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Tumor necrosis factor-α-induced protein-8 like 2 regulates lipopolysaccharide-induced rat rheumatoid arthritis immune responses and is associated with Rac activation and interferon regulatory factor 3 phosphorylation

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Abstract. The endogenously activated rheumatoid arthritis (RA) synovial fibroblasts (RSFs) are likely to be the key to curing the disease. RSFs express Toll-like receptors (TLRs) rendering them prone to activation by exogenous and endogenous TLR ligands, resulting in the production of chemokines and cytokines Germline deletion of tumor necrosis factor-α-induced protein-8 like 2 (TIPE2, also known as TNFAIP8L2) results in fatal inflammation and hypersensitivity to TLR and T cell receptor stimulation. The present study demonstrates an inverse association between TIPE2 and cytokine gene expression in RSFs following lipopolysaccharide (LPS) stimulation. Enhanced TIPE2 expression decreased Ras-related C3 botulinum toxin substrate (Rac) activation and interferon regulatory factor 3 phosphorylation, and phosphoinositide 3-kinase and Rac inhibition significantly diminished LPS-induced cytokine gene expression in RSFs. In conclusion, the findings of the present study demonstrate that TIPE2 serves a negative role in activating the Rac signaling pathway and in the initiation of the immune response by decreasing the activity of proinflammatory cytokines. These results may be useful in designing novel strategies for the prevention and treatment of RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by articular inflammation and leads to joint destruction (1,2). RA pathogenesis involves a complex humoral and cellular immune response, including the infiltration of lymphocytes and monocytes into the synovium. These infiltrating cells and synoviocytes release numerous proinflammatory cytokine and chemokines, including interleukin IL-6, IL-1 and tumor necrosis factor-α (TNF-α), which may serve a significant role in RA pathogenesis (3,4), and may determine the activation and proliferation of the synovial lining and the recruitment of inflammatory cells that induce inflammation and promote destruction. Elevated levels of proinflammatory cytokines have been observed in the sera and synovial fluids of patients with RA, such as IL-6, IL-8, TNF-α, IL-20, IL-17 and IL-33 (5-9). Neutralizing these cytokines with monoclonal antibodies or soluble receptors has previously been developed as a novel treatment for RA (10). The TNF-α-inducible protein 8 (TNFAIP8) family consists of TNFAIP8, TNF-α-induced protein 8-like 1 (TIPE1, also termed TNFAIP8L1), TIPE2 (also termed TNFAIP8L2) and TIPE3 (also termed TNFAIP8L3) (11). These are recently identified proteins that share considerable sequence homology for the regulation of cellular and immune homeostasis (12). TIPE2 is preferentially expressed in lymphoid tissues and hematopoietic cells, and negatively regulates immunity (12,13). TIPE2 deletion in mice leads to multi-organ inflammation, splenomegaly and premature death (14-16). TIPE2-deficient cells hyperactivate toll-like receptors (TLRs) and T cell receptors (11,12). TIPE2 is significantly downregulated in during infection or autoimmune disease (17,18). TIPE2-deficient cells lead to the production of various kinds of proinflammatory cytokines and activation of the phosphoinositide 3-kinase (PI3K)-Ras-related C3 botulinum toxin substrate (Rac) signaling pathway, which enhances protein kinase B (AKT), Rac and interferon (IFN) regulatory factor 3 activities (19-21).

In the present study, TIPE2 regulated lipopolysaccharide (LPS)-induced RA immune responses by targeting Rac

GTPases in a PI3K-dependent manner. Tipe2 protein has the potential to be used to treated RA (19,21-25).

Materials and methods

Cell lines and plasmids. The induction of adjuvant arthritis in rats, the preparation of rheumatoid arthritis (RA) synovial fibroblasts (RSF) and identification techniques were performed according to previous publications (10,26). Normal synovial fibroblasts (NSFs) and RSFs were grown in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), penicillin and streptomycin. To generate stable cell lines, $5x10^5$ adjuvant arthritis fibroblast-like synoviocytes were transduced with MIGR1/TIPE2-overexpression recombinant lentiviral vectors and a MIGR1 lentiviral vector was used as the negative control (gift from Dr Chen Youhai, University of Pennsylvania School of Medicine, Philadelphia, PA, USA). The lentiviral vectors contained human and rat inserts for the gene encoding TIPE2 and the sequences are at: http://www.addgene.org/27490/sequences/#addgene-partial. The cells were cultured at 37˚C and the culture medium was replaced 24 h after transduction. After an additional 48 h culture at 37˚C, transduced cells were observed visually under a fluorescent microscope, 12 fields were counted and infected cells constituted >90% of the total cell count. The infected cells were selected by flow cytometry and identified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis (11,26).

RT-qPCR. Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) from NSFs and RSFs and purified with RNeasy Mini kits (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol (11,26).

