ARTICLE
Journal of Cellular Biochemistry 114:1315–1321 (2013)

Journal of Cellular Biochemistry

Role of JAK2–STAT3 in TLR2-Mediated Tissue Factor Expression

Dae-Weon Park,[1] Ji Hyo Lyu,[1] Jin-Sik Kim,[1] Haemin Chin,[1] Yoe-Sik Bae,[2] and Suk-Hwan Baek[1*]

[1] *Department of Biochemistry and Molecular Biology, Aging-Associated Vascular Disease Research Center, College of Medicine, Yeungnam University, Daegu 705-802, Republic of Korea*

[2] *Department of Biological Science, Sungkyunkwan University, Suwon 440-746, Republic of Korea*

ABSTRACT

Tissue factor (TF) is a core protein with an essential function in the coagulation cascade that maintains the homeostasis of the blood vessels. TF not only participates in neointima formation, but also causes the development of atherosclerosis. This study investigated the mechanism regulating TF expression in macrophages using Pam_3CSK_4, a TLR2 ligand. Pam_3CSK_4 induced TF expression in two types of macrophages (Raw264.7 and BMDM), but not in TLR2 KO mice derived BMDM. Pam_3CSK_4 induced TF expression was inhibited by pretreatment with pan-JAK inhibitor or JAK2 inhibitor AG490. JAK2 knock-down by siRNA inhibited Pam_3CSK_4 induced TF expression. Pam_3CSK_4 stimulated STAT3 phosphorylation (S727), while STAT3 knock-down by siRNA reduced Pam_3CSK_4 induced TF expression. These results suggest that Pam_3CSK_4 induced TF expression is regulated by the JAK2–STAT3 signaling pathway. Pam_3CSK_4, unlike increased TF expression, significantly decreased RGS2 expression, while RGS2 overexpression decreased Pam_3CSK_4 induced TF expression. Inhibition of TF by RGS2 WT did not occur in mutants with flawed RGS domains. We also investigated the correlation between RGS2 and STAT3 phosphorylation. RGS2 knock-down elevated Pam_3CSK_4 induced STAT3 phosphorylation, but RGS2 overexpression had the opposite effect on STAT3 phosphorylation. These results suggest that, while Pam_3CSK_4 induced TF expression is regulated by JAK2–STAT3 signaling, RGS2 is a negative regulator targeted to STAT3. J. Cell. Biochem. 114: 1315–1321, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: PAM_3CSK_4; TLR2; TF; JAK2; STAT3; RGS2

Innate immune response performs as a primary defense mechanism for bacterial or viral infection and is initiated via pattern recognition receptors such as toll-like receptor (TLR) in macrophages, dendritic cells, and epithelial cells [Akira et al., 2006]. TLRs are primarily expressed in cells responsible for immune response, and transduce signals to the inside of cells by recognizing specific structures of microbial products. There are currently 13 known types of TLRs. When a TLR is stimulated, the expression of cytokines, chemokines, and adhesion proteins increases and induces various immune responses [Newton and Dixit, 2012]. However, overactivation of immune responses by TLR stimulation can cause diseases such as atherosclerosis. TLR2 and TLR4 are the most well studied TLRs involved in atherosclerosis. Lipoproteins recognize TLR2 present in the plasma membrane. The activation of TLR2 switches macrophages to foam cells, and induces atherosclerosis [Cole et al., 2010].

It has been suggested that tissue factor (TF) is important among various proteins regulating atherosclerosis. TF, which is also known as thromboplastin, is a transmembrane glycoprotein of 47 kDa that is expressed in vascular or non-vascular cells. TF is constantly expressed in subendothelial cells such as vascular smooth muscle cells (VSMC) and cooperates with various factors to induce rapid initiation of blood coagulation when vascular damage occurs. Monocytes in the physiological environment expresses very low levels of TF; therefore, it is difficult to understand the function of TF in circulating vascular cells. However, when exposed to lipopolysaccharide, they improve TF expression and elevate activation. Since TF mRNA was first found in plaque and shown to contribute to

Grant sponsor: Korea Science and Engineering Foundation; Grant sponsor: Korean Government (MEST); Grant numbers: 2011-0013206, 2011-0006181.

*Correspondence to: Suk-Hwan Baek, PhD, Department of Biochemistry and Molecular Biology, Aging-Associated Vascular Disease Research Center, 210 Main Building, College of Medicine, Yeungnam University, 170 Hyeonchung-ro (317-1 Daemyung-5 Dong), Nam-gu, Daegu 705-802, Republic of Korea. E-mail: sbaek@med.yu.ac.kr

Manuscript Received: 15 October 2012; Manuscript Accepted: 27 November 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 December 2012

DOI 10.1002/jcb.24472 • © 2012 Wiley Periodicals, Inc.

atherosclerosis [ten Cate, 2012], many TF inducing factors have been identified, including lipopolysaccharide (LPS), interleukin-1 (IL-1), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and oxidized low-density lipoprotein (oxLDL).

