

# Sulforaphane inhibits the interferon- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 pancreatic $\beta$ -cells through the downregulation of IRF-1, STAT-1 and PKB

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**Abstract.** Sulforaphane (SFN) is a dietary isothiocyanate abundantly available in cruciferous vegetables and has been shown to possess anti-inflammatory and immunomodulatory activities. Chemokines are important mediators of inflammation and immune responses due to their ability to recruit and activate macrophages and leukocytes. To date, little is known about the SFN-mediated regulation of chemokine expression in pancreatic  $\beta$ -cells. In this study, we investigated the inhibitory effects and mechanisms of SFN on the interferon- $\gamma$  (IFN- $\gamma$ )-induced expression of a subset of chemokines, including monokine induced by IFN- $\gamma$  (MIG), IFN-inducible protein of 10 kDa (IP-10) and IFN-inducible T-cell alpha chemoattractant (I-TAC), in INS-1 cells, a rat pancreatic  $\beta$ -cell line. Notably, IFN- $\gamma$  treatment led to an increase in the mRNA expression levels of MIG, IP-10 and I-TAC in the INS-1 cells. However, SFN strongly blocked the mRNA expressions of MIG, IP-10 and I-TAC induced by IFN- $\gamma$  in INS-1 cells. On the mechanistic level, SFN significantly decreased not only the mRNA expression levels of interferon regulatory factor-1 (IRF-1), but also the phosphorylation levels of signal transducer and activator of transcription-1 (STAT-1) and protein kinase B (PKB) which were induced by IFN- $\gamma$  in the INS-1 cells. Pharmacological inhibition experiments further revealed that treatment with JAK inhibitor I weakly inhibited the IFN- $\gamma$ -induced expression of IP-10, whereas it strongly suppressed the IFN- $\gamma$ -induced expression of MIG and I-TAC

in the INS-1 cells. Moreover, treatment with LY294002, a PI3K/PKB inhibitor, was able to slightly repress IFN- $\gamma$ -induced expressions of MIG and I-TAC, but not IP-10, in INS-1 cells. Importantly, the IFN- $\gamma$ -induced increase in the expression levels of MIG, IP-10 and I-TAC in the INS-1 cells was strongly inhibited by SFN, but not by other natural substances, such as curcumin, sanguinarine, resveratrol, triptolide and epigallocatechin gallate (EGCG), suggesting the specificity of SFN in downregulating the levels of these chemokines. To the best of our knowledge, these results collectively demonstrate for the first time that SFN strongly inhibits the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells and this inhibition is, at least in part, mediated through the reduced expression and phosphorylation levels of IRF-1, STAT-1 and PKB.

## Introduction

Chemokines have been shown to control the migratory behavior of several cell types, including lymphocytes. Helper T-lymphocytes (Th1), cellular mediators of adaptive immune responses, produce interferon- $\gamma$  (IFN- $\gamma$ ) that can stimulate macrophages, epithelial cells and tissue parenchymal cells to express CXC chemokines, such as monokine induced by IFN- $\gamma$  (MIG), IFN-inducible protein of 10 kDa (IP-10) and IFN-inducible T-cell alpha chemoattractant (I-TAC) (1-5). It has also been shown that Th1 cells preferentially express CXC chemokine receptor 3 (CXCR3), which binds IP-10, I-TAC and MIG with high affinity, and the binding of CXCR3 ligands to the receptor produces cellular signals important for chemotaxis and the activation of T-cells (6,7), suggesting that the expression of IP-10, MIG and I-TAC is important in T-lymphocyte recruitment and host defense following various infections.