After processing with RNase-free DNase I (Invitrogen; Thermo Fisher Scientific, Inc.), RNA samples were reverse transcribed with oligo (dT) and SuperScript II transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). PCR was performed using an Applied Biosystems 7500 system and Power SYBR-Green PCR Master mix (Applied Biosystems, Thermo Fisher Scientific, Inc.). Relative gene expression levels were determined using GAPDH as the control. The PCR products were run in an agarose gel and were in all cases confined to a single band of the expected size. A melting-curve analysis was also performed to ensure specificity of the products (11,24). Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta Cq}$ method (27).

TLR2, *TLR3* and *TLR4* mRNA expression levels were analyzed by RT-qPCR. The cycling conditions used were as follows: An initial predenaturation step at 94˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 30 sec, extension at 72˚C for 1 min and a final extension step at 72˚C for 5 min. Each sample was normalized to the expression levels of β-actin or GAPDH. (11,26).

TNFAIP8L2, *IL-1*, and *GAPDH* primers were purchased from Qiagen, Inc. Other primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc., and the sequences were as follows: 5'-GTTGACAGCCACTGCCTTCC-3' (forward) and 5'-CTGACAGTGC ATCATCGCTG-3' (reverse) for *IL-6*; 5'-CCTCTCCATCGACTACAAGC-3' (forward) and 5'-CTCTTCTCCATCTGTGACGG-3' (reverse) for *IFNG*; 5'-CCAGGAGAAAGTCAGCCTCC-3' (forward) and 5'-GTTGACCTCAGCGCTGAGC-3' (reverse) for *TNF*; 5'-CTGTGATAGGCCTCTCAAGG-3' (forward) and 5'-CACCATGGCCAATGTAGGTG-3' (reverse) for *TLR2*; 5'-CCAGCTGTTAGCAACCAGC-3' (forward) and 5'-CGAGGGACAGATACTTCAGG-3' (reverse) for *TLR3*; 5'-GTAGCCGCTCTGGCATCATC-3' (forward) and 5'-CTCCCCAGAGCATTGTCCTC-3' (reverse) for *TLR4*; 5'-TGCGTGACATCAAAGAGAAG-3' (forward) and 5'-TCCATACCCAAGAAGGAAGG-3' (reverse) for β-actin 5'-TGATTCTACCCACGGCAAGTT-3' (forward) and 5'-TGATGGGTTTCCCATTGATGA-3' (reverse) for GAPDH; 5'-CCTTGTGCAAGTGTCTGAAGC-3' (forward) and 5'-CCCAAGTCAAGGGCTTGGAA-3' (reverse) for IL-1; and, 5'-GGGAACATCCAAGGCAAG-3' (forward) and 5'-AGCTCATCTAGCACCTCACT-3' (reverse) for TNFAIP8L2.

LPS stimulation assay. Control RFSs and TIPE2-overexpressed RSFs were treated by LPS (100 ng/ml; 9001-62-1; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 3 time-points (0 min, 2, 8 h). After LPS treatment the cells were collected and Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta Cq}$ method (27).

PI3K and Rac inhibition assay. Control RSFs were treated with or without the indicated concentrations of LY294002 or NSC23766 inhibitors for 20 min prior to stimulation with LPS (100 ng/ml) for 2 h. Cytokine expression was determined using RT-qPCR (19,26).

Western blot analysis. The protein was isolated from RSFs and NSFs using the ProteoPrep Total Extraction Sample kit (PROTTOT-1KT; Sigma-Aldrich; Merck KGaA), and the protein concentration was determined by BCA assay (p0012 BCA; Beyotime Institute of Biotechnology; Shanghai, China). Then, 0.6 μg aliquots of synovial cell lysates were loaded and separated by 12% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, blocked by 5% FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) for 1 h at room temperature and probed with the following primary antibodies for 75 min at room temperature: rabbit anti-β-actin (m1210-5; 1:1,000; Hangzhou Huaan Biotechnology Co., Ltd., Hangzhou, China), rabbit anti-TIPE2 (ab110389; 1:1,000; Abcam, Cambridge, UK), rabbit anti-caspase-8 (p18; ab25901; 1:1,000; Abcam), rabbit anti-p21 activated kinase 1 protein (PAK1; ab40852; 1:1,000 Abcam), rabbit anti-phosphorylated pPAK1 (phospho; ab40795; 1:1,000; Abcam), rabbit anti-pTANK-binding kinase 1 (phospho; NAK; ab40676; 1:1,000; Abcam), rabbit anti-pAKT (phospho; ab81283; 1:1,000; Abcam) and rabbit anti-Rac (Cell Signaling Technology, Inc., Danvers, MA, USA). The second antibodies (goat anti rabbit; A32732; 1;1,000; Thermo Fisher Scientific, Inc.) were added for 1 h at room temperature. The protein bands were visualized with an

Enhanced Chemiluminescence substrate (NCI4106; Thermo Fisher Scientific, Inc.). Immunoblot analysis was performed as previously described (19,26). Densitometric analysis was performed with ImageJ software (version 1.50; National Institutes of Health, Bethesda, MD, USA).