We recently identified the function of janus kinase (JAK) and signal transducer and activator of transcription (STAT) according to TLR2 stimulation. Cooperation of JAK1 and JAK3 contributed to Nox1 expression and ROS production stimulated by TLR2. In this process, STAT3 activation was necessary for NADPH oxidase 1 (Nox1) expression [Lee et al., 2012]. JAK2 function was also observed in TLR2 induced MCP-1 production. JAK2 activation regulated the Akt (also known as protein kinase B)-glycogen synthase kinase 3 beta (GSK3β) pathway, thereby inducing MCP-1 production [Park et al., 2012b]. However, there have been no studies of the role of JAK and STAT in TF expression to date.

The JAK–STAT signaling pathway is related to various immune diseases. Four types of JAKs (JAK1, 2, 3, and TYK2) in mammals are currently known. It can be phosphorylated by directly binding with tyrosine kinase receptor. The main mechanism of JAK is to induce STAT protein phosphorylation and increase the transcription of other genes [Rane and Reddy, 2000; Butterbach et al., 2011]. STAT is an intracellular transcription factor in an inactive form that is transformed to an active form via various cytokines or growth factors. To date, seven types of STAT proteins have been identified (STAT1, 2, 3, 4, 5A, 5B, and 6), most of which are activated through tyrosine and serine residue phosphorylation. Activated STAT forms mono- or hetero-dimers, which are transferred to the nucleus where they bind to the γ-interferon activation sequence (GAS) or interferon-stimulated response element (ISRE) and induce gene transcription [Katsoulidis et al., 2008].

Regulators of G-protein signaling (RGS) proteins can set thresholds for the activation of heterotrimeric G-proteins, function as feedback inhibitors, sharpen signal termination following stimulus removal, and interact with other components in G-protein signaling pathways. Recent studies have elucidated some of the biochemical pathways and physiological processes regulated by RGS proteins. One of these proteins, RGS2, potently inhibits signaling through receptors that couple to Gq and substantially inhibits the activation of certain adenylyl cyclase isoforms, thereby interfering with Gs signaling [Kehrl and Sinnarajah, 2002].

In the present study, a novel TF expression mechanism in the process of atherosclerosis caused by TLR2 activation was verified. The results presented herein suggest the importance of the JAK2–STAT3 signaling pathway in TF expression, as well as the involvement of RGS2, making it a new target for regulation of further atherosclerosis.

MATERIALS AND METHODS

REAGENTS

Cell-culture reagents including fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY). α-RGS2 antibody was obtained from Abnova (Walnut, CA). α-STAT3 and α-phospho-STAT3 (S727) were obtained from Cell Signaling (Danvers, MA). α-Flag and α-β-actin (anti-β-actin) antibodies were purchased from Sigma–Aldrich (St. Louis, MO). Reverse transcription polymerase chain reaction (RT-PCR) kits were acquired from Takara Bio (Kyoto, Japan). STAT3, RGS2 siRNA, and TRIzol were purchased from Invitrogen (Carlsbad, CA). JAK2 siRNA was obtained from Bioneer (Daejeon, Korea). AG490 was purchased from Biomol (Plymouth Meeting, PA). pan-JAK inhibitor (JAK inhibitor I) was purchased from Merck/Calbiochem (Darmstadt, Germany). Pam_3CSK_4 was purchased from InvivoGen (San Diego, CA).

CELL CULTURE AND MOUSE BMDM PREPARATION

The Raw264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2. Primary BMDMs were isolated from WT or TLR2 KO mouse bone marrow and differentiated for 5–7 days in media containing M-CSF. The culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% L929 cell-conditioned medium (as a source of M-CSF). This study was conducted in accordance with the guidelines for the care and use of laboratory animals provided by Yeungnam University and all experimental protocols were approved by the Ethics Committee of Yeungnam University, Republic of Korea.

REVERSE-TRANSCRIPTION POLYMERASE-CHAIN REACTION (RT-PCR) AND REAL-TIME PCR

Total RNA was extracted from cells using TRIzol. First-strand cDNA was synthesized from 1 μg of total RNA in the presence of random primers, oligo(dT) and reverse transcriptase (Takara Bio). The conditions for PCR were as follows: 95°C for 5 min, followed by 26–33 cycles of 95°C for 1 min, 60–63°C for 1 min and 72°C for 1 min. For semi-quantitative PCR, target genes were normalized for β-actin transcription. The sequences of PCR primers used in the present study were as follows: (i) tissue factor forward: 5′-GAA GGA TGT GAC CTG GGC C-3′, reverse: 5′-GTA CCA TTC TTT CTG ACT AA-3′; (ii) JAK2 primer forward: 5′-AAG AGC AAC GGA AGA TTG C-3′, reverse: 5′-CGT CAC AGT TTC TTC TGC CT-3′; (iii) RGS2 primer forward: 5′-ATG CAA AGT GCC ATG TTC CTG G-3′, reverse: 5′-TCA TGT AGC ATG GGG CTC CG-3′; (iv) β-actin primer forward: 5′-TCC TTC GTT GCC GGT CCA CA-3′, reverse: 5′-CGT CTC CGG AGT CCA TCA CA-3′. Real-time PCR was performed using a LightCycler 1.5 Thermal Cycler (Roche Diagnostics, Almere, The Netherlands) with SYBR-Green I as the florescent dye according to the manufacturer's instructions. The sequences of PCR primers used in the present study were as follows: (i) Tissue factor forward, 5′-GAA GGA TGT GAC CTG GGC C-3′, reverse, 5′-GTA CCA TTC TTT CTG ACT AA-3′ (ii) β-actin primer forward, 5′-AGA GGG AAA TCG TGC GTG AC-3′, reverse, 5′-CAA TAG TGA TGA CCT GGC CGT-3′.