Immune responses that contribute to host defense are also capable of causing tissue injury and disease under pathological conditions. There is increasing evidence to indicate that during the development of type 1 diabetes, autoreactive T-cells transverse the endothelium and matrix barriers to infiltrate pancreatic islets and these autoreactive T-cells target a number of islet cell autoantigens, including insulin (8), glutamic acid decarboxylase (9) and protein tyrosine phosphatase (10). The infiltration of immune cells in islets, termed insulinitis, increases

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progressively once it begins and leads to  $\beta$ -cell destruction, insulin deficiency and clinical type 1 diabetes (11). It has been reported that insulinitic lesions are characterized by the presence of  $\beta$ -cells, elevated levels of chemokines, such as IP-10, and the infiltration of lymphocytes expressing CXCR3 (12), which may suggest that IFN- $\gamma$ -inducible chemokines may play crucial roles in the initiation of insulinitis.

Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane, SFN) is a dietary isothiocyanate found abundantly in cruciferous vegetables and has been shown to possess anti-inflammatory and immune modulatory activities (13,14). For instance, SFN has been shown to suppress the bacterial lipopolysaccharide-mediated expression of inducible nitric oxide synthase, cyclooxygenase 2, interleukin-1 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in RAW 264.7 and peritoneal macrophages (13,15,16). It has also been reported that SFN inhibits TNF- $\alpha$ -induced nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation, which leads to the reduced expression of NF- $\kappa$ B-regulated gene products, such as matrix metalloproteinase-9 (17), further supporting its anti-inflammatory activity. As MIG, IP-10 and I-TAC play an important role in T-cell recruitment for the initiation of adaptive immunity, therapies aiming at reducing the levels of these chemokines are of great interest.

In this study, we primarily investigated the effects of IFN- $\gamma$  on the expression levels of MIG, IP-10 and I-TAC in INS-1 cells, a rat pancreatic  $\beta$ -cell line, and secondly determined whether SFN modulates the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells. To the best of our knowledge, we report for the first time, the ability of SFN to strongly inhibit the IFN- $\gamma$ -induced mRNA expression of MIG, IP-10 and I-TAC in INS-1 cells through the modulation of the expression and/or phosphorylation levels of interferon regulatory factor (IRF)-1, signal transducer and activator of transcription-1 (STAT-1) and protein kinase B (PKB).

## Materials and methods

**Materials.** Recombinant rat IFN- $\gamma$  was obtained from R&D Systems (Minneapolis, MN, USA). JAK inhibitor I (a pan-JAK inhibitor) and LY294002 (a PI3K/PKB inhibitor) were obtained from Calbiochem (Billerica, MA, USA) and Biomol (Plymouth Meeting, PA, USA), respectively. Anti-p-STAT-1 (#9171), anti-STAT-1 (#9175), anti-p-PKB (#9271) and anti-PKB (#9272) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).  $\beta$ -actin antibody (A5441) was purchased from Sigma (St. Louis, MO, USA). PCR primers were purchased from Bioneer (Daejeon, Korea). RPMI-1640 was purchased from Gibco-BRL (Carlsbad, CA, USA). Other chemicals, including SFN, were obtained from Sigma.

**Cell culture.** The INS-1 cells, an immortalized rat pancreatic  $\beta$ -cell line (Korean Cell Line Bank, Seoul, Korea), were cultured in RPMI-1640 medium containing 11.2 mM glucose, 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum, 1 mM pyruvate, 10 mM HEPES, 50  $\mu$ M 2-mercaptoethanol and 100  $\mu$ g/ml streptomycin.

**Treatment of the INS-1 cells with IFN- $\gamma$ , SFN, and/or other agents.** The INS-1 cells were treated with or without IFN- $\gamma$  at various concentrations (0, 0.25, 0.5, 1, 2, 5 or 10 ng/ml) and