ELISA analysis. The levels of inflammatory cytokines in supernatants were measured with commercially available ELISA kits for IL-6 (BMS625TWO), IL-1 (BMS627TWO), IFN-γ (BMS621) and TNF-α (BMS622TWO; all from eBioscience; Thermo Fisher Scientific, Inc.), in accordance with the manufacturers' protocols. The absorbance was measured at a wavelength of 450 nm using a 680XR microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All samples were analyzed in duplicate. The standard curve for interpolating the protein concentration in each sample was generated using linear regression analysis, and was performed as previously described (11,28).

Rac pulldown assay. To assess Rac activation, cells ($2x10^8$/ml) were incubated with PAK-glutathione-S-transferase fusion protein beads (Cytoskeleton, Inc., Denver, CO, USA) at 4˚C for 60 min. The collected beads were then washed three times and resuspended in SDS protein sample buffer. Bound proteins and total cell lysates were analyzed by western blotting using an anti-Rac antibody (Cell Signaling Technology, Inc.). The Rac pulldown assay was performed as previously described (26,29).

Statistical analysis. Statistical analysis was performed using SPSS version 11.5 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation of three experiments. The significance of the differences in the mean values between or within multiple groups was determined with a Student's t-test test and two-way analysis of variance followed by Tukey's post hoc test, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inverse association between TIPE2 and cytokine gene expression in RSFs following TLR stimulation with LPS. RSFs serve important roles in immunity to RA pathogens. To explore the roles of TIPE2 in RSFs-mediated proinflammatory cytokine secretion, *TNFAIP8L2* and TIPE2 expression was examined in rat RSFs by RT-qPCR and western blotting, respectively. The results demonstrated that *TNFAIP8L2* mRNA (Fig. 1A) and TIPE2 protein expression (Fig. 1B) was lower rat RSFs than in NSFs. Upon stimulation with LPS (the TLR4 ligand), the mRNA expression levels of the cytokines *IL-6*, *IL-1*, *TNF* and *IFNG* (Fig. 1C) and their protein products (IL-6, IL-1, TNF-α and IFN-γ, respectively; Fig. 1D), were significantly increased in RSFs. This inverse association between TIPE2 and cytokine levels in RSFs treated with the TLR ligand LPS, suggested a role for TIPE2 in regulating RA inflammatory responses.

Enhanced TIPE2 expression in RSFs decreases cytokine expression. To determine whether TIPE2 expression affected the production of IL-1, IL-6 and TNF-α by RSFs, a TIPE2 overexpression plasmid was transfected with MIGRI retrovirus which contained the lentiviral constructs or with MIGRI retrovirus negative control. TIPE2 overexpression in rat RSFs was termed TIPE2-overexpressed RSFs, whereas RSFs transfected with the MIGRI retrovirus were named control RSFs. Subsequently, expression levels of *TNFAIP8L2* and its TIPE2 protein product were measured by RT-qPCR and western blotting, respectively. The results revealed that *TNFAIP8L* and TIPE2 expression levels were elevated in the TIPE2-overexpressed group compared with the control (Fig. 2A). By contrast, mRNA expression levels of the cytokines *IL-6*, *IL-1*, *TNF* and *IFNG* (Fig. 2B) and their protein products (IL-6, IL-1, TNF-α and IFN-γ, respectively; Fig. 2C), were significantly increased in the control group compared with the TIPE2-overexpressed group.

Enhanced TIPE2 expression in RSFs decreases Rac activation. To explore the potential underlying mechanism of TIPE2 in LPS-induced cytokine expression, TLR mRNA expression levels between the TIPE2-overexpressed RSFs and control RSFs were compared (Fig. 3A), and results suggested that decreased cytokine expression in TIPE2-overexpressed cells was not due to the decreased expression of the *TLR2*, *TLR3* or *TLR4* (16). Endogenous TIPE2 may constitutively bind to the small GTPase, Rac, in immune cells. Therefore, Rac activation between LPS-treated TIPE2-overexpressed RSFs and control RSFs was compared. Elevated constitutive Rac activation was observed in the control RSFs (Fig. 3B), suggesting that Rac was activated and involved in LPS-mediated cytokine expression. PAK and AKT are downstream effectors of Rac and PI3K, respectively. As demonstrated in Fig. 3C, following LPS-treatment (30, 60 and 120 min), the levels of phosphorylation and activation in control RSFs were higher compared with $TIPE2^{+/+}$ RSFs at corresponding time points. Rac1 and PAK1 have been reported to act upstream of TBK1/inhibitor of κ B (IκB) kinase-ε in the viral activation of interferon regulatory factor 3 (IRF3) (17). Decreased phosphorylation and activation of NAK was also observed in the control RSFs at 60 min LPS treatment compared with the TIPE2-overexpressed RSFs (Fig. 3D), suggesting that TIPE2 regulates NAK via the Rac/PAK signaling pathway.