SIRNA AND TRANSFECTION

Stealth RNAi Negative control (Invitrogen, 12935-300) and gene-specific siRNA against the following target genes were designed using the Block-IT Stealth RNAi designer (Invitrogen): (i) JAK2, 5′-CGG GUC GGC GCA ACC UAA GAU UAA U -3′; (ii) STAT3, 5′-AAA CGU GAG CGA CUC AAA CUG CCC U-3′; (iii) RGS2, 5′-AGA AGU AGC UCA AAC GGG UCU UCC A-3′. For transfection experiments,

Raw264.7 cells were plated in 35 mm plates and transfected with siRNA at a final concentration of 150–200 pM using nucleoporation reagents from Lonza (Allendale, NJ). Cells were nucleoporated according to the manufacturer's protocols and incubated for 18–24 h before Pam_3CSK_4 stimulation.

PLASMIDS

To create RGS2 expression constructs (pFLAG-CMV2 empty vector or mouse RGS2 (pEGFP-C1)), cDNA was PCR-amplified using the following primers: forward, 5′-CGC AAG CTT ATA GAA TGC AAA GTG CCA TG-3′; reverse, 5′-GGG GGA TCC TCC TGG TCT CAT GTA GCA TG-3′. The RGS2 PCR product and vectors (pFLAG-CMV2) (Sigma–Aldrich) were digested with *Hin*dIII and *Bam*HI before ligation of the vector and T4 ligase was inserted. For FLAG-RGS2 plasmids, cDNA was PCR-amplified with the following primers: forward, 5′-CGC AGA TCT AAT GCA AAG TGC CAT GTT C-3′; reverse, 5′-GGG GGA TCC TCC TGG TCT CAT GTA GCA TG-3′. PCR products were digested with *Bgl*II and *Bam*HI and ligated into pFLAG-CMV2 (Sigma–Aldrich). Ligated vectors were transformed into DH5α cells and the plasmid sequence was confirmed by Sanger sequencing. Macrophages were plated in 35 mm plates and transfected with empty pFLAG-CMV2 vector or that expressing RGS2 (1 μg) using nucleoporation reagents from Lonza (Allendale, NJ). Cells were nucleoporated according to the manufacturer's protocols and incubated for 18–24 h before Pam_3CSK_4 stimulation.

WESTERN BLOT ANALYSIS

Macrophages were cultured in 35 mm Petri dishes and treated with Pam_3CSK_4 for the times indicated. Cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride; and protease inhibitor cocktail). Next, the proteins were separated by 8–10% reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted onto nitrocellulose membranes in 20% methanol, 25 mM Tris, and 192 mM glycine. Membranes were then blocked with 5% non-fat dry milk and incubated with primary antibody for 18 h. The membranes were subsequently washed, incubated for 90 min with secondary antibody conjugated to horseradish peroxidase, rewashed, and finally developed using an enhanced chemiluminescence system (LAS 3000, GE Healthcare, Buckinghamshire, UK).

RESULTS

PAM_3CSK_4 INDUCES TF EXPRESSION VIA TLR2

Pam_3CSK_4 is a synthetic tripalmitoylated modifying bacterial lipopeptide that initiates cell signaling via recognition of TLR1 and TLR2 cooperation. Raw264.7 cells were treated with 100 ng/ml Pam_3CSK_4, and the change in TF mRNA expression was measured. Pam_3CSK_4 induced TF expression increased from 30 min, showed the maximum effect at 2 h, and was maintained until 6 h (Fig. 1A). TLR2 KO mice were used to confirm whether TF mRNA expression is TLR2 dependent or not. BMDMs differentiated from bone marrow-derived monocytes with M-CSF were used for the experiment. In WT BMDM, TF expression by Pam_3CSK_4 increased similarly to in Raw264.7 cells, but was not induced in TLR2 KO BMDM (Fig. 1B).

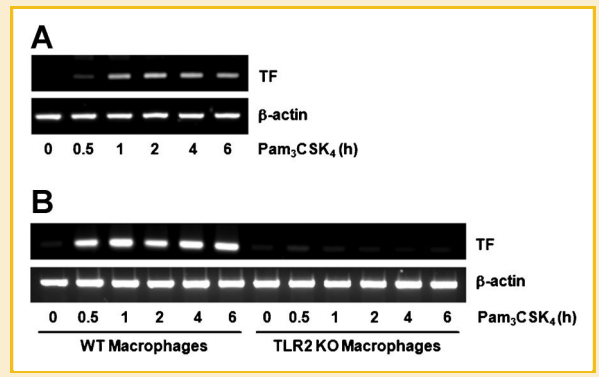

Fig. 1. Pam_3CSK_4 induces TF expression via a TLR2-dependent pathway. A: Raw264.7 cells were treated with Pam_3CSK_4 (100 ng/ml) for the times indicated, after which TF mRNA expression was determined by RT-PCR and normalized to a β-actin control. B: BMDMs were isolated from WT or TLR2 KO mouse bone marrow and treated with Pam_3CSK_4 (100 ng/ml) for the times indicated, after which TF mRNA expression was determined by RT-PCR and normalized to a β-actin control.