for different periods of time (0, 0.5, 2, 6 or 24 h). The INS-1 cells were also treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of SFN at various concentrations (0, 5, 10 or 25  $\mu$ M) for 6 h. For pharmacological inhibition experiments, the INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of JAK inhibitor I (JI, 0.1  $\mu$ M), a pan-JAK inhibitor, or LY294002 (LY, 25  $\mu$ M), a PI3K/PKB inhibitor, for 6 h. For comparison, the INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of SFN (25  $\mu$ M), curcumin (10  $\mu$ M), sanguinarine (500 nM), resveratrol (25  $\mu$ M), triptolide (40 nM) or EGCG (25  $\mu$ M) for 6 h.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA), and cDNA was prepared using M-MLV reverse transcriptase (Gibco-BRL, Carlsbad, CA, USA) according to the manufacturers' instructions. The following primers were used for the amplification of rat MIG, IP-10, I-TAC, IRF-1, IRF-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): MIG sense, 5'-CAG CCA AGG CAC ATT CCA CT-3' and antisense, 5'-GAT GCA GAG CGC TTG TTG GT-3'; IP-10 sense, 5'-CAA GTG CTG CTG TCG TTC TC-3' and antisense, 5'-TCT CTC TGC TGT CCA TCG GT-3'; I-TAC sense, 5'-AGA TCA CCA GAG CCA CAG CA-3' and antisense, 5'-ATC CGA CCT CCT AGC GAG TT-3'; IRF-1 sense, 5'-AAG TGA AGG ACC AGA GCA GG-3' and antisense, 5'-CTG TTG CAG CTT CAG AGG TG-3'; IRF-2 sense, 5'-ACA ACG CCT TCA GAG TCT AC-3' and antisense, 5'-TGC ATA GGA AGA CAC AGG AG-3'; and GAPDH sense, 5'-CCG TAT CGG ACG CCT GGT TA-3' and antisense, 5'-TGG TGG TGC AGG ATG TAT TG-3'. The PCR amplification was carried out using the following cycling conditions: 94°C for 3 min followed by 17-25 cycles of 94°C for 45 sec, 58°C for 45 sec, 72°C for 45 sec, and a final extension at 72°C for 5 min. The amplified products were separated by electrophoresis on a 1.5% agarose gel and detected under UV light.

**Preparation of whole cell lysates.** The INS-1 cells were washed with cold phosphate-buffered saline (PBS) and lysed in a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM  $\text{Na}_3\text{VO}_4$  and 1 mM NaF) containing protease inhibitors (100  $\mu$ M phenyl-methylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin and 2 mM EDTA) on ice for 15 min. The cell lysates were collected and centrifuged at 10,000  $\times$  g at 4°C for 10 min. The supernatant was saved and protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA, USA).

**Western blot analysis.** Proteins (50  $\mu$ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and transferred onto Immobilon-P membranes. The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween-20 (TBST) followed by blocking with TBST containing 5% (w/v) non-fat dried milk. The membranes were incubated overnight with antibodies specific for p-STAT-1 (1:1,000), STAT-1 (1:1,000), p-PKB (1:2,000) or PKB (1:1,000) at 4°C. The membranes were then exposed to secondary antibodies [anti-rabbit IgG (sc-2004) and anti-mouse IgG (sc-2005); purchased from Santa Cruz Biotechnology, Delaware, CA, USA]

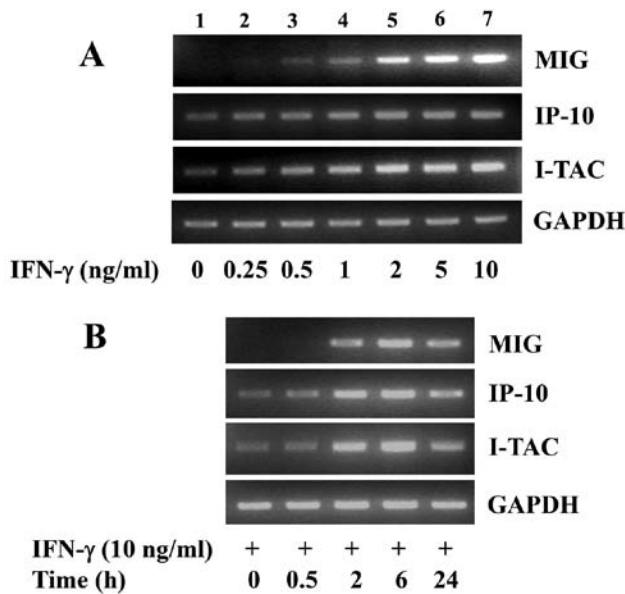


Figure 1. Effect of IFN- $\gamma$  on the mRNA expression levels of monokine induced by IFN- $\gamma$  (MIG), IFN-inducible protein of 10 kDa (IP-10) and IFN-inducible T cell alpha chemoattractant (I-TAC) in INS-1 cells. (A) INS-1 cells were treated with or without interferon- $\gamma$  (IFN- $\gamma$ ) at the indicated concentrations for 6 h. Total RNA was prepared and used for RT-PCR with specific primers for MIG, IP-10, I-TAC or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) for the indicated time periods. At each time point, total RNA was prepared and used for RT-PCR with specific primers for MIG, IP-10, I-TAC or GAPDH. The blots in (A or B) are representative of 3 independent experiments.

coupled to horseradish peroxidase for 2 h at room temperature.  $\beta$ -actin (dilution, 1:10,000) was used as an internal control. The membranes were washed 3 times with TBST at room temperature. Immunoreactivities were detected by ECL reagents.

## Results

**Dose- and time-dependent increase in the mRNA levels of the T-cell-specific chemokines, MIG, IP-10 and I-TAC, in INS-1 cells treated with IFN- $\gamma$ .** Initially, we investigated the effects of treatment with various concentrations of IFN- $\gamma$  for 6 h on the expression of T-cell-specific chemokines, including MIG, IP-10 and I-TAC in INS-1 cells. As shown in Fig. 1A, compared with the control (lane 1), 6 h of treatment with IFN- $\gamma$  led to a concentration-dependent increase in the mRNA levels of MIG, IP-10 and I-TAC in the INS-1 cells (lanes 2-7). Due to the strong induction of the MIG, IP-10 and I-TAC mRNA expression levels in INS-1 cells, the concentration of 10 ng/ml of IFN- $\gamma$  was selected for use in further experiments. Time course experiments were then carried out to determine the time of induction of chemokine expression in INS-1 cells in response to IFN- $\gamma$  (10 ng/ml). As shown in Fig. 1B, treatment with IFN- $\gamma$  led to a time-dependent increase in the mRNA levels of MIG, IP-10 and I-TAC in the INS-1 cells, in which 6 h of treatment with IFN- $\gamma$  was maximal for the induction of the expression of these chemokines. The mRNA expression of the control, GAPDH, remained constant under these experimental conditions.

**Blockage of the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells by SFN.** We then determined the effects

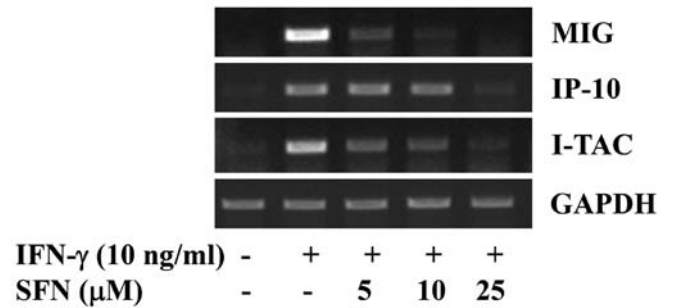


Figure 2. Effect of sulforaphane (SFN) on interferon- $\gamma$  the (IFN- $\gamma$ )-induced mRNA expression of monokine induced by IFN- $\gamma$  (MIG), IFN-inducible protein of 10 kDa (IP-10) and IFN-inducible T cell alpha chemoattractant (I-TAC) in INS-1 cells. INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of SFN at the indicated concentrations for 6 h. Total RNA was prepared and used for RT-PCR with specific primers for MIG, IP-10, I-TAC or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The blots are representative of 3 independent experiments.

of treatment with various concentrations of SFN on the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells. As shown in Fig. 2, treatment with SFN resulted in a dose-dependent blockage of the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in the INS-1 cells. Of note, treatment with SFN at 25  $\mu$ M was able to almost completely inhibit the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC. The mRNA expression of the control, GAPDH, remained unaltered under these experimental conditions.