PI3K and Rac inhibition significantly diminishes LPS-induced cytokine gene expression. TIPE2 is a negative regulator in immune response and TIPE2-overexpression cells have low LPS-induced cytokine gene expression. To further dissect the pathways involved in LPS-mediated cytokine production in RSFs, inhibitors of various signaling pathways were used. As presented in Fig. 4A, the PI3K inhibitor LY294002 and the Rac inhibitor NSC 23,766 effectively inhibited *TNF* and *IL-6* expression levels in a dose-dependent manner. Next, it was examined whether LY294002 may also affect Rac activation. As demonstrated in Fig. 4B, LY294002 partially blocked Rac activation. PAK, AKT, and NAK expression levels were also compared in the control RSFs with and without PI3K inhibitor LY294002 and Rac inhibitor NSC 23,766 added to cells 30 min prior to LPS stimulation. The expression level of pAKT was reduced in the control RSFs with PI3K inhibitor LY294002 compared with those without the inhibitor present. The expression levels of pPAK and pNAK were also diminished. (Fig. 4C) Reduced

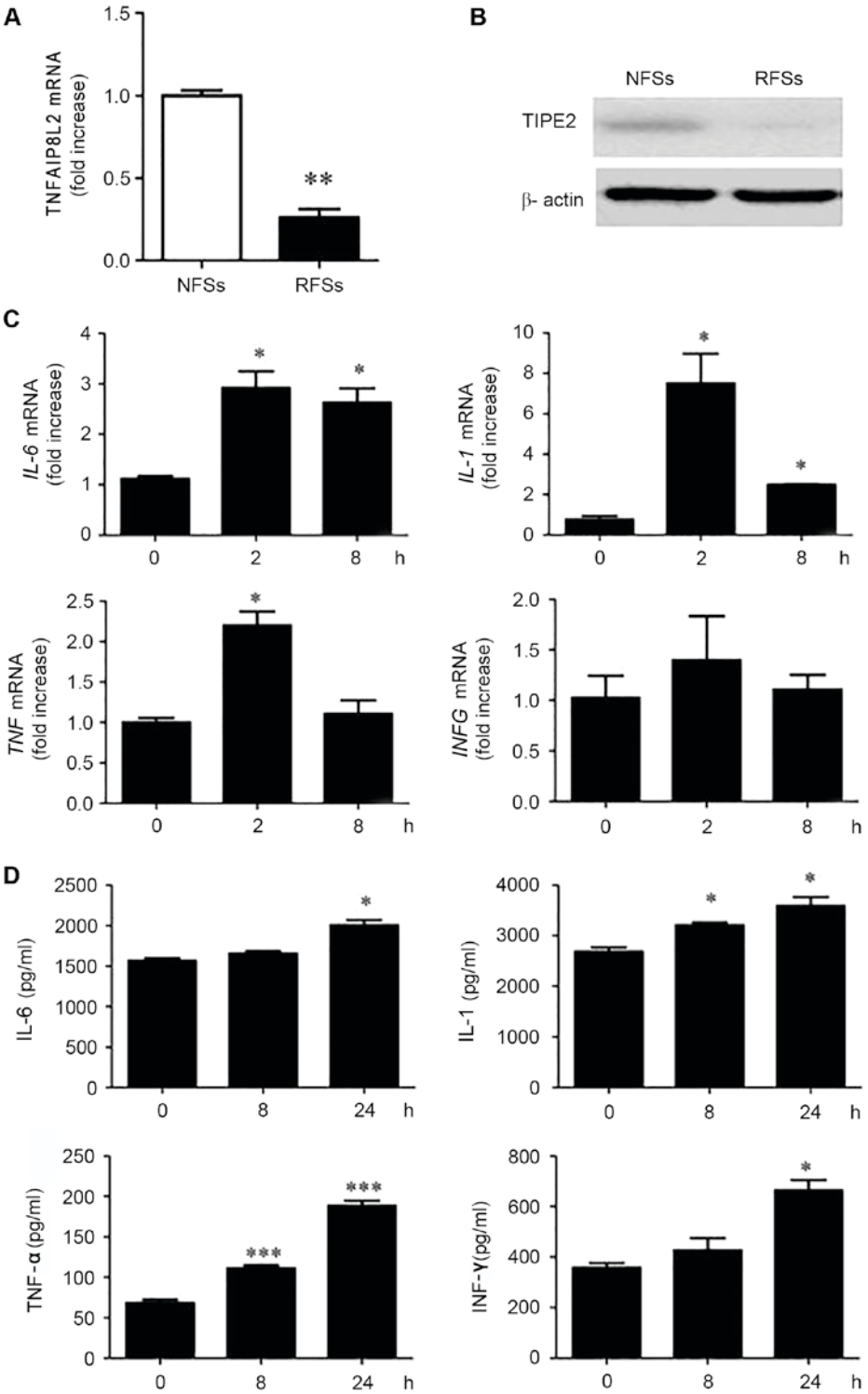

Figure 1. Inverse association between the gene encoding TIPE2 and cytokine expression levels in RSFs following TLR stimulation with LPS. (A) *TNFAIP8L2* expression levels, as measured by RT-qPCR. **P<0.01 vs. NSFs. (B) TIPE2 protein expression by western blot analysis, in freshly harvested NSFs and RSFs. (C) RSFs were stimulated with LPS (100 ng/ml) for 2 or 8 h and *IL-6*, *IL-1*, *TNF* and *IFNG* mRNA expression levels were determined by RT-qPCR. (D) RSFs were stimulated with LPS (100 ng/ml) for 2 or 8 h and IL-6, IL-1, TNF-α and IFN-γ concentrations were determined by ELISA. *P<0.05, **P<0.01 ***P<0.001 vs. 0 h. Data are presented as the mean ± standard deviation (n=3). RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TIPE2, tumor necrosis factor-α-induced protein-8 like 2; *TNFAIP8L2*, tumor necrosis factor-α-induced protein-8 like 2 gene; RSFs, rheumatoid arthritis synovial fibroblasts; NSFs, normal synovial fibroblasts; LPS, lipopolysaccharide; *IL-6*, interleukin 6 gene; *IL-1*, interleukin 1 gene; *TNF,* tumor necrosis factor-α gene; *IFNG,* interferon-γ gene; IL-6, interleukin-6; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

expression levels of pPAK, pAKT and pNAK were observed in the control RSFs with Rac inhibitor NSC 23,766 compared with those without, (Fig. 4D), suggesting that Rac was activated significantly and involved in LPS-mediated cytokine expression. PI3K was segmental activated and involved in LPS-mediated cytokine expression. These results suggested that Rac activation is directly involved in LPS-mediated cytokine expression.

