ARTICLE
Journal of Cellular Biochemistry 117:483–490 (2016)

Journal of Cellular Biochemistry

Regulation of PHLDA1 Expression by JAK2-ERK1/2-STAT3 Signaling Pathway

Ji Hyo Lyu, Bin Huang, Dae-Weon Park, and Suk-Hwan Baek*

Department of Biochemistry and Molecular Biology, College of Medicine, Yeungnam University, 170 Hyeonchung-ro, Nam-gu, Daegu 705-703, South Korea

ABSTRACT

Toll-like receptor 2 (TLR2)-mediated signaling cascades and gene regulation are mainly involved in diseases, such as immunity and inflammation. In this study, microarray analysis was performed using bone marrow-derived macrophages (BMDM) and Raw 264.7 cells to identify novel proteins involved in the TLR2-mediated cellular response. We found that pleckstrin homology-like domain family, member 1 (PHLDA1) is a novel gene up-regulated by TLR2 stimulation and determined the unique signaling pathway for its expression. Treatment with TLR2 agonist Pam_3CSK_4 increased mRNA, protein, and fluorescence staining of PHLDA1. Induction of PHLDA1 by TLR2 stimulation disappeared from TLR2 KO mice-derived BMDM. Among janus kinase (JAK) family members, JAK2 was involved in TLR2-stimulated PHLDA1 expression. Signal transducer and activator of transcription 3 (STAT3) also participated in PHLDA1 expression downstream of the JAK2. Interestingly, ERK1/2 was an intermediate between JAK2 and STAT3. In silico analysis revealed the presence of highly conserved γ-activated sites within mouse PHLDA1 promoter and confirmed the JAK2-STAT3 pathway is important to Pam_3CSK_4-induced PHLDA1 transcription. These findings suggest that the JAK2-ERK1/2-STAT3 pathway is an important signaling pathway for PHLDA1 expression and that these proteins may play a critical role in eliciting TLR2-mediated immune and inflammatory response. J. Cell. Biochem. 117: 483–490, 2016.

KEY WORDS: PHLDA1; TLR2; JAK2; STAT3

The innate immune system, which is the primary defense mechanism against pathogens, is activated swiftly upon recognition of non-self matter by factors such as toll-like receptor (TLR). TLR recognizes a vast range of ligands including lipopeptides or lipopolysaccharide that represent pathogen-associated molecular signatures. In mammals, 13 homologues of TLR have been identified to date [Bowie and O'Neill, 2000; Takeda et al., 2003]. Bacterial lipopeptides are predominantly recognized by the membrane receptor TLR2, but differ in their affinities for TLR1 and TLR6 according to the number and length of their fatty acids and the amino acid sequences of their peptide tails [Jin et al., 2007]. Stimulation of TLR2 by Pam_3CSK_4 leads to activation of a series of signaling cascades that culminate in the expression of inflammatory cytokines and regulators [Manetsch et al., 2012].

The PHLDA1 gene encodes an evolutionarily conserved proline-histidine rich protein and a 401-amino acid protein. PHLDA1 was initially identified in T-cell hybridoma, where it mediates apoptosis by inducing Fas expression, and was thus named T-cell death-associated gene 51 [Park et al., 1996]. The role of PHLDA1 in the regulation of apoptosis is quite controversial. For example, it has been reported that PHLDA1 is a crucial mediator of the anti-apoptotic effect of insulin-like growth factor-I (IGF-I) in NIH3T3 cells [Toyoshima et al., 2004]. However, another study showed that PHLDA1 played no role in T-cell apoptosis [Oberg et al., 2004]. Furthermore, it was reported to have a different function including atherogenesis regulation [Hossain et al., 2003,2013], and follicular stem cell marker [Sellheyer and Krahl, 2011]. Basseri et al. suggested that PHLDA1 is involved in energy homeostasis, at least in part, by regulating lipogenesis in liver, and white adipose tissue [Basseri et al., 2013]. Therefore, the results of these studies assume that PHLDA1 protein plays multiple roles in cells. Although PHLDA1 protein plays a variety of roles in different cells, little is known about specific signaling pathway for its expression.