**Downregulation of the IFN- $\gamma$ -induced mRNA expression levels of IRF-1 and phosphorylation levels of STAT-1 and PKB in INS-1 cells by SFN.** It has been shown that IFN- $\gamma$  activity is mediated through the activation of the JAK/STAT signaling pathway, which further triggers the activation of many downstream effectors, such as PKB, and the activation of these signaling pathways or components is necessary for the transcriptional induction of IFN- $\gamma$  target genes, including chemokines (18,19). There is also strong evidence to indicate that the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC occurs through an STAT-1/IRF-1-dependent mechanism, which may play an important role in the infiltration of leukocytes into tissue (20). This promptly led us to investigate the effects of IFN- $\gamma$  and/or SFN on the activation (phosphorylation) of STAT-1 and PKB, and on the expression of IRF-1 and IRF-2 in the INS-1 cells. As shown in Fig. 3A, compared with the control (lane 1), treatment with IFN- $\gamma$  increased the phosphorylation levels of both STAT-1 and PKB in the INS-1 cells (lane 2). However, treatment with SFN led to a concentration-dependent reduction in the phosphorylation levels of these proteins induced by IFN- $\gamma$  in the INS-1 cells (lanes 3-5). The maximal inhibition of the IFN- $\gamma$ -induced phosphorylation of STAT-1 and PKB was observed by treatment with SFN at 25  $\mu$ M. The total protein expression levels of STAT-1 and PKB remained constant by treatment with or without IFN- $\gamma$  in the absence or presence of SFN, suggesting that IFN- $\gamma$  or SFN treatment alters the phosphorylation levels of pre-existing STAT-1 and PKB in the INS-1 cells without leading to *de novo* protein synthesis. As shown in Fig. 3B, compared with control (lane 1), treatment with IFN- $\gamma$  increased the mRNA expression levels of both

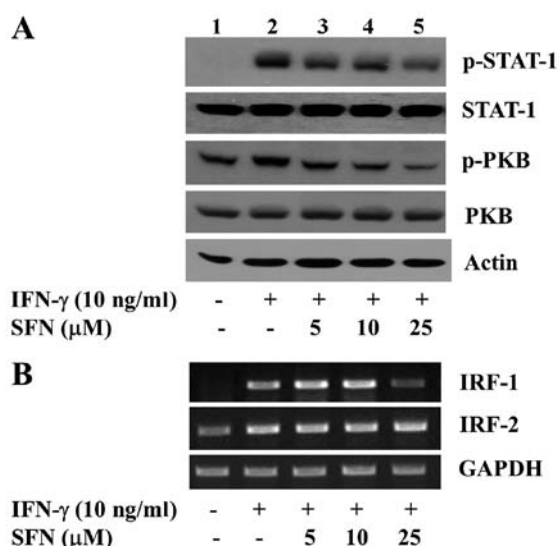


Figure 3. Effect of sulforaphane (SFN) on the interferon- $\gamma$  (IFN- $\gamma$ )-induced activation (phosphorylation) of signal transducer and activator of transcription-1 (STAT-1) and protein kinase B (PKB) and the mRNA expression of interferon regulatory factor-1/2 (IRF-1/2) in INS-1 cells. (A) INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of SFN at the indicated concentrations for 6 h. Whole cell lysates were prepared and analyzed by western blot analysis with specific antibodies to p-STAT-1, STAT-1, p-PKB or PKB. p-STAT-1, phosphorylated STAT-1; STAT-1, total STAT-1; p-PKB, phosphorylated PKB; PKB, total PKB. The blots in (A) are representative of 3 independent experiments. (B) INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of SFN at the indicated concentrations for 6 h. Total RNA was prepared and used for RT-PCR with specific primers for interferon regulatory factor-1 (IRF-1), IRF-2 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The blots in (B) are representative of 3 independent experiments.