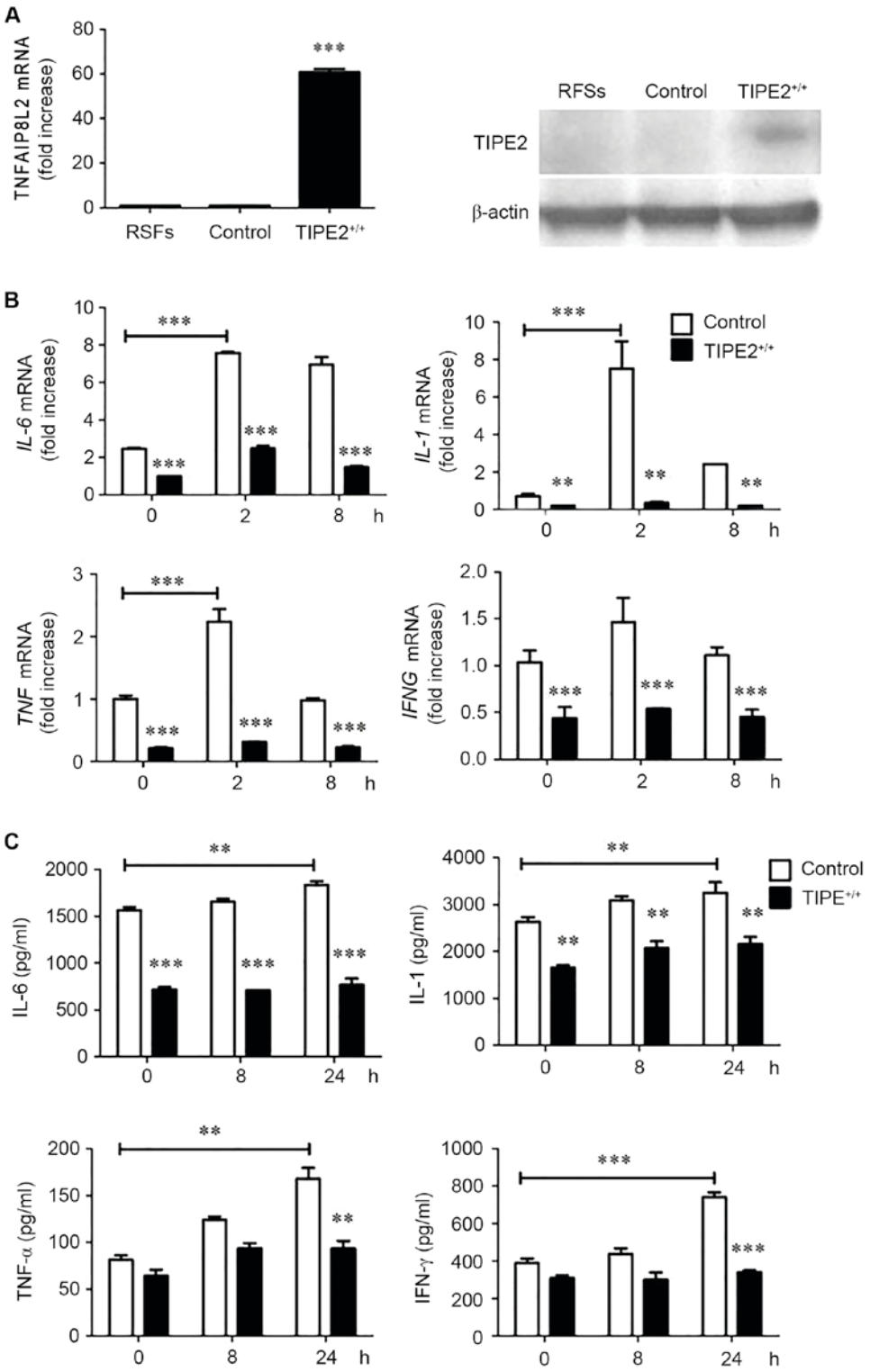

Figure 2. Enhanced *TNFAIP8L2* expression levels in RSFs associates with decreased cytokine expression. (A) RT-qPCR analysis of *TNFAIP8L2* expression and western blot analysis of TIPE2 expression, in freshly harvested control RSFs and TIPE$^{+/+}$ (TIPE2-overexpressed) RSFs stimulated with LPS (100 ng/ml) for the indicated times. (B) *IL-6*, *IL-1*, *TNF* and *IFNG* mRNA expression levels and (C) IL-6, IL-1, TNF-α, and IFN-γ expression levels, as determined by ELISA. Data are presented as the mean ± standard deviation (n=3). **P<0.01 and ***P<0.001 vs. control at each time point, unless otherwise indicated. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TIPE2, tumor necrosis factor-α-induced protein-8 like 2; *TNFAIP8L2*, tumor necrosis factor-α-induced protein-8 like 2 gene; RSFs, rheumatoid arthritis synovial fibroblasts; LPS, lipopolysaccharide; *IL-6*, interleukin 6 gene; *IL-1*, interleukin 1 gene; *TNF,* tumor necrosis factor-α gene; *IFNG,* interferon-γ gene; IL-6, interleukin-6; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

Discussion

TLRs recognize pathogen-associated molecular patterns, which are microbial and viral products that induce cell activation (30,31). Exogenous TLR ligands include lipoteichoic acid, LPS, CpG motifs of bacterial DNA and viral RNA (32,33). TLR2, TLR3, TLR4, TLR7 and various other ligands were demonstrated to be highly expressed in synovial

