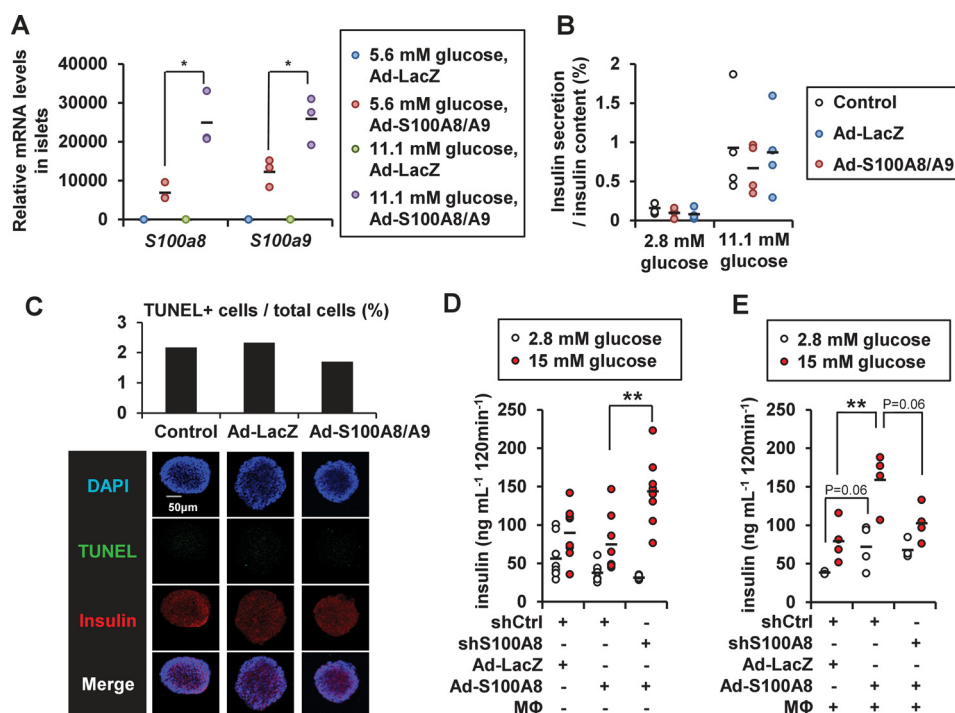
Figure 8. Effects of S100A8 on insulin secretion from pancreatic islets. *A*, islets were treated with adenoviral LacZ or adenoviral S100A8/A9 for 48 h. The mRNA expression levels in the adenoviral S100A8/A9-transduced islets (50 islets) cultured in the presence of 5.6 or 11.1 mmol/liter glucose are shown. *Horizontal bars*, mean values. *, $p \leq 0.05$ ($n = 3$). *B*, glucose-stimulated insulin secretion analysis from the adenoviral S100A8/A9-transduced islets (50 islets). *Horizontal bars*, mean values ($n = 4$). *C*, TUNEL assay in the adenoviral S100A8/A9-transduced islets (200 islets). *Horizontal bars*, mean values. *D* and *E*, glucose-stimulated insulin secretion analysis from shRNA-treated MIN6K8 cells. MIN6K8 cells were cultured for 24 h for adenoviral S100A8 transduction with/without peritoneal macrophages. *D*, without macrophages. *Horizontal bars*, mean values. **, $p \leq 0.01$ ($n = 8$). *E*, with macrophages. *Horizontal bars*, mean values. **, $p \leq 0.01$ ($n = 4$).

Experimental procedures

Animals and animal care

Animal handling procedures were in accordance with institutional animal care and use committee protocols approved by Yokohama City University. Animals were housed in rooms maintained at a constant room temperature (25 °C) under a 12-h light (07:00 h)/12-h dark (19:00 h) cycle, and the animals were given free access to food and water. C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). All of the mice used in this study belonged to the C57BL/6J background. db/db mice and db/+ mice (BKS.Cg-*Lepr*^{db}/*Lepr*^{db} and BKS.Cg-*Lepr*^{db}/*Dock7*^{m}) were purchased from Charles River Laboratories Japan (Yokohama, Japan). TLR4$^{-/-}$ mice were purchased from Oriental Bio Service (Kyoto, Japan).

Reagents, viruses, and cells

The S100A8 and S100A9 ELISA kits were purchased from Cloud-Clone Corp. (Houston, TX). GKA Cpd A was purchased from Merck (Darmstadt, Germany). Collagenase L was purchased from Nitta-Gelatin (Osaka, Japan). Thapsigargin was purchased from Sigma. Collagenase XI and LPS were purchased from Sigma-Aldrich. The TLR4 peptide inhibitor VIPER (40) was purchased from Novus Biologicals, LLC (Littleton, CO). TAK-242 was purchased from Chemscene, LLC (Monmouth Junction, NJ). D-Mannoheptulose was purchased from Carbosynth Ltd. (Compton, Berkshire, UK). Adenoviruses containing S100A8, S100A9, or LacZ were generated using the Virapower adenoviral expression system (Invitrogen).
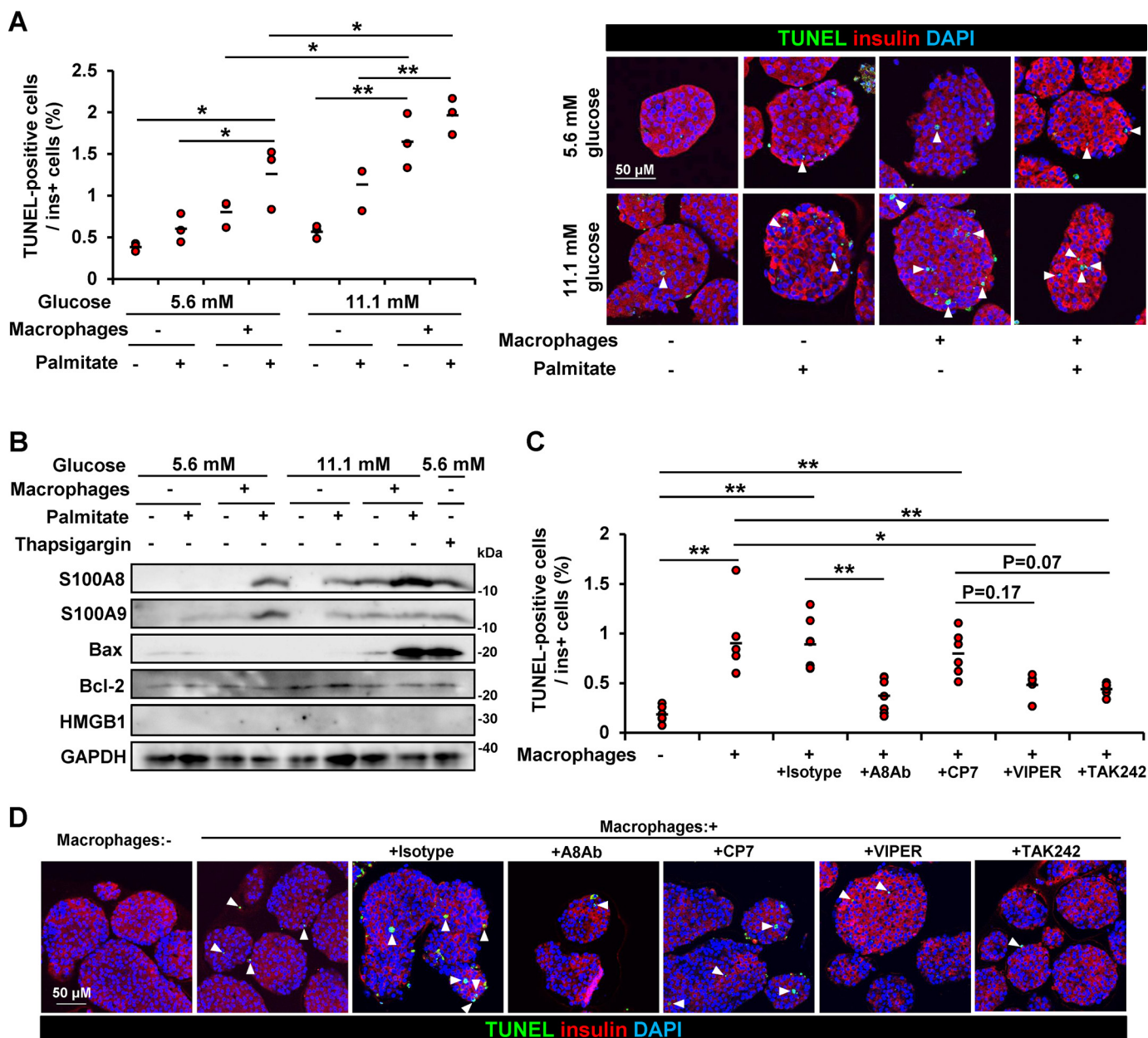
Five micrograms of adenoviral constructs were digested with PacI, and the linearized DNA was transfected into HEK293A cells. The adenovirus produced by these cells was then collected and subjected to three cycles of freezing and thawing to release the adenovirus. The resulting adenovirus was stored at −80 °C for later use. Viral titers were determined by plaque assays using cultured HEK293A cells, according to the manufacturer's instructions. Monoclonal rat IgG$_{2B}$ anti-mouse S100A8 antibody and monoclonal rat IgG$_{2B}$ isotype control were purchased from R&D Systems (Abingdon, UK). Calcein-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). The MIN6K8 cell line was provided by Dr. Susumu Seino (Kobe University). Lentivirus particles expressing short hairpin RNA for S100A8 were purchased from Santa Cruz Biotechnology, Inc.

Isolation and co-culture of islets, resident peritoneal macrophages, and white adipocytes

Islets and peritoneal macrophages were isolated as described elsewhere (41, 42). The proportion of F4/80- and CD11b-positive macrophages was more than 90%, as confirmed by flow cytometry (Fig. 2*A*). Adipocytes were prepared by collagenase digestion (Nitta Gelatin) of epididymal fat tissue, as described previously (43). Glucose-stimulated insulin secretion from the islets was induced as described previously (44). The co-culture was performed at 37 °C in a Krebs–Ringer bicarbonate HEPES buffer, pH 7.4, containing 0.2% BSA. Isolated pancreatic islets and peritoneal macrophages were plated in a Netwell insert

Figure 9. S100A8-stimulated macrophages induced β-cell apoptosis. Isolated pancreatic islets (200 islets) were co-cultured with peritoneal macrophages (5×10^5 cells) in medium containing BSA in the presence/absence of palmitate for 48 h. *A* and *C*, the islets were subjected to the TUNEL assay. The proportion of TUNEL-positive β cells is shown as a percentage of the co-cultured islets. Insulin is stained *red*, nuclei are stained *blue*, and TUNEL-positive nuclei are stained *green*. *A*, *left*, co-cultivation in the presence of 5.6 or 11.1 mmol/liter glucose. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 3$). *Right*, representative images. *B*, isolated islets were co-cultured with macrophages in the presence of 5.6 or 11.1 mmol/liter glucose with/without palmitate for 48 h. Total cell extracts from the islets were subjected to immunoblotting as indicated. *C*, isolated islets were co-cultured with macrophages in the presence of 11.1 mmol/liter glucose with palmitate for 24 h. Co-cultivation in the presence of anti-S100A8 neutralizing antibody, IgG$_{2B}$ isotype control, the control peptide CP7, the TLR4 inhibitory peptide VIPER, or the TLR4 inhibitor TAK-242. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 6$). *D*, representative images of *C*.

with a 74-μm mesh size polyester membrane (Corning, Inc.) and in the bottom wells, respectively, and the cultures were incubated for 24 or 48 h in RPMI1640 containing fatal bovine serum, Krebs–Ringer bicarbonate containing BSA, or 500 μmol/liter palmitate (Fig. 2*A*). Adipocytes (from 25 mg of epididymal fat) were co-cultured above the co-culture Netwell insert.

Real-time PCR

Total RNA isolation from pancreatic islets, cDNA synthesis, and quantitative PCR were performed as described previously

(25, 45). Data were normalized according to the expression level of β-actin, 18S rRNA, or GAPDH. The primers used for the real-time PCR are listed in Table S2.

Immunohistochemical analysis

Pancreases and islets were fixed and immunostained as reported previously (25, 45). Pancreatic tissue sections were immunostained with antibodies to S100A8 (Santa Cruz Biotechnology, Abcam), S100A9 (Abcam), insulin (Santa Cruz Biotechnology), or glucagon (Abcam). Alexa Fluor 488-, 555-, and 647-conjugated secondary antibodies (Invitrogen) were used
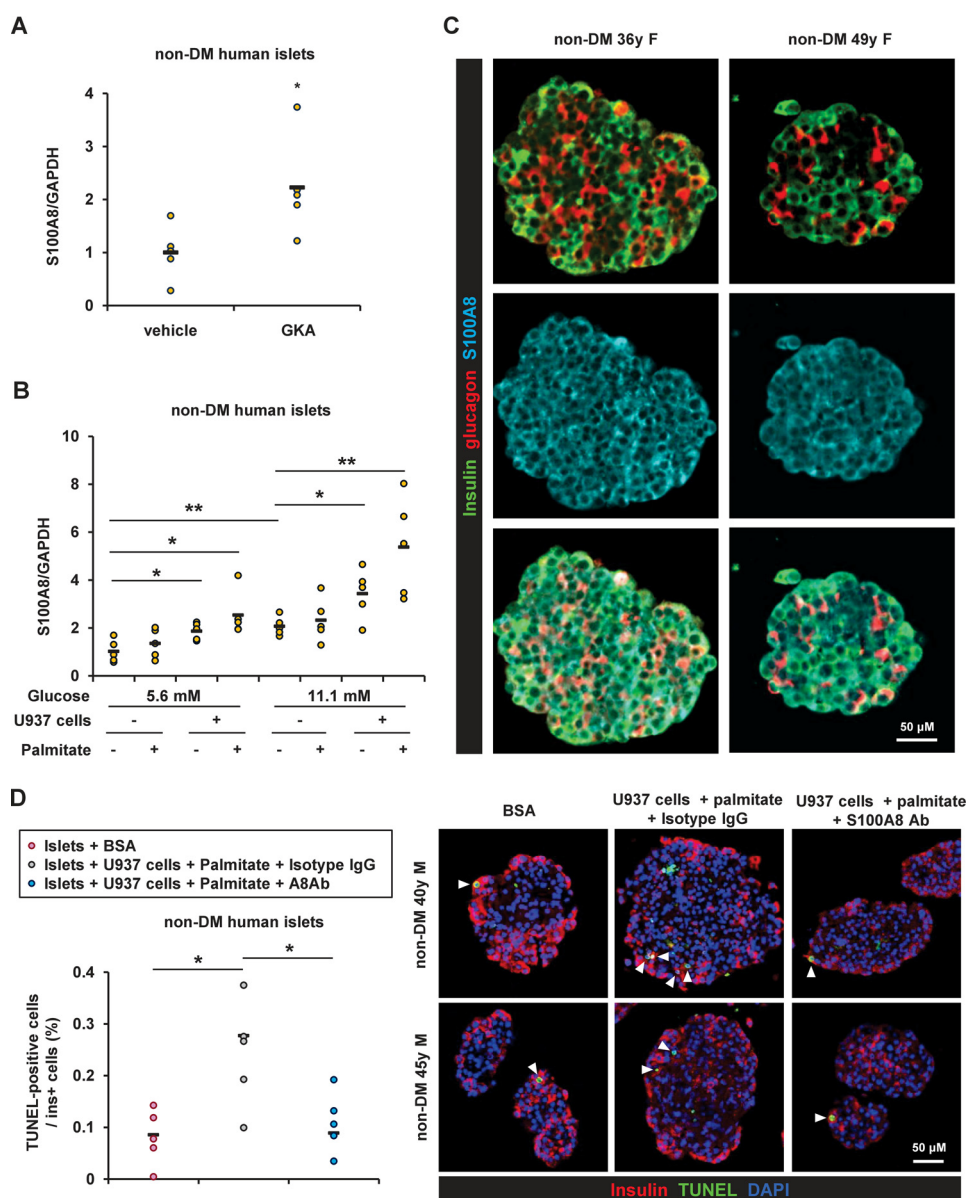
Figure 10. The expression of S100A8 was induced by glucose stimulation, monocytes, and palmitate in human islets. *A*, mRNA expression levels in human islets from nondiabetes (*non-DM*) donors after stimulation with GKA Cpd A (glucokinase activator; 30 μmol/liter) for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 5$). *B*, mRNA expression levels in human islets co-cultured with U937 human monocyte cell line (1×10^5 cells) in the presence of BSA (0.5%) or palmitate (500 μmol/liter) for 24 h. *Horizontal bars*, mean values. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 5$). *C*, embedded human islets from nondiabetes donors were stained with antibodies to insulin (*green*), glucagon (*red*), and S100A8 (*blue*). *Scale bar*, 50 μm. *D*, human islets from nondiabetes donors were co-cultured with U937 cells in the presence of 11.1 mmol/liter glucose with palmitate for 24 h. They were co-cultivated in the presence of anti-S100A8 neutralizing antibody or IgG$_{2B}$ isotype control. The proportion of TUNEL-positive β cells is shown as a percentage of the co-cultured islets. *Horizontal bars*, mean values. *, $p \leq 0.05$ ($n = 5$).

for the fluorescence microscopic analysis. β-cell apoptosis was evaluated using a TdT-mediated dUTP nick-end labeling (TUNEL) assay of the co-cultured β cells. Co-cultured islets (200 islets) were attached to poly-L-lysine–coated coverslips (Falcon) and subjected to a TUNEL assay using the ApopTag *in situ* detection kit (EMD Millipore, MA). All of the images were acquired using a FluoView FV1000-D confocal laser-scanning microscope (Olympus, Tokyo, Japan).

Immunoblotting

For immunoblotting, isolated islets (200 islets) were lysed in radioimmune precipitation buffer (Cell Signaling Technology, Danvers, MA) with complete protease inhibitor mixture

(Roche Diagnostics). After centrifugation, the extracts were subjected to immunoblotting with antibodies. The primary antibodies used were Calgranulin A (S100A8), Calgranulin B (S100A9), Bcl-2–associated X protein (BAX), Bcl-2 (Santa Cruz Biotechnology), high-mobility group box 1 (HMGB1), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (Abcam).

GST-fused proteins

GST-fused constructs comprising the mouse S100A8 and S100A9 proteins were generated in pGEX4T-1 and were received as kind gifts from Dr. Sachie Hiratsuka and Dr. Yoshihiro Maru (Department of Pharmacology, Tokyo Women's Medical University) (46). The proteins were
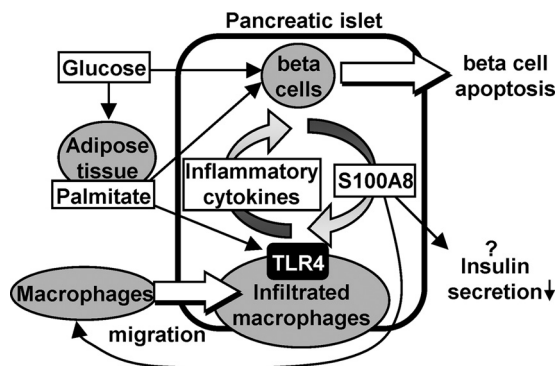
Figure 11. An illustrative model of islet inflammation–induced β-cell apoptosis via S100A8 in diabetes. A combination of infiltrating macrophages, saturated fatty acids (palmitate), and hyperglycemia augmented the production of S100A8. S100A8 secreted from the islets induced further macrophage migration and inflammation through TLR4. This positive feedback loop potentiates islet inflammation and β-cell death.

expressed in *Escherichia coli* BL21 and purified on a GSH-Sepharose column.

Macrophage migration assay

The migrated macrophages were labeled with Calcein-AM (WAKO, Osaka, Japan) and measured using an ARVO™ MX plate reader (PerkinElmer Life Sciences) at an excitation wavelength of 485 nm and emission filter of 535 nm. The migration of macrophages was evaluated using 8-μm pore Falcon BD FluoroBlok™ inserts and plates (BD Biosciences) with isolated pancreatic islets (50 islets) or S100A8-GST in the presence or absence of anti-S100A8 antibody, isotype control IgG2b, VIPER/CP7, or TAK-242. Macrophages were seeded onto the insert mesh and incubated for 24 or 48 h at 37 °C under 5% CO_2.

Human islets

Human islets were obtained from the Integrated Islet Distribution Program (National Institutes of Health). All studies and protocols used were approved by the Joslin Diabetes Center's Committee on Human Studies (approval CHS#5-05). Details of human islets are described in Table S3. Upon receipt, islets were cultured overnight in Miami Medium 1A (Cellgro). Co-culture was performed at 37 °C in final wash/culture medium (Cellgro) or RPMI1640 medium. Cadaveric human islets and U937 human monocyte cell line (ATCC) were plated in a Netwell insert with a 74-μm mesh size polyester membrane (Corning) and in the bottom wells, respectively.

Statistical analyses

All experiments were independently repeated at least three times. *Horizontal bars* indicate mean values. Statistical analyses were conducted using IBM SPSS Statistics version 19. Equality of variances was determined by using an F-test or Levene's test. Statistical comparisons between groups were analyzed for significance by an unpaired two-tailed Student's *t* test and one-way analysis of variance with post hoc Tukey tests for a parametric test or Welch's *t* test or Games–Howell test for a nonparametric test. Differences were considered significant at $p < 0.05$.

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