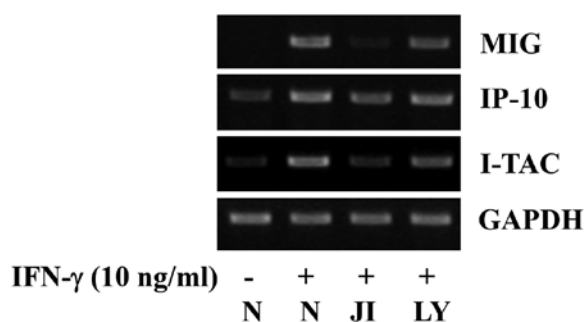


Figure 4. Effect of interferon- $\gamma$  (IFN- $\gamma$ ) and/or JAK inhibitor I or LY294002 on the mRNA expression of monokine induced by IFN- $\gamma$  (MIG), IFN-inducible protein of 10 kDa (IP-10), and IFN-inducible T cell alpha chemoattractant (I-TAC) in INS-1 cells. INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of JAK inhibitor I (JI, 0.1  $\mu$ M), a pan-JAK inhibitor, or LY294002 (LY, 25  $\mu$ M), a PI3K/PKB inhibitor, for 6 h. Total RNA was prepared and used for RT-PCR with specific primers for MIG, IP-10, I-TAC or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The blots are representative of 3 independent experiments.

IRF-1 and IRF-2 in the INS-1 cells (lane 2). However, treatment with SFN, particularly at 25  $\mu$ M, strongly reduced the mRNA levels of IRF-1 in the INS-1 cells (lane 5). SFN at the concentrations tested had no effect on the mRNA expression of IRF-2 induced by IFN- $\gamma$  in the cells. The mRNA expression of the control, GAPDH, remained constant by treatment without or with IFN- $\gamma$  in the absence or presence of SFN.

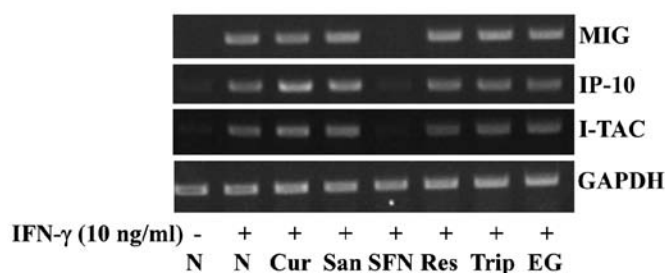


Figure 5. Effect of sulforaphane (SFN), curcumin (Cur), sanguinarine (San), resveratrol (Res), triptolide (Trip) or epigallocatechin gallate (EGCG; EG) on the interferon- $\gamma$  (IFN- $\gamma$ )-induced expression of monokine induced by IFN- $\gamma$  (MIG), IFN-inducible protein of 10 kDa (IP-10) and IFN-inducible T cell alpha chemoattractant (I-TAC) in INS-1 cells. INS-1 cells were treated with or without IFN- $\gamma$  (10 ng/ml) in the absence or presence of SFN (25  $\mu$ M), curcumin (10  $\mu$ M), sanguinarine (500 nM), resveratrol (25  $\mu$ M), triptolide (40 nM) or EGCG (25  $\mu$ M) for 6 h. Total RNA was prepared and used for RT-PCR with specific primers for MIG, IP-10, I-TAC or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The blots are representative of 3 independent experiments.