These results suggest that TF expression by Pam_3CSK_4 is TLR2 dependent.

JAK2 INVOLVED IN PAM_3CSK_4-INDUCED TF EXPRESSION

We studied the signaling pathway of TF expression induced by Pam_3CSK_4. Screening with various blockers showed the function of JAK. Specifically, pretreatment of macrophages with pan-JAK inhibitor strongly inhibited TF mRNA expression (Fig. 2A), and TF expression was also inhibited by the JAK2 specific inhibitor, AG490 (Fig. 2B). siRNA was then used to confirm the role of JAK2 in TF expression. The application of siRNA led to a significant decrease in JAK2 mRNA as well as TF mRNA expression (Fig. 2C). Real-time PCR showed results similar to RT-PCR in that TF mRNA expression was inhibited by JAK2 siRNA (Fig. 2D).

STAT3 MEDIATES PAM_3CSK_4-INDUCED TF EXPRESSION

Since we confirmed the function of JAK2 in TF expression, we investigated the effects of STAT3, which is closely related to JAK2. Treatment with Pam_3CSK_4 elevated STAT3 phosphorylation, which occurred at between 15 and 30 min of Pam_3CSK_4 treatment (Fig. 3A). Serine 727 (S727) phosphorylation was clearly confirmed, but tyrosine 705 (Y705) phosphorylation was weakly detected (data not shown). When STAT3 siRNA was used to decrease endogenous STAT3 protein expression, TF mRNA expression also decreased (Fig. 3B). Taken together, these results suggest that the JAK2–STAT3 pathway is essential to TF expression by Pam_3CSK_4.

RGS2 IS A NEGATIVE REGULATOR OF TLR2-MEDIATED TF EXPRESSION

Our previous studies showed that TLR2 stimulation decreases macrophage RGS2 expression, and that this is one of the essential mechanisms of STAT3 signaling regulation [Lee et al., 2012]. Therefore, we investigated the effects of RGS2 in TLR2-mediated TF expression. When Raw264.7 cells were treated with Pam_3CSK_4,

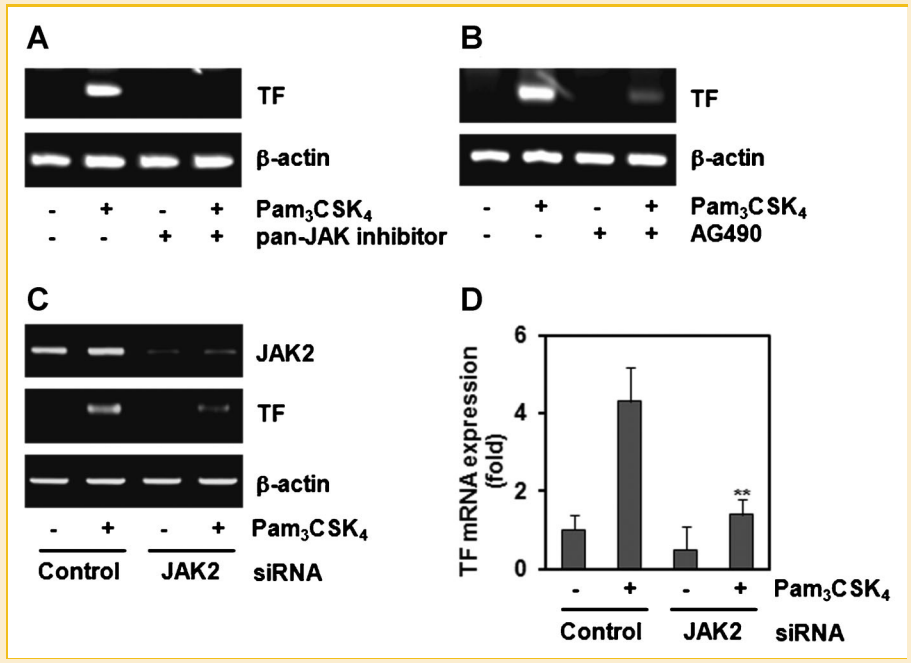

Fig. 2. JAK2 mediates TF induction by Pam_3CSK_4. A,B: Raw264.7 cells were pretreated with pan-JAK inhibitor (2 μM) or AG490 (20 μM) and stimulated with Pam_3CSK_4, after which TF mRNA expression was determined by RT-PCR and normalized to a β-actin control. C,D: Raw264.7 cells were transfected with either control or JAK2 siRNA (200 pM) for 24 h. Cells were then stimulated with vehicle or Pam_3CSK_4 (100 ng/ml). After 1 h, JAK2 and TF mRNA expression was determined by RT-PCR (C) or real-time PCR (D) and normalized to a β-actin control. Each data point represents the mean ± standard deviation of three independent experiments ($^{**}P < 0.01$).