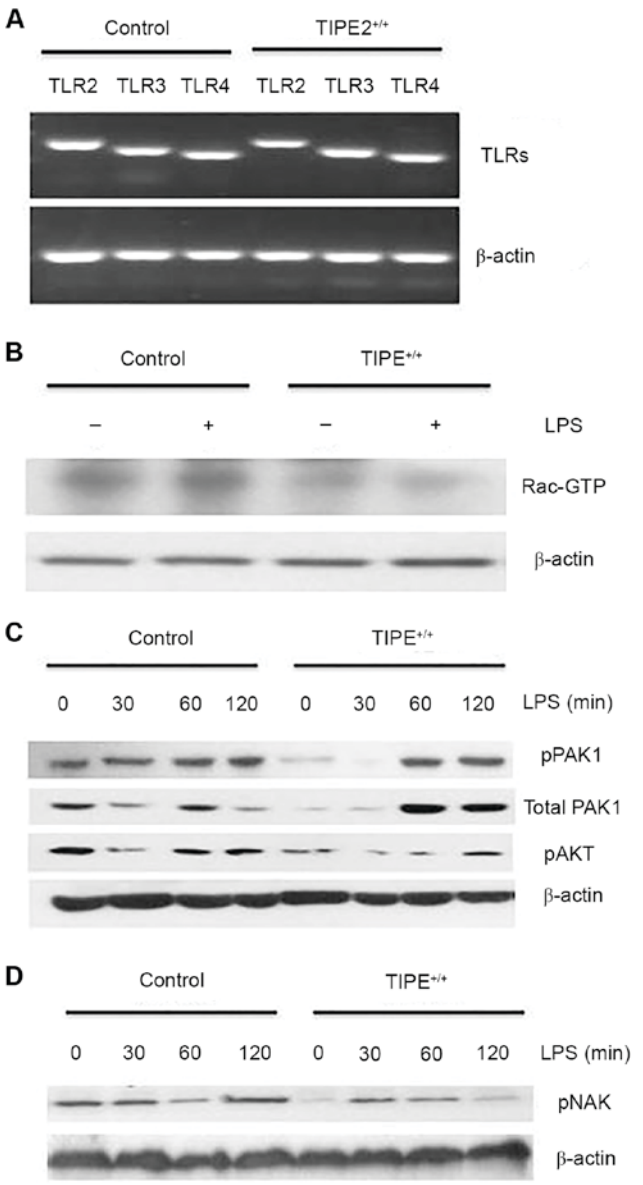

Figure 3. Enhanced TIPE2 expression in RSFs decreases Rac activation. (A) *TLR2*, *TLR3* and *TLR4* mRNA expression levels in freshly harvested in control RSFs and TIPE$^{+/+}$ (TIPE2-overexpressed) RSFs, as determined by reverse transcription-quantitative polymerase chain reaction. (B) Rac is activated in control RSFs. Cells were lysed and incubated with PAK-glutathione-S-transferase fusion protein beads and activated Rac was detected by western blotting. (C and D) Increased pNAK, pPAK and pAKT in control RSFs. Control RSFs and TIPE$^{+/+}$ RSFs were treated with LPS (100 ng/ml) for the indicated times. The levels of the total proteins and phosphorylated proteins were determined by western blotting. Data presented in this figure are representative of at least three independent experiments. TIPE2, tumor necrosis factor-α-induced protein-8 like 2; *TLR2*, toll-like receptor 2; *TLR3*, toll-like receptor 3; *TLR4*, toll-like receptor 4; RSFs, rheumatoid arthritis synovial fibroblasts; PAK, p21 activated kinase 1 protein; pNAK, phosphorylated TANK-binding kinase 1; pPAK, phosphorylated p21 activated kinase 1 protein; pAKT, phosphorylated protein kinase B; LPS, lipopolysaccharide.

tissue from RA patients compared with that from healthy donors (22,23). TLR-mediated activation of RSFs from patients with RA leads to significantly higher levels of key proinflammatory cytokines, including IL-8, IL-6 and IL-15, compared with fibroblast-like synoviocytes from healthy counterparts (5,11,31).

TIPE2 is preferentially expressed in lymphoid tissues such as the thymus and lymph nodes. Although TIPE2 is not expressed in the NIH 3T3 fibroblast cell line, following stimulation with TNF-α, NIH 3T3 fibroblasts express detectable mRNA expression levels of the gene encoding TIPE2, suggesting that TIPE2 may be expressed in other cell types to establish equilibrium during an inflammatory response (11,34). Increasing experimental evidence suggests that TIPE2 is closely associated with the occurrence and development of inflammatory diseases and autoimmune diseases such as lung injury, acute-on-chronic hepatitis B liver failure, hepatocellular carcinoma, colitis, type 2 diabetes and systemic lupus erythematosus (26,35,36). However, the present study demonstrated that TIPE2 serves an inhibitory role in RA. The results revealed that TIPE2 expression was lower in rat RSFs compared