**Role of JAK/STAT-1 and PI3K/PKB in the IFN- $\gamma$ -induced expressions of MIG, IP-10 and I-TAC in the INS-1 cells.** In order to determine the role of STAT-1 and/or PKB in the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC, as well as in the suppressive effects of SFN on the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in the INS-1 cells, we then performed pharmacological inhibition experiments with the pan-JAK inhibitor, JAK inhibitor I and the PI3K/PKB inhibitor, LY294002. As shown in Fig. 4, treatment with JAK inhibitor I strongly blocked the IFN- $\gamma$ -induced expression of MIG and I-TAC, but weakly inhibited the IFN- $\gamma$ -induced expression of IP-10 in the INS-1 cells. Treatment with LY294002 also slightly reduced the mRNA levels of MIG and I-TAC, but not those of IP-10, which were induced by IFN- $\gamma$  in the INS-1 cells. The mRNA expression of the control, GAPDH, remained constant by treatment with or without IFN- $\gamma$  in the absence or presence of JAK inhibitor I or LY294002.

**Comparison of the effects of SFN and other natural substances on the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells.** To examine the specificity, we then compared the effects of SFN and other natural substances, including curcumin, sanguinarine, resveratrol, triptolide and EGCG, on IFN- $\gamma$ -induced expressions of MIG, IP-10 and I-TAC in INS-1 cells. As shown in Fig. 5, as expected, treatment with SFN strongly inhibited the mRNA expression levels of MIG, IP-10 and I-TAC induced by IFN- $\gamma$  in the INS-1 cells. However, treatment with curcumin (Cur), sanguinarine (San), resveratrol (Res), triptolide (Trip) or epigallocatechin gallate (EGCG; EG) did not affect the IFN- $\gamma$ -induced expressions of MIG, IP-10 and I-TAC in the INS-1 cells.

## Discussion

Evidence suggests elevated levels of CXC chemokines in pathological conditions of the pancreas, including pancreatic infection (21) or autoimmune insulinitis (22). The increased expression of IP-10 and the infiltration of lymphocytes



expressing CXCR3 has been observed in all pancreatic lesions of patients with type 1 diabetes, compared with no or low expression of IP-10 and CXCR3 in the pancreas of non-diabetic control subjects (12). The enhanced expression of chemokines within pancreatic islets is likely to contribute to islet inflammation by controlling the recruitment and the activation of macrophages, neutrophils and Th1. Thus, any compound that can inhibit the excessive expression of chemokines in pancreatic  $\beta$ -cells may have preventive and/or therapeutic potential against islet inflammation and related diseases.

IFN- $\gamma$  is a pro-inflammatory cytokine and mediates inflammatory and immune responses. Reportedly, type I and II interferons, including IFN- $\gamma$ , are also associated with islet inflammation and  $\beta$ -cell death and dysfunction (23-25). At present, the IFN- $\gamma$ -mediated induction of chemokine expression in pancreatic  $\beta$ -cells is not well known. In this study, we demonstrated that treatment with IFN- $\gamma$  largely stimulated the mRNA expression levels of MIG, IP-10 and I-TAC in INS-1 cells at the concentration of 10 ng/ml (Fig. 1). IFN- $\gamma$  activity is mediated through its cognate receptors, IFN- $\gamma$ R1 and IFN- $\gamma$ R2, which further trigger the activation of the JAK-STAT signaling pathway (26). Upon IFN- $\gamma$  binding to the IFN- $\gamma$ R1 and IFN- $\gamma$ R2, JAK1 and JAK2 associated with the receptors are activated, leading to STAT-1 phosphorylation (on tyrosine 701) and activation. Active STAT-1 then undergoes dimerization and the dimeric complex translocates to the nucleus where it regulates gene expression by binding to  $\gamma$ -activated sequence elements in the promoters of IFN- $\gamma$ -regulated genes, including chemokines (18,27,28). These results point out the critical role of JAK-STAT-1 activity in the transcriptional upregulation of chemokines in response to IFN- $\gamma$  exposure. In addition to JAK-STAT activity, the PI3K/PKB signaling pathway has been reported to be important for IP-10 gene expression (19). In the present study, IFN- $\gamma$  treatment largely increased the phosphorylation levels of STAT-1 and PKB in the INS-1 cells (Fig. 3A, lane 2), but the blockage of JAK/STAT-1 activity by JAK inhibitor I or PI3K/PKB activity by LY294002 strongly or weakly abrogated the ability of IFN- $\gamma$  to induce the mRNA expression of MIG, IP-10 and I-TAC in INS-1 cells (Fig. 4, lanes 3 or 4). These results suggest that the activation of the JAK/STAT-1 and PI3K/PKB signaling proteins is critical for the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells.

SFN is an isothiocyanate substance abundantly found in cruciferous vegetables, such as broccoli and brussel sprouts, and has been shown to possess anti-inflammatory, anti-cancerous, and immunomodulatory effects (13,14,29,30). Previously, the SFN-mediated regulation of chemokines, such as CCL17 and CCL22, through heme oxygenase-1 and NF- $\kappa$ B in human keratinocytes has been reported (31). At present, neither the inhibitory effect, nor the mechanisms of action of SFN as regards the expression of T-cell chemokines induced by IFN- $\gamma$  in pancreatic  $\beta$ -cells are known. In this study, we demonstrated that SFN at the 25  $\mu$ M concentration strongly inhibited not only the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC (Fig. 2), but also the IFN- $\gamma$ -induced activation (phosphorylation) of STAT-1 and PKB signaling proteins (Fig. 3A, lane 5) in INS-1 cells. Considering the positive role of STAT-1 and PKB activities in the IFN- $\gamma$ -induced expression of MIG, IP-10, and/or I-TAC in INS-1 cells herein (Fig. 4), it is evident that the

suppressive effects of SFN on the IFN- $\gamma$ -induced expression of MIG, IP-10, and I-TAC in INS-1 cells is, at least in part, attributable to the inhibition of STAT-1 and PKB signaling proteins.

The activation of STAT-1 induced by IFN- $\gamma$  leads to the transcriptional induction of a number of genes, including IRF-1, a transcription factor involved in the transcription of many antiviral and anti-apoptotic genes (32). There is of interest recent evidence suggesting that the transcriptional upregulation of MIG, IP-10 and I-TAC genes occurs through an STAT-1/IRF-1-dependent mechanism (20). The present study revealed that IFN- $\gamma$  treatment largely upregulated the transcript levels of IRF-1 and IRF-2, while SFN treatment, particularly at 25  $\mu$ M strongly suppressed the IFN- $\gamma$ -induced mRNA expression of IRF-1, but not that of IRF-2 in the INS-1 cells (Fig. 3B). It is thus likely that the downregulation of the STAT-1/IRF-1 signaling pathways further contributes to the suppressive effects of SFN on the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells.

An interesting finding of the present study is the specificity of SFN in inhibiting the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells, as evidenced by that unlike SFN, other natural substances with known anti-inflammatory and/or immune modulatory activities, such as curcumin (33), sanguinarine (34), resveratrol (35), triptolide (36) or EGCG (37), had no effect on IFN- $\gamma$ -induced expressions of MIG, IP-10 and I-TAC in INS-1 cells (Fig. 5). These results indicate that SFN may have a unique structural moiety leading to a strong inhibitory effect on the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells.

In conclusion, the findings of our study collectively demonstrate the ability of SFN to strongly inhibit the IFN- $\gamma$ -induced expression of MIG, IP-10 and I-TAC in INS-1 cells through the reduced expression and phosphorylation levels of IRF-1, STAT-1 and PKB. Although there are still important issues that remain to be resolved, including the suppressive effects of SFN on the expression of chemokines in animal models of islet inflammation, the present findings suggest that SFN may be a promising natural product for use against pancreatic islet inflammation in which the overexpression of T-cell chemokines is problematic.

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