RGS2 expression decreased; however, its expression was recovered at 2 h (Fig. 4A). Similar to the results of previous studies, RGS2 expression by Pam_3CSK_4 treatment decreased in WT BMDMs, while no changes were observed in TLR2 KO BMDMs (data not shown). To identify the function of RGS2 in TF expression, FLAG-RGS2 was overexpressed in Raw264.7 cells and the inhibited TF expression increased in response to treatment with Pam_3CSK_4 (Fig. 4B). These results suggest that RGS2 is a negative regulator of TF expression by Pam_3CSK_4. RGS2 contains the RGS domain in the C-terminal and regulates various functions that utilize GAP activity in GPCR signaling [Langer et al., 2009]. We cloned a deletion mutant (1–82) with the RGS domain eliminated and compared the effects of TF expression at WT RGS2 (1–211). WT RGS2 overexpression inhibited Pam_3CSK_4 induced TF expression, but mutant RGS2 overexpression had no effect (Fig. 4C). These results suggest that the RGS domain is not involved in regulation of TF expression, and that GAP activity of RGS2 is unrelated.

RGS2 SUPPRESSES TLR2-MEDIATED STAT3 PHOSPHORYLATION

STAT3 has been confirmed as an essential transcription factor in Pam_3CSK_4 induced TF expression; therefore, we investigated the effects of RGS2 on STAT3 phosphorylation. RGS2 siRNA transfection decreased endogenous RGS2 expression by about 70%. STAT3 phosphorylation by Pam_3CSK_4 in control siRNA cells and RGS2 siRNA cells was compared. STAT3 phosphorylation increased at 60 min of Pam_3CSK_4 treatment in control siRNA transfected cells. RGS2 knock-down cells already had high basal STAT3 phosphorylation levels, which increased further in response to Pam_3CSK_4 treatment (Fig. 5A). We also compared STAT3 phosphorylation in RGS2 overexpression cells. STAT3 phosphorylation increased with Pam_3CSK_4 treatment in FLAG vector transfected cells, as expected. However, RGS2 overexpression cells had low basal levels of STAT3 phosphorylation, which did not increase in response to treatment with Pam_3CSK_4 (Fig. 5B). These results suggest that when RGS2 acts as a negative regulator at TF expression, it occurs via STAT3 phosphorylation.

DISCUSSION

TF expression and activation appeared in vascular smooth muscle cells and fibroblasts, as well as to a small degree in endothelial cells [Wilcox et al., 1989; Edgington et al., 1991]. TF is also found in small amounts in vascular circulating mononuclear cells, and this protein usually participates in blood coagulation, although it also has other functions [Steffel et al., 2006]. For example, TF has been reported as a transmembrane protein that participates in neointima formation [Ott et al., 1998], and it has been suggested that TF expression is upregulated in patients with cardiovascular risk factors or coronary artery disease [Moons et al., 2002].

TF expression is very weak in macrophages; however, it increases in response to cytokines, CRP, CD40 ligand, angiotensin II, and oxLDL [Cermak et al., 1993; Wada et al., 1994; Mach et al., 1997; He et al., 2006]. LPS is known as a strong stimulant of TF expression [Guha and Mackman, 2002]. It is very important to identify the proteins involved in the TF expression induced by such stimulants. Mitogen-activated protein kinase (MAPK) is the most well known

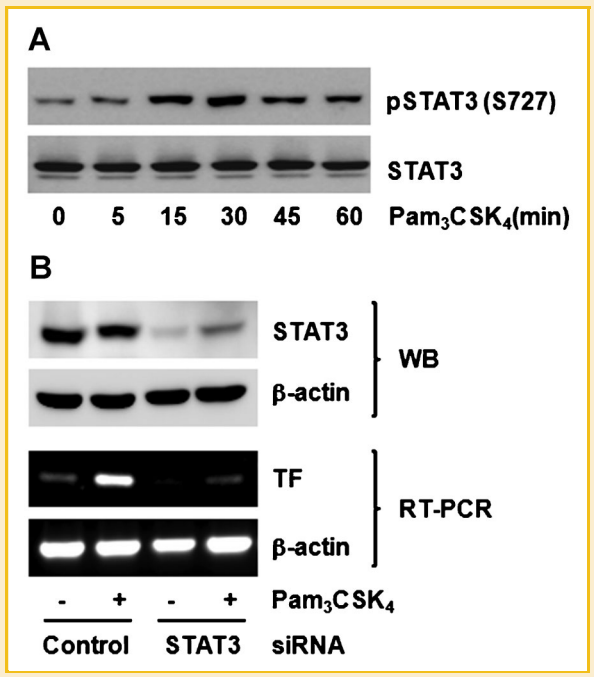

Fig. 3. STAT3 is a transcription factor of TLR2-induced TF expression. A: Raw264.7 cells were stimulated with vehicle or Pam_3CSK_4 (100 ng/ml) for the times indicated, after which STAT3 phosphorylation was determined by Western blotting using α-phospho-STAT3 antibody (S727) and normalized to STAT3 total protein. B: Raw264.7 cells were transfected with control or STAT3 siRNA (200 pM) for 24 h, after which cells were stimulated with vehicle or Pam_3CSK_4 (100 ng/ml). STAT3 protein expression was determined by Western blotting and normalized to a β-actin control, while TF mRNA expression was determined by RT-PCR and normalized to a β-actin control.