with NSFs, whereas cytokine expression levels of IL-6, IL-1, IFN-γ and TNF-α were significantly increased in rat RSFs upon stimulation with LPS. In addition, Rac activation between TIPE2-overexpressed RSFs and control RSFs were compared, and elevated Rac activation was observed in control RSFs, whereas IL-6, IL-1, TNF-α and IFN-γ were significantly increased in the control group compared with the TIPE2-overexpressed group. The Rac inhibitor NSC 23,766 effectively inhibited TNF-α and IL-6 production in a dose-dependent manner. Taken together, these results suggested that TIPE2 regulates cytokines secretion in RSFs via the Rac signaling pathway.

The TIPE2 protein, which constitutively binds to the Rac small GTPase in immune cells, serves as a negative regulator of phagocytosis and oxidative burst during infection in immune cells (19), which are two fundamental effector mechanisms of innate immunity. These effector mechanisms are activated by TLRs and Rac GTPases, that work in unison to eliminate infectious microbes (24,25). PAK and AKT are downstream effectors of Rac and PI3K, respectively (19). Previous studies have suggested that Rac1 and PAK1 act upstream of NAK (also known as TBK1/IκB kinase-ε) in the viral activation of IRF3 (20). In addition, Joung *et al* (21) reported that AKT contributes to the activation of the TIR-domain containing adapter-inducing IFN-β-dependent signaling pathways of TLRs by interacting with NAK. In the present study, PAK and AKT were also activated in control RSFs compared with TIPE$^{+/+}$ RSFs (37,38). Increased activation of NAK was also observed in control RSFs compared with TIPE2-overexpressed RSFs, indicating that TIPE2 regulates NAK via the Rac/PAK pathway (39,40).

The present study also revealed that the PI3K inhibitor LY294002 effectively inhibited TNF-α and IL-6 production in control RSFs in a dose-dependent manner, similar to the Rac inhibitor NSC 23,766. LY294002 also significantly blocked Rac activation. In addition, less PAK, AKT and NAK activation was observed in the control RSFs and partial PAK, AKT and NAK activation was found when the Rac inhibitor NSC 23,766 and LY294002 were added before LPS stimulation, indicating that Rac and PI3K may be activated and involved in LPS-mediated cytokine expression. These results suggested that Rac activation may be directly involved in LPS-mediated cytokine expression in a PI3K dependent manner (19-21).