PHLDA1 expression is induced by homocysteine or heat shock [Hossain et al., 2003; Hayashida et al., 2006]. IGF-I is also strong inducer of PHLDA1 expression [Toyoshima et al., 2004; Wu et al., 2010]. However, there are not many known specific signaling

Ji Hyo Lyu and Bin Huang contributed equally to this work.
Conflicts of interest: The authors declare no conflicts of interest.
Grant sponsor: Yeungnam University Research; Grant number: 212A345025.
*Correspondence to: Prof. Suk-Hwan Baek, Ph.D., Department of Biochemistry and Molecular Biology, 210 Main Building, College of Medicine, Yeungnam University, 170 Hyeonchung-ro, Nam-gu, Daegu 705-703, South Korea.
E-mail: sbaek@med.yu.ac.kr
Manuscript Received: 21 May 2015; Manuscript Accepted: 30 July 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 August 2015
DOI 10.1002/jcb.25296 • © 2015 Wiley Periodicals, Inc.

proteins associated with PHLDA1 expression. The ERK1/2 is currently the most well-known signaling protein for PHLDA1 expression [Toyoshima et al., 2004; Oberst et al., 2008]. Recently, Johnson et al. found that Aurora A, known as serine/threonine-protein kinase, negatively regulates PHLDA1 protein levels by directly phosphorylating Ser98 in breast cancer cells [Johnson et al., 2011]. Nevertheless, few proteins that control the expression of PHLDA1 have been identified, and information regarding the transcription factor has not been reported.

The janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is stimulated by cytokines, vasoactive agents, hormones, and growth factors and provides a direct mechanism to translate extracellular signal into a transcriptional response, despite immunoregulation. Activated STATs bind specific regulatory sequences, such as interferon-stimulated response element or γ-activated sequence (GAS), to induce or repress transcription of target genes [O′Shea and Plenge, 2012]. Although some reports suggest the role of STAT3 in cytokine production in response to TLR, the importance of JAK-STAT pathway has mainly been reported to occur via indirect mode [Liljeroos et al., 2008; Samavati et al., 2009; Greenhill et al., 2011]. However, we attempted to identify a direct mode of action of the JAK-STAT pathway in TLR-mediated gene regulation or response.

Here, we report that PHLDA1 expression is regulated by the JAK2-STAT3 signaling pathway and identify the putative STAT3 binding site for PHLDA1 transcription. Interestingly, ERK1/2 is an important mediator of the connection between JAK2 and STAT3. This study provides the first insight into how PHLDA1 expression is regulated by the JAK2-STAT3 pathway, adding to the diversity of known PHLDA1 protein function beyond its ability to regulate apoptosis or atherogenesis.

MATERIALS AND METHODS

MATERIALS

Anti-pERK1/2, ERK1/2, pSTAT3, STAT3, and IκBα antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-PHLDA1 antibody was acquired from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-β-actin antibody was obtained from Sigma–Aldrich (St. Louis, MO). JAK inhibitor, AG490, U0126, SB203580, SP600125 were purchased from Calbiochem (San Diego, CA). DAPI were purchased from Sigma–Aldrich. Mouse TLR1-9 agonist kit were purchased from in vivo Gen (San Diego, CA). Reverse-transcription polymerase chain reaction (RT-PCR) kits were obtained from Takara Bio (Ohtsu, Japan). TRIzol and siRNAs (JAK1, JAK2, JAK3, TYK2, and STAT3) were purchased from Invitrogen (Carlsbad, CA). Wild-type (WT) mice (C57BL6) were purchased from Central Lab Animal (Seoul, South Korea). TLR2 deficient mice were kindly provided by Dr. S.J. Lee (Seoul National University, South Korea).

CELL CULTURE AND TRANSFECTION

Raw 264.7 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2.Primary bone marrow-derived monocytes were differentiated into bone marrow-derived macrophages (BMDM) by incubation 5–7 days in DMEM supplemented with 10% L929 cell-conditioned medium (as a source of macrophage colony-stimulating factor). Raw 264.7 cells were plated in 35 mm diameter plates at an appropriate density and grown overnight before transfection using commercial reagents (Amaxa, Lonza, Switzerland),according to themanufacturer′s instructions.

MICROARRAY AND DATA ANALYSIS

BMDMs and Raw 264.7 cells were treated with vehicle or Pam_3CSK_4 (1 μg/ml). At 2 h after stimulation, total RNA was obtained, and used for microarray analysis, which was performed by D&P Biotech (Daegu, Korea). Briefly, 1 μg RNA was used to generate biotin-labeled single-stranded cDNA by in vitro transcription. The biotinylated single-stranded cDNA was then hybridized to an Affymetrix Human HG 1.0 ST Array GeneChip (Affymetrix, Santa Clara, CA). Each stained probe array was scanned with a GeneChip® Scanner 3,000 (Affymetrix) 7G at 570 nm. The signal intensity of the gene expression level was calculated using Expression Console™ software, Version 1.1 (Affymetrix). To remove false positive signal, we applied cut-off value of 300 to the detection signal. Gene ontology was analyzed using NetAffyx.

PLASMID CONSTRUCTS AND siRNAs

Flag-PHLDA1 expression plasmids were constructed using the following primers: Flag-PHLDA1 (pFLAG-CMV2 vector): forward primer, 5′-CGC GAA TTC AAT GCT GGA GAA CAG CGG CT-3′; reverse primer, 5′-CCC GGA TCC TCT TCA GGC AGA GTT-3′. The plasmid constructs were systematically checked by sequence analyses. Stealth control and gene-specific siRNAs against the following target genes were designed using the Block-IT Stealth RNAi designer (Invitrogen): JAK1, 5′-UCA CCG GGA CUU AGC AGC AAG AAA U-3′; JAK2, 5′-CGG GUC GGC GCA ACC UAA GAU UAA U-3′; JAK3, 5′-CAC AUG ACU CGG AAG UCC UCC UGA A-3′; TYK2, 5′-GCG AGG AGG AGA UCC ACC ACU UUA A-3′; STAT3, 5′-AAA CGU GAG CGA CUC AAA CUG CCC U-3′.

WESTERN-BLOT

Following SDS–PAGE, the proteins were transferred to nitrocellulose membranes, after which the membranes were blocked with Tris-buffered saline TBS containing 5% skimmed milk for 1 h at room temperature. After blocking, the membranes were-incubated with primary antibodies overnight at 4°C. Following three washes, the membranes were incubated with HRP-conjugated secondary antibodies (anti-mouse or -rabbit 1:10,000; Santa Cruz Biotechnology) for 1 h at room temperature and visualized using an enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK).

RT-PCR

Total RNA was extracted from cells using TRIzol. First-strand cDNA was synthesized from 1 μg total RNA by employing random

primers, oligo-dT, and reverse transcriptase (Promega, Madison, WI). Cycling conditions for the PCR were 95°C for 5 min, followed by 26–33 cycles at 95°C for 1 min, 60°C–62°C for 1 min, and 72°C for 1 min. Target gene expression was normalized to β-actin transcription. The specific primers for mouse PHLDA1, JAK1, JAK2, JAK3, TYK2, and β-actin, which were designed as follows: PHLDA1: Tm 60°C, 290 bp, 30 cycles, forward primer 5′-CAT ACC GCC CAA GCA GCT AC-3′ and reverse primer 5′-GGC GAT TTT TGT ACT GCA CC-3′; JAK1: Tm 60°C, 736 bp, 33 cycles, forward primer 5′-ATG GAA GAC GGA GGC AAT GGT-3′ and reverse primer 5′-GGA ACT TTA GAG GCA GAA TAC-3′; JAK2: Tm 60°C, 260 bp, 28 cycles, forward primer 5′-AAG AGC AAC GGA AGA TTG C-3′ and reverse primer 5′-CGT CAC AGT TTC TTC TGC CT-3′; JAK3: Tm 60°C, 412 bp, 28 cycles, forward primer 5′-CAC ACC TGG CAT CCC GAA TC-3′ and reverse primer 5′-AGC AGT AGG CGG TCG GTG TG-3′; TYK2: Tm 60°C, 413 bp, 28 cycles, forward primer 5′-CCT GGC CAT GAC CTG AAC AG-3′ and reverse primer 5′-TGT GCC CTT CAC TGA CGG AG-3′; β-actin: Tm 62°C, 509 bp, 26 cycles, forward primer 5′-TCC TTC GTT GCC GGT CCA CA-3′ and reverse primer 5′-CGT CTC CGG AGT CCA TCA CA-3′.

IMMUNOHISTOCHEMISTRY

Raw 264.7 cells were grown and then fixed with 4% paraformaldehyde in PBS. Subsequently, the fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min, and washed three times in PBS. The cells were blocked with 5% BSA in PBS for 1 h at room temperature, then incubated overnight at 4°C with PHLDA1 antibody (1:200). The cells were washed three times and incubated with Alexa 546-conjugated secondary antibody for 1 h at room temperature. Next, the cells were washed three times, stained with DAPI (1 μg/ml), mounted on glass slides, and viewed under a Leica TCS SP2 AOBS spectral confocal microscope (Mannheim, Germany).

REPORTER CONSTRUCT AND LUCIFERASE ASSAY

The promoter regions of the mouse PHLDA1 gene (~1,200 bp) were amplified by PCR from genomic DNA and inserted into the multicloning site of the pGL3 basic vector (Promega). Deletion mutants were generated by progressive removal of putative GAS sites from PHLDA1 promoters and cloned in the same reporter vector. The construct was subjected to sequence analyses to verify the orientations and fidelity of the insert. Raw 264.7 cells were transfected with individual plasmids using commercial reagents (Amaxa). After transfection, the cells were incubated with Pam_3CSK_4 for 8 h. The cell lysates were then assayed for dual luciferase activity using the luciferase reporter assay system (Promega) according to the manufacturer's protocol. Luciferase values were corrected for transfection efficiency by determining the ratio between plasmids and renilla luciferase. All data were normalized to untreated pGL3 basic.

STATISTICAL ANALYSIS

Results are expressed as the mean ± SD of at least three independent assays. Comparisons between the two groups were made by nonpaired two-tailed Student's *t*-test using SPSS v12.0 (SPSS, Chicago, IL). A P-value < 0.05 was considered significant.

RESULTS

PHLDA1 INDUCTION BY Pam_3CSK_4 IS TLR2-DEPENDENT

To identify specific genes involved in the progression of TLR2 signaling, we conducted microarray analysis using BMDMs or Raw 264.7 cells, and found the PHLDA1 gene. We confirmed whether the TLR2 agonist Pam_3CSK_4 can induce PHLDA1 mRNA and protein expression in Raw 264.7 cells (Fig. 1A and B). To examine specific localization of PHLDA1 in the cells, we performed immunostaining using Ab. When cells were treated with Pam_3CSK_4 for 12 h, a marked increase in immunofluorescence with predominant localization in the cytosol was observed for PHLDA1 when compared with that of the control (Fig. 1C). To confirm the importance of TLR2, we tested the effects of Pam_3CSK_4 after isolating BMDMs from WT or TLR2 knockout mouse bone marrow. Although PHLDA1 expression was induced by Pam_3CSK_4 in BMDMs from WT mice, its expression was not affected in BMDMs from mice with deletions in TLR2 (TLR2 KO; Fig. 1D and E). To prove the general effect of TLRs for PHLDA1 expression, we tested other types of TLR agonists. All the tested agonists (including TLR1/2, TLR2, TLR3, TLR4, TLR5, TLR6/2, TLR8, and TLR9) induced the PHLDA1 gene expression except TLR3 and TLR8 (Fig. 1F). These results suggest that PHLDA1 is a novel gene up-regulated by TLR2 stimulation in macrophages and its expression is dependent on TLR2.

THE JAK2-STAT3 PATHWAY IS KEY SIGNALING PATHWAY IN Pam_3CSK_4-INDUCED PHLDA1 EXPRESSION

We investigated the identity of signaling proteins that control the expression of PHLDA1 and found that JAK was involved in the Pam_3CSK_4-mediated PHLDA1 induction. Pretreatment of Raw 264.7 cells with overall JAK inhibitor, a general suppressor of JAK activity, strongly blocked PHLDA1 mRNA, and protein induction in a dose-dependent manner (Fig. 2A). Therefore, we investigated the identity of JAK specific to PHLDA1 gene expression by siRNA knockdown of individual JAKs in cells stimulated with TLR2 agonist. The induction of PHLDA1 by Pam_3CSK_4 was partially inhibited by JAK2 or JAK3 siRNA, however, JAK2 is stronger than JAK3 knockