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ORIGINAL PAPER

c-Jun N-terminal kinases inhibitor suppresses the TNF-α induced MCP-1 expression in human umbilical vein endothelial cells

Rania Abdel Muneem Ahmed · Koji Murao · Hitomi Imachi · Kazuya Yoshida · Hiroaki Dobashi · Naohisa Hosomi · Toshihiko Ishida

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Abstract Monocyte chemoattractant protein-1 (MCP-1) is a 76-amino-acid chemokine that is considered to be an important chemotactic factor for monocytes. MCP-1 is expressed in the macrophage-rich areas of atherosclerotic lesions. A recent report indicated that MCP-1 expression in human umbilical vein endothelial cells (HUVECs) is induced by the stimulation of tumor necrosis factor (TNF)-α via the c-Jun N-terminal kinases (JNK) pathway. In this study, we examined the effects of JNK inhibitor (JNKI-1), on MCP-1 expression. The results of this study indicated that the expression of MCP-1 mRNA and protein were stimulated in the presence of TNF-α. TNF-α stimulated the phosphrylation of JNK, however, JNKI-1 inhibited the TNF-α stimulated MCP-1 secretion and gene expression. As expected, JNKI-1 blocked the stimulatory effect of TNF-α on the MCP-1 promoter activity. In conclusion, JNKI-1 partially inhibits the TNF-α-induced MCP-1 expression in HUVECs, and therefore JNKI-1 may be of therapeutic value in the treatment of diseases such as atherosclerosis.

Keywords JNKI-1 · MCP-1 · TNF-α · JNK · HUVEC

R. A. M. Ahmed · K. Murao (✉) · H. Imachi · K. Yoshida · H. Dobashi · T. Ishida
Division of Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe Miki-cho, Kita-gun, Kagawa 761-0793, Japan
e-mail: mkoji@kms.ac.jp

N. Hosomi
Department of Cardiorenal and Cerebrovascular Medicine, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe Miki-cho, Kita-gun, Kagawa 761-0793, Japan

Introduction

Chemokines belong to the family of low molecular weight proteins (8–16 kD); they have been classified thus because they have a conserved 4-cysteine motif and can facilitate the directional migration of leukocytes in vitro [1]. Further, chemotactic cytokines or chemokines partly mediate recruitment of monocytes and lymphocytes into the sites of atherosclerotic lesions [2]. It has been demonstrated that monocytes are selectively attracted to specific chemokines that predominantly belong to the C–C family of chemoattractants that includes human monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a 76-amino-acid chemokine that is considered to be an important chemotactic factor for monocytes [3]. Several reports have indicated that it is one of the key factors for initiating the inflammatory process during atherogenesis [2–5]. MCP-1 is expressed in a variety of cells including monocytes, smooth muscle cells, and vascular endothelial cells in response to several different stimuli such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α [6, 7]. In a previous study, we reported that TNF-α stimulates the expression of MCP-1 in human umbilical vein endothelial cells (HUVECs) [8, 9], however the mechanism of the signal transduction remains to be clarified.

Mitogen-activated protein kinases (MAPKs) are activated in response to the inflammatory mediators. Three important members of the MAPK family, are extracellular signal regulated kinase (ERK), p38, and c-Jun N-terminal kinases (JNK) [10]. TNF-α is a pleiotropic cytokine that plays a pivotal role in inflammatory responses. It activates 2 transcription factors, i.e., activator protein 1 (AP-1) and nuclear factor-κB (NF-κB). Both the transcription factors are activated through the phosphorylation of JNK and IκB kinases, respectively [11]. Further, JNK interacting

protein-1 (JIP-1) was initially identified as an inhibitor of JNK activation [12]. Hence, overexpression of JIP-1 leads to the retention of JNK in the cell cytoplasm and inhibits its signaling activity [12].

In this study, we have investigated the role of JNK signaling pathway on the TNF-α stimulated expression of MCP-1 gene expression in HUVECs.

Materials and methods

Cell culture

HUVECs were purchased from Clonetics (San Diego, CA) and used between passages 1–6. HUVECs were maintained in the M199 medium (Sigma) that was supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical Co., Tokyo, Japan), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Real-time reverse-transcriptase–polymerase chain reaction

Polymerase chain reactions (PCRs) were performed in a final volume of 20 μl in LightCycler (Roche, Mannheim, Germany) glass capillaries as described previously [13]. The reaction mixture consisted of 2 μl LightCycler-Fast-Start DNA Master SYBR Green I (Roche), 2.4 μl 25 mM MgCl2 stock solution, 11.6 μl sterile PCR-grade H_2O, 2 μl of the cDNA template for each gene of interest, and 1 μl of 10 μM of each primer. The forward and reverse primer sequences of the human MCP-1 gene were 5′-AATAG-GAAGATCTCAGTGCA-3′ and 5′-TCAAGTCTTCGGA GTTTGGG-3′, respectively. The cycling program consisted of initial denaturation for 600 s at 95°C followed by 55 cycles of 95°C for 5 s, 62°C for 5 s, and 72°C for 15 s, with 20°C/s slope. Each set of PCR reactions included water as a negative control and five dilutions of standard. Known amounts of DNA were then diluted to provide standards and a regression curve of crossing points versus concentration generated with the LightCycler. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping standard, as described previously [14].

Transfection of HUVECs and luciferase reporter gene assay

In order to confirm the transcriptional regulation of MCP-1 expression by TNF-α, we used a reporter gene construct that was controlled by the MCP-1 gene promoter [5, 8, 9]. The reporter gene construct contained the human MCP-1 gene sequence spanning the region from −515 to +44; this was amplified by PCR and cloned into the luciferase reporter gene (pMCP-LUC). Purified plasmids containing the reporter genes were transfected into HUVECs (at 60% confluence) by using conventional cationic liposome transfection methods (Lipofectamine, Life Technologies, Gaithersburg, MD). Rous sarcoma virus-β-galactosidase was added to all transfection mediums in order to monitor the efficiency of DNA uptake by HUVECs as described previously [15]. All the assay mixtures were corrected for β-galactosidase activity and the total amounts of protein obtained per reaction were identical. After the transfected cells were harvested, an aliquot of the cytoplasmic fraction was used for the measuring the β-galactosidase activity. 20-μl aliquots of the cytoplasmic fraction were used for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

Immunoblotting of JNK

The cells were lysed by homogenizing them for 10 min in ice-cold buffer A (50 mM Tris-HCl, pH 7.5/1 mM EDTA/1 mM EGTA/0.5 mM Na_3VO_4/0.1% 2-mercaptoethanol/1% Triton X-100/50 mM NaF/5 mM sodium pyrophosphate/10 mM Na-glycerophosphate/0.1 mM PMSF/1 μM microcystin/1 μg/ml each pepstatin, aprotinin, and leupeptin). The supernatants containing protein concentration of 20 μg/ml were used for immunoblotting according to standard procedures. Levels of phosphorylated and total JNK were detected with a 1:1,000 dilution of each specific antiserum (New England Biolabs, Beverly, MA) as described previously [16]. The protein bands were visualized by chemiluminescence.

MCP-1 enzyme-linked immunosorbent assay (ELISA)

The levels of immunoreactive MCP-1 were quantified by using a commercially available sandwich-type ELISA (R&D Systems Inc., Minneapolis, MN) as described previously [17]. ELISA plates were coated with a specific murine monoclonal antibody (mAb) against human MCP-1. Dilutions of cell-free supernatants were added in duplicate, followed by the addition of a second horseradish peroxidase-conjugated goat polyclonal Ab against MCP-1. After washing to remove any unbound Ab-enzyme reagent, a substrate solution (a 1:1 solution of hydrogen peroxide and tetramethylbenzidine) was added to the wells. The color development was stopped with 2 N sulfuric acid, and the intensity of the color was measured at 540 nm on a spectrophotometer. This ELISA is sensitive to 2.5 pg/ml MCP-1, and it has an intraassay coefficient of variation of <0.5% and an interassay coefficient of variation of <10%.

Statistical analysis

Statistical comparisons were made by one-way analysis of variance and Student's *t*-test, with $P < 0.05$ considered significant.

Results

JNKI-1 blocks the MCP-1 protein secretion induced by TNF-α in HUVECs

Previously we reported that TNF-α stimulated the secretion of MCP-1 protein in a dose-dependent manner (Fig. 1a); in HUVECs, the maximal stimulatory effect was observed at a concentration of 10 ng/ml of TNF-α [5, 8]. In this study, we used the 10 ng/ml of TNF-α as a concentration for positive stimulation. We examined the effect of JNKI-1 on TNF-α-stimulated MCP-1 secretion in HUVECs. The MCP-1 levels in culture supernatants were quantified by ELISA. TNF-α significantly increased the amount of MCP-1 secreted into the cultured medium, as shown in Fig. 1. When the cells were incubated with JNKI-1, the MCP-1 secretion was less as compared to those incubated with TNF-α. This inhibitory effect of JNKI-1 was observed in a dose-dependent manner (Fig. 1); in HUVECs, the maximal inhibitory effect was observed at a concentration 1 nM of JNKI-1.

JNKI-1 inhibits the expression of MCP-1 mRNA in HUVECs

The effects of JNKI-1 on the TNF-α-stimulated MCP-1 gene expression were analyzed by measuring the levels of endogenous MCP-1 mRNA in HUVECs by using real-time PCR method. As expected, TNF-α stimulated the expression of MCP-1 in HUVECs (Fig. 2). When HUVECs were cultured with 1 nM JNKI-1 and TNF-α, the increase in MCP-1 mRNA was suppressed unlike in the case of cells that were treated with TNF-α alone.

Kinetics of JNK phosphorylation by TNF-α treatment

The preceding results suggest that JNK may be required for inducing the stimulatory effects of TNF-α on MCP-1 expression. If this is true, TNF-α should induce JNK activity. This assumption can be verified by analyzing the kinetics of Thr183/Tyr185 phosphorylation by performing western blot analysis. This phosphorylation is a prerequisite for the induction of JNK catalytic activity. The results of the western blot analysis (Fig. 3) revealed that the phosphorylation of JNK increased when the HUVECs were incubated with TNF-α. This finding confirmed the above results that JNKI-1 competes with TNF-α for regulating MCP-1 expression.

Effect of JNKI-1 on TNF-α-stimulated MCP-1 promoter activity

Next, we measured the transcriptional activity of the MCP-1 promoter in HUVECs. HUVECs that were transfected with pMCP-LUC were incubated with TNF-α and JNKI-1 or with TNF-α alone (Fig. 3). Consistent with the results observed for the effects of TNF-α on the levels of MCP-1 protein and mRNA, TNF-α stimulated the activity of the MCP-1 promoter as well. Further, we examined the

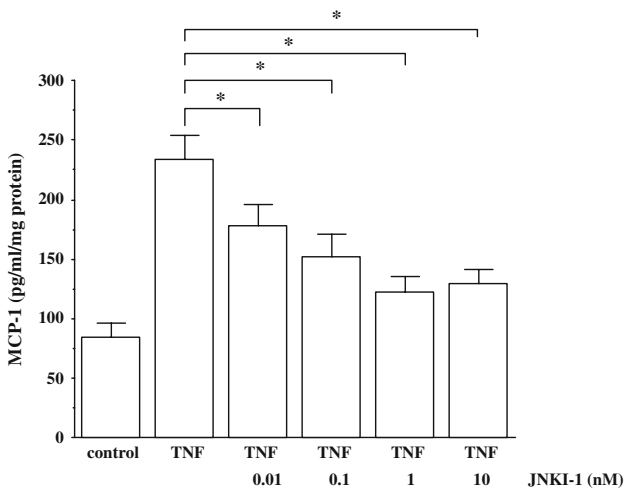

Fig. 1 Effect of JNKI-1 on MCP-1 release by TNF-α in HUVECs. HUVECs were coincubated with indicated amount of JNKI-1 and TNF-α, and MCP-1 concentration was measured by the ELISA. TNF; 10 ng/ml TNF-α. The asterisk denotes a significant difference ($P < 0.05$). Error bars show SEM of four determinations

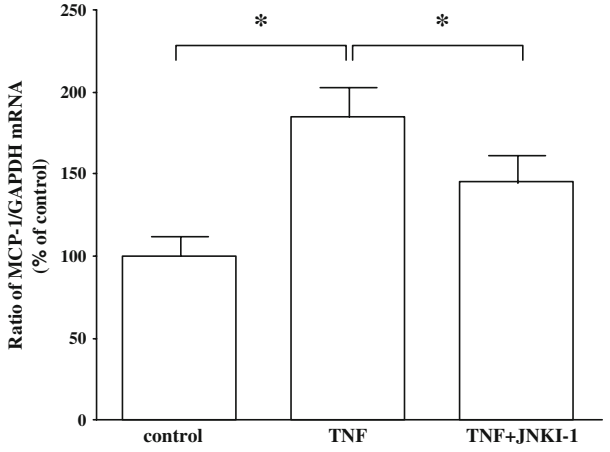

Fig. 2 Effect of JNKI-1 on MCP-1 mRNA expression by TNF-α in HUVECs. HUVECs were cultured for 24 h with 10 ng/ml TNF-α and/or JNKI-1. The steady-state expression of MCP-1 mRNA was quantified by real-time PCR method. Abundance of GAPDH served as a control. The ratio of MCP-1/GAPDH is shown as % of control in the figure. Each data point shows the mean and SEM ($n = 3$) of separate experiments. The asterisk denotes a significant difference ($P < 0.05$)

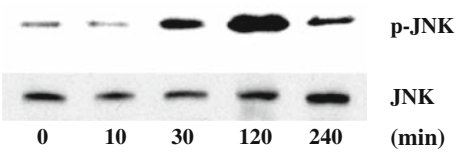

Fig. 3 JNK phosphorylation in HUVECs exposed to TNF-α. HUVECs were exposed to 10 ng/ml TNF-α for 2 min before harvest at indicated time periods. The total cell extracts were subjected to western blot analysis using an anti-phospho-JNK antibody (upper insert). Total cell lysates were also blotted using JNK antibody as a control (lower insert). An identical experiment independently performed gave similar results

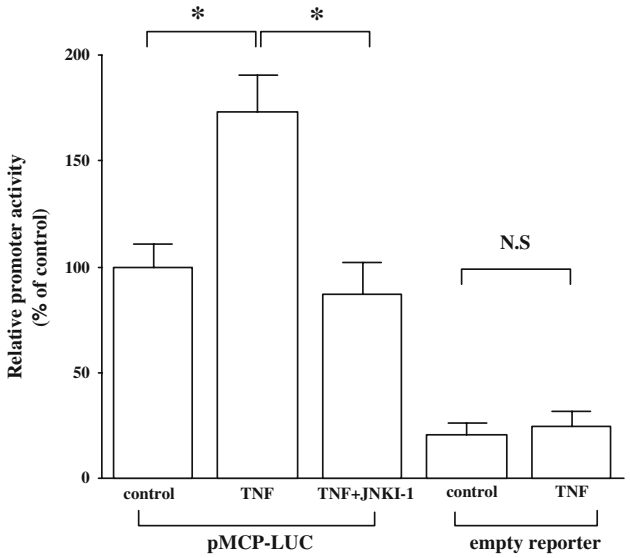

Fig. 4 Effect of JNKI-1 on TNF-α-induced MCP-1 transcriptional activity in HUVECs. HUVECs were transfected with 1 μg of pMCP-LUC or an empty reporter gene plasmid (empty reporter), and treated with 10 ng/ml TNF-α and/or JNKI-1 for 48 h prior to cell harvest. All assays were corrected for ß-galactosidase activity and total amount of protein per reaction were identical. The results were expressed as relative luciferase activity compared to control cells arbitrarily set at 100. Each data point shows the mean ± SEM of 3 separate transfections. The asterisk denotes a significant ($P < 0.05$). N.S., no significant difference

mechanisms by with TNF-α increased the activity of MCP-1 gene. From our observation, we postulated the potential involvement of signaling pathways. We analyzed the effects of JNKI-1 against the TNF-α stimulation of MCP-1 promoter activity. The results of this analysis (Fig. 4) revealed that the stimulatory effect of TNF-α on MCP-1 promoter activity was inhibited in the presence of JNKI-1, the inhibitor of JNK pathway.

Discussion

In this report, we have summarized the results of the studies indicating that TNF-α stimulates MCP-1 gene transcription and JNKI-1 suppresses this stimulatory effect. The suppressive effects of TNF-α-stimulated MCP-1 promoter activity in HUVECs require the participation of the JNK cascade. The results of the treatment of HUVECs with the JNKI-1 revealed that it has inhibitory effects on TNF-α inducing MCP-1 gene expression. Taken together, these findings seem to suggest that TNF-α induced MCP-1 expression may be the underlying reason for the higher risk of atherosclerotic diseases; hence, JNKI-1 may have therapeutic value in the treatment of diseases such as atherosclerosis.

MCP-1 is expressed in a variety of cells, including monocytes, vascular smooth muscle cells, and HUVECs, in response to several different stimuli such as TNF-α [5–7, 18]. Besides inducing chemoattraction through MCP-1 expression, TNF-α also causes increased adhesion of monocytes to the endothelial cell surface, probably through the expression of specific endothelial monocyte adhesion molecules [19]. Boring et al. [20] used mutant mice that lacked chemokine receptor (CCR2), the receptor for MCP-1, and crossed them with apolipoprotein (apo) E-null mice that develop severe atherosclerosis, thus demonstrating that the selective absence of CCR2 markedly decreases the formation of atherosclerotic lesions in apoE−/− mice. These results provide a strong evidence for the direct involvement of MCP-1 in macrophage recruitment and atherogenesis progression.

TNF-α has been implicated in the pathogenesis of non-insulin-dependent diabetes as well as in acute and chronic inflammatory diseases such as atherosclerosis [11]. The activation of JNK by TNF-α is considered to be mediated by a family of intracellular signaling molecules known as TNF-α receptor-associated factors (TRAFs) [21]. Several studies performed using TRAF2 transgenic and knockout mice have demonstrated that TRAF2 is essential for the activation of JNK in response to TNF-α induction [22, 23]. In this study, we demonstrate the intermediary role of JNK pathway in the TNF-α-stimulated MCP-1 promoter activity by using the JNKI-1 (Fig. 4). Our data support the model that TNF-α activates the JNK pathway, which in turn triggers a series of events that stimulate MCP-1 promoter activity. In agreement with this hypothesis, we found that JNKI-1 mimics the stimulatory action of TNF-α on MCP-1 promoter activity, and also on its secretion and gene expression. However, the inhibitor JNKI-1 (Fig. 1) did not completely blocked the effect of TNF-α-stimulated MCP-1 expression, our studies did not exclude the participation of other factors that might add to the net capacity of the cell to induce MCP-1 expression by TNF-α.

JNKI-1 is a peptide that is derived from the JNK binding domain of JNK-interacting protein-1 (JIP-1) and has been reported to function as a dominant inhibitor of the JNK pathway [24]. In order to convert the minimal JNK-binding

domain into a bioactive cell-permeable compound, we covalently linked a 20-amino-acid sequence derived from the JNK-binding domain of JIP-1 (RPK RPT TLN LFP QVP RSQ DT) at its C terminal to a 10-amino-acid carrier peptide derived from the HIV-TAT sequence (GRK KRR QRR R). A recent report has suggested a potential new therapy for diabetes by using the cell-permeable JNK-inhibitory peptide [21]. Intraperitoneal administration of this peptide in diabetic mice led to its transduction into various tissues in vivo, and this treatment markedly improved insulin resistance and ameliorated glucose tolerance. Our results revealed that JNKI-1 might be effective in regulating MCP-1 expression in several diseases such as atherosclerosis. Further studies need to be performed for elucidating the effect of JNKI-1 for preventing atherosclerosis.

In summary, the results of this study indicate that TNF-α treatment stimulates the expression of endogenous MCP-1 in HUVECs. This stimulatory effect of TNF-α treatment on MCP-1 promoter is partly mediated by the JNK pathway. JNKI-1 partially inhibits the TNF-α-induced MCP-1 expression, and hence, JNKI-1 may have therapeutic value in the treatment of diseases such as atherosclerosis.

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References

1. T.J. Schall, K.B. Bacon, Curr. Opin. Immunol. **6**, 865–873 (1994)
2. R. Terkeltaub, W.A. Boisvert, L.K. Curtiss, Curr. Opin. Lipidol. **9**, 397–405 (1998)
3. M. Navab, S.S. Imes, S.Y. Hama, G.P. Hough, L.A. Ross, R.W. Bork, A.J. Valente, J.A. Berliner, D.C. Drinkwater, H. Laks, A.M. Fogelman, J. Clin. Invest. **88**, 2039–2046 (1991)
4. S. Yla-Herttuala, B.A. Lipton, M.E. Rosenfeld, T. Sarkioja, T. Yoshimura, E.J. Leonard, J.L. Witztum, D. Steinberg, Proc. Natl. Acad. Sci. USA **88**, 5252–5256 (1991)
5. K. Murao, X. Yu, W.M. Cao, H. Imachi, K. Chen, T. Muraoka, N. Kitanaka, J. Li, R.A. Ahmed, K. Matsumoto, T. Nishiuchi, M. Tokuda, T. Ishida, Life Sci. **81**, 592–599 (2007)
6. E.J. Leonard, T. Yoshimura, Immunol. Today **11**, 97–101 (1990)
7. B.J. Rollins, T. Yoshimura, E.J. Leonard, J.S. Pober, Am. J. Pathol. **136**, 1229–1233 (1990)
8. A. Momoi, K. Murao, H. Imachi, T. Ishida, W.M. Cao, M. Sato, J. Takahara, Chest **120**, 1293–1300 (2001)
9. K. Murao, T. Ohyama, H. Imachi, T. Ishida, W.M. Cao, H. Namihira, M. Sato, N.C.W. Wong, J. Takahara, Biochem. Biophys. Res. Commun. **276**, 791–796 (2000)
10. G.L. Johnson, R. Lapadat, Science **298**, 1911–1912 (2002)
11. B.B. Aggarwal, Nat. Rev. Immunol. **3**, 745–756 (2003)
12. M. Dickens, J.S. Rogers, J. Cavanagh, A. Raitano, Z. Xia, J.R. Halpern, M.E. Greenberg, C.L. Sawyers, R.J. Davis, Science **277**, 693–696 (1997)
13. X. Yu, K. Murao, H. Imachi, W.M. Cao, J. Li, K. Matsumoto, T. Nishiuchi, R.A. Ahmed, N.C.W. Wong, H. Kosaka, T.G. Unterman, T. Ishida, Hypertension **49**, 1378–1384 (2007)
14. K. Murao, H. Imachi, X. Yu, W.M. Cao, T. Nishiuchi, K. Chen, J. Li, R.A. Ahmed, N.C.W. Wong, T. Ishida, Gut **57**, 664–671 (2008)
15. K. Murao, Y. Wada, T. Nakamura, A.H. Taylor, A.D. Mooradian, N.C.W. Wong, J. Biol. Chem. **273**, 18959–18965 (1998)
16. H. Abe, K. Murao, H. Imachi, W.M. Cao, X. Yu, K. Yoshida, N.C.W. Wong, M.A. Shupnik, J.A. Haefliger, G. Waeber, T. Ishida, Endocrinology **145**, 5623–5628 (2004)
17. R. Tangirara, K. Murao, O. Quenquenberger, J. Biol. Chem. **272**, 8050–8056 (1997)
18. A. Sica, J.M. Wang, F. Colotta, E. Dejana, A. Mantovani, J.J. Oppenheim, C.G. Larsen, C.O. Zachariae, K. Matsushima, J. Immunol. **144**, 3034–3038 (1990)
19. M.P. Bevilacqua, J.S. Pober, M.E. Wheeler, R.S. Cotran, M.A. Gimbrone Jr., J. Clin. Invest. **76**, 2003–2011 (1985)
20. L. Boring, J. Gosling, M. Cleary, I.F. Charo, Nature **394**, 894–897 (1998)
21. H. Kaneto, Y. Nakatani, T. Miyatsuka, D. Kawamori, T.A. Matsuoka, M. Matsuhisa, Y. Kajimoto, H. Ichijo, Y. Yamasaki, M. Hori, Nat. Med. **10**, 1128–1132 (2004)
22. S.Y. Lee, A. Reichlin, A. Santana, K.A. Sokol, M.C. Nussenzweig, Y. Choi, Immunity **7**, 703–713 (1997)
23. W.C. Yeh, A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J.L. de la Pompa, D. Ferrick, B. Hum, N. Iscove, Immunity **7**, 715–725 (1997)
24. C. Bonny, A. Oberson, S. Negri, C. Sauser, D.F. Schorderet, Diabetes **50**, 77–82 (2001)