In conclusion, the findings of the present study demonstrated that TIPE2 serves a negative role in activating the Rac

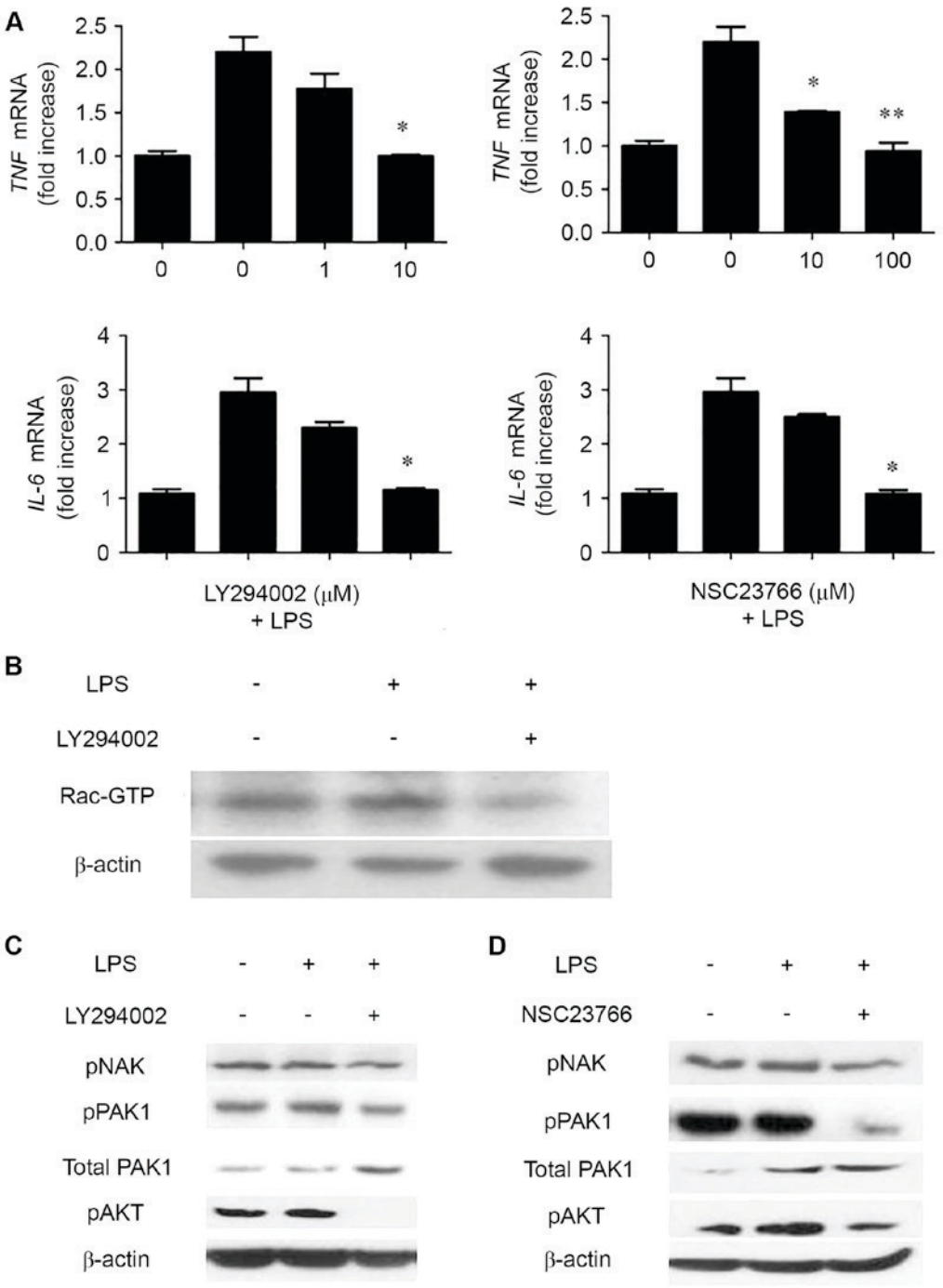

Figure 4. PI3K and Rac inhibition significantly diminishes LPS-induced cytokine gene expression in control RSFs. (A) Control RSFs were treated with or without the indicated concentrations of LY294002 (left) or NSC23766 (right) inhibitors for 20 min prior to stimulation with LPS (100 ng/ml) for 2 h. Cytokine expression was determined with reverse transcriptase-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. LPS treated NSFs. (B) Control RSFs were treated with or without LY294002 (10 mM) for 30 min prior to stimulation with LPS (100 ng/ml) for 2 h. Cells were lysed, and activated Rac-GTP was detected via the pulldown assay and western blotting. (C) Control RSFs were treated with or without LY294002 (10 μM) and (D) NSC23766 (100 μM) for 30 min prior to stimulation with LPS (100 ng/ml) for 2 h. Cells were lysed, and pNAK, pPAK and pAKT were detected. PI3K, phosphoinositide 3-kinase; LPS, lipopolysaccharide; RSFs, rheumatoid arthritis synovial fibroblasts; GTP, guanosine triphosphate; pNAK, phosphorylated TANK-binding kinase 1; pPAK, phosphorylated p21 activated kinase 1 protein; pAKT, phosphorylated protein kinase B.

signaling pathway and in the initiation of the immune response via the activity of proinflammatory cytokines. These findings uncovered some of the underlying mechanisms involved in RA with the large number of inflammatory cytokines produced by RSFs. These results may be useful in designing novel strategies for preventing and treating RA.

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