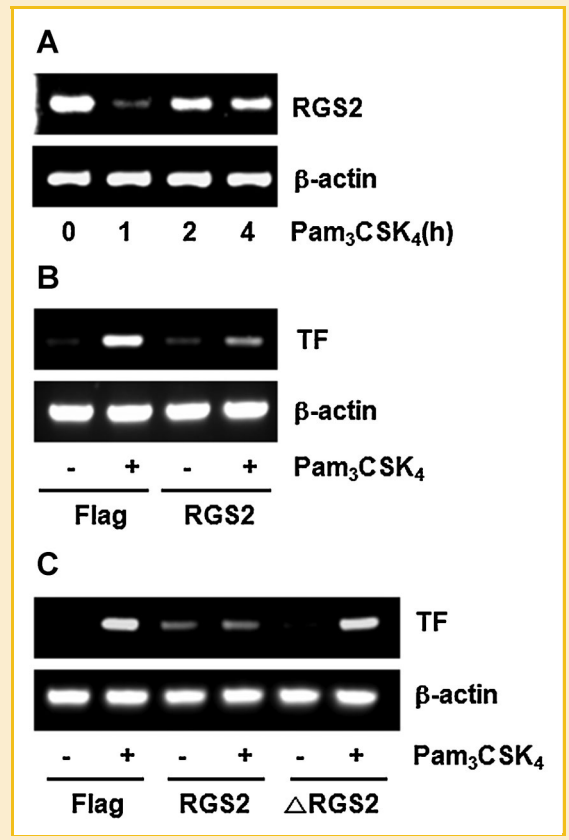

Fig. 4. RGS2 is a negative regulator of TLR2-induced TF expression. A: Raw264.7 cells were treated with Pam_3CSK_4 (100 ng/ml) for the times indicated, after which RGS2 mRNA expression was determined by RT-PCR and normalized to a β-actin control. B: Raw264.7 cells were transfected with empty pFLAG vector or that expressing RGS2 (1 μg), after which cells were stimulated with vehicle or Pam_3CSK_4 (100 ng/ml) and TF mRNA expression was determined by RT-PCR and normalized to a β-actin control. C: Raw264.7 cells were transfected with control vector, WT RGS2 (1–211), or mutant RGS2 (1–82) for 24 h, after which cells were stimulated with vehicle or Pam_3CSK_4 (100 ng/ml) for 1 h and TF mRNA expression was determined by RT-PCR and normalized to a β-actin control.

signaling protein related to LPS induced TF expression [Guha and Mackman, 2002]. TF expression has also been reported to be regulated by cooperation of the phosphatidylinositol 3-kinase (PI3 kinase) and MAP kinase pathway in endothelial cells [Guha and Mackman, 2002]. However, not many signaling proteins are known to regulate TF expression. Therefore, our finding of JAK2 involvement in TF expression is of great importance. JAK function in TLR signaling has only recently been reported, and its precise functions are not yet known. We recently reported the correlation of JAK and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) in TLR signaling. Specifically, TLR2 stimulation causes JAK2 phosphorylation, which leads to ERK1/2 phosphorylation [Park et al., 2012a]. This signaling mechanism is a very unique, unexpected reaction. However, the mechanism through which TLR activation phosphorylates JAK is not yet known.

It is crucial to have information pertaining to the transcription factors regulating TF expression. JAK2 function in TF expression is estimated to must have STAT3's essential role. Previous studies have shown that various transcription factors are related to TF expression, including early growth response protein-1, *c-Fos*/*c-Jun*, and *c-Rel*/p65 [Oeth et al., 1997]. However, there is insufficient evidence that STAT3 is a key transcription factor regulating TF expression. Therefore, verification of the role of STAT3 in TF regulation can be a meaningful discovery towards another transcription factor. Pam_3CSK_4 induced phosphorylation of STAT3 at the S727 residue, while treatment with JAK inhibitor inhibited this phosphorylation (data not shown). However, phosphorylation of the Y705 residue, which is required in STAT3 dimerization, was weakly detected when compared to S727 phosphorylation (data not shown). While STAT3 tyrosine phosphorylation is most well known for its direct activation by JAK2, MAPK is most well known for STAT3 serine phosphorylation. In previous studies of TLR signaling, we found that treatment with JAK inhibitor inhibited ERK1/2 and STAT3 (S727) phosphorylation, and that MAPK/ERK (MEK) inhibitor U0126 treatment significantly inhibited STAT3 (S727) phosphorylation [Batra et al., 2007; Lee et al., 2012]. These results suggest that, in TLR2 induced TF expression, S727 phosphorylation is more important than Y705 phosphorylation in the JAK2-ERK1/2-STAT3 signaling pathway.

Through our previous efforts to identify proteins regulating STAT3 activation according to TLR stimulation, we discovered the importance of RGS2. Inhibition of Nox1 expression by TLR2

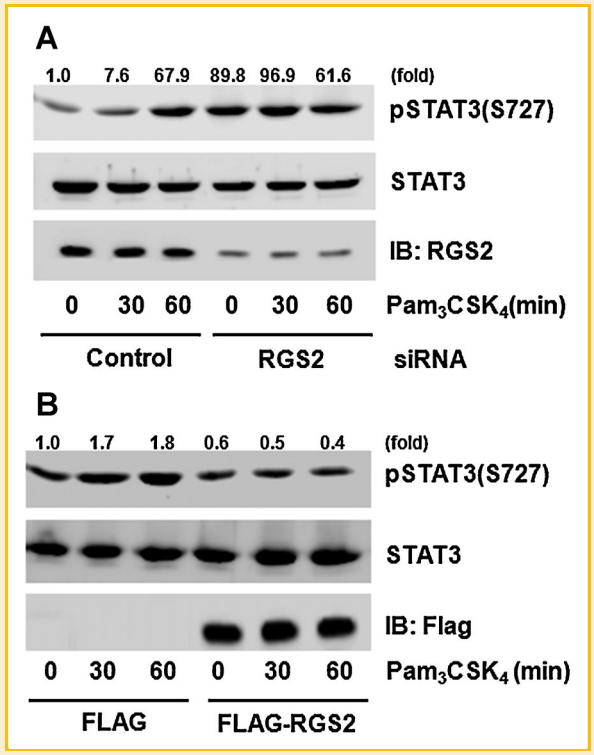

Fig. 5. RGS2 blocked Pam_3CSK_4-stimulated STAT3 phosphorylation. A: Raw264.7 cells were transfected with control or RGS2 siRNA (200 pM) for 24 h and then stimulated with Pam_3CSK_4 (100 ng/ml) for the times indicated, after which STAT3 phosphorylation was determined by Western blotting using α-phospho-STAT3 antibody (S727) and normalized to STAT3 total protein. RGS2 protein expression was determined by Western blotting using α-RGS2 antibody. B: Raw264.7 cells were transfected with empty pFLAG vector or that expressing RGS2 (1 μg), after which cells were stimulated with Pam_3CSK_4 (100 ng/ml) for the times indicated and STAT3 phosphorylation was determined by Western blotting using α-phospho-STAT3 antibody (S727) and normalized to STAT3 total protein. In addition, RGS2 protein expression was determined by Western blotting using α-Flag antibody.

activation was confirmed by direct binding of RGS2 and STAT3 [Lee et al., 2012]. Therefore, we investigated whether RGS2 causes a change in STAT3 phosphorylation. RGS2 overexpression inhibits Pam_3CSK_4 induced STAT3 phosphorylation, and RGS2 knock-down increases STAT3 phosphorylation. These results suggest that the combination of these proteins regulates STAT3 activity. RGS protein promotes hydrolysis of GTP and regulates G-protein coupled receptor (GPCR) signaling [Kimple et al., 2009]. RGS protein was first identified as a negative regulator of G-protein, but is currently recognized as a protein with more functions. Over 30 types of RGS and RGS-like proteins have been identified, and these have been divided into six different subgroups according to their amino acid arrangement [Manzur and Ganss, 2009]. Among these, RGS2 correlates with Gq/11, acts as an essential regulator of cardiovascular disease, and prevents Gq-mediated vascular contraction [Osei-Owusu et al., 2012]. RGS2 has also been recognized as a protein involved in blood pressure regulation [Sun et al., 2005], and some studies have suggested that it is related to atherosclerosis. They show that RGS2 expression increased by peroxisome proliferator-activated receptor delta (PPARδ) ligand blocks chemokine signaling, and inhibits atherosclerosis. However, it is still not known how RGS2 proteins regulate atherosclerosis. Therefore, the inhibitory function of RGS2 in TF expression should be investigated in future studies.

In this study, we demonstrate that the JAK2–STAT3 pathway is involved in Pam_3CSK_4-induced TF expression. In JAK2 or STAT3 knockdown cells, TF expression by Pam_3CSK_4 was greatly reduced. Furthermore, we found that RGS2 is participated in inactivation of STAT3 phosphorylation and inhibition of STAT3-mediated TF expression. In RGS2 overexpressed cells, the TF expression and STAT3 phosphorylation were reduced, suggesting that RGS2-mediated phosphorylation of STAT3 inhibition negatively impacts TLR2 signaling.

ACKNOWLEDGMENTS

This study was supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (MEST) (2011-0013206 and 2011-0006181).

REFERENCES

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell 124:783–801.

Batra A, Pietsch J, Fedke I, Glauben R, Okur B, Stroh T, Zeitz M, Siegmund B. 2007. Leptin-dependent toll-like receptor expression and responsiveness in preadipocytes and adipocytes. Am J Pathol 170:1931–1941.

Butterbach K, Beckmann L, de Sanjose S, Benavente Y, Becker N, Foretova L, Maynadie M, Cocco P, Staines A, Boffetta P, Brennan P, Nieters A. 2011. Association of JAK–STAT pathway related genes with lymphoma risk: Results of a European case–control study (EpiLymph). Br J Haematol 153:318–333.

Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. 1993. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. Blood 82:513–520.

Cole JE, Georgiou E, Monaco C. 2010. The expression and functions of toll-like receptors in atherosclerosis. Mediators Inflamm 2010:393946.

Edgington TS, Mackman N, Brand K, Ruf W. 1991. The structural biology of expression and function of tissue factor. Thromb Haemost 66:67–79.

Guha M, Mackman N. 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 277:32124–32132.

He M, He X, Xie Q, Chen F, He S. 2006. Angiotensin II induces the expression of tissue factor and its mechanism in human monocytes. Thromb Res 117:579–590.

Katsoulidis E, Sassano A, Majchrzak-Kita B, Carayol N, Yoon P, Jordan A, Druker BJ, Fish EN, Platanias LC. 2008. Suppression of interferon (IFN)-inducible genes and IFN-mediated functional responses in BCR-ABL-expressing cells. J Biol Chem 283:10793–10803.

Kehrl JH, Sinnarajah S. 2002. RG S2: A multifunctional regulator of G-protein signaling. Int J Biochem Cell Biol 34:432–438.

Kimple AJ, Soundararajan M, Hutsell SQ, Roos AK, Urban DJ, Setola V, Temple BR, Roth BL, Knapp S, Willard FS, Siderovski DP. 2009. Structural determinants of G-protein alpha subunit selectivity by regulator of G-protein signaling 2 (RGS2). J Biol Chem 284:19402–19411.

Langer I, Tikhonova IG, Boulegue C, Esteve JP, Vatinel S, Ferrand A, Moroder L, Robberecht P, Fourmy D. 2009. Evidence for a direct and functional

interaction between the regulators of G protein signaling-2 and phosphorylated C terminus of cholecystokinin-2 receptor. Mol Pharmacol 75: 502–513.

Lee HK, Park DW, Bae JH, Kim HJ, Shin DG, Park JS, Lee JG, Lee SJ, Bae YS, Baek SH. 2012. RGS2 is a negative regulator of STAT3-mediated Nox1 expression. Cell Signal 24:803–809.

Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. 1997. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of C D40: Induction of collagenase, stromelysin, and tissue factor. Circulation 96:396–399.

Manzur M, Ganss R. 2009. Regulator of G protein signaling 5: A new player in vascular remodeling. Trends Cardiovasc Med 19:26–30.

Moons AH, Levi M, Peters RJ. 2002. Tissue factor and coronary artery disease. Cardiovasc Res 53:313–325.

Newton K, Dixit VM. 2012. Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol 4:a006049.

Oeth P, Parry GC, Mackman N. 1997. Regulation of the tissue factor gene in human monocytic cells. Role of AP-1, NF-kappa B/Rel, and Sp1 proteins in uninduced and lipopolysaccharide-induced expression. Arterioscler Thromb Vasc Biol 17:365–374.

Osei-Owusu P, Sabharwal R, Kaltenbronn KM, Rhee MH, Chapleau MW, Dietrich HH, Blumer KJ. 2012. Regulator of G protein signaling 2 deficiency causes endothelial dysfunction and impaired endothelium-derived hyperpolarizing factor-mediated relaxation by dysregulating Gi/o signaling. J Biol Chem 287:12541–12549.

Ott I, Fischer EG, Miyagi Y, Mueller BM, Ruf W. 1998. A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. J Cell Biol 140:1241–1253.

Park DW, Kim JS, Chin BR, Baek SH. 2012a. Resveratrol inhibits inflammation induced by heat-killed listeria monocytogenes. J Med Food 15:788–794.

Park DW, Lee HK, Jeong TW, Kim JS, Bae YS, Chin BR, Baek SH. 2012b. The JAK2-Akt-glycogen synthase kinase-3beta signaling pathway is involved in toll-like receptor 2-induced monocyte chemoattractant protein-1 regulation. Mol Med Rep 5:1063–1067.

Rane SG, Reddy EP. 2000. Janus kinases: Components of multiple signaling pathways. Oncogene 19:5662–5679.

Steffel J, Luscher TF, Tanner FC. 2006. Tissue factor in cardiovascular diseases: Molecular mechanisms and clinical implications. Circulation 113:722–731.

Sun X, Kaltenbronn KM, Steinberg TH, Blumer KJ. 2005. RGS2 is a mediator of nitric oxide action on blood pressure and vasoconstrictor signaling. Mol Pharmacol 67:631–639.

ten Cate H. 2012. Tissue factor-driven thrombin generation and inflammation in atherosclerosis. Thromb Res 129(Suppl 2):S38–S40.

Wada H, Kaneko T, Wakita Y, Minamikawa K, Nagaya S, Tamaki S, Deguchi K, Shirakawa S. 1994. Effect of lipoproteins on tissue factor activity and PAI-II antigen in human monocytes and macrophages. Int J Cardiol 47:S21–S25.

Wilcox JN, Smith KM, Schwartz SM, Gordon D. 1989. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci USA 86:2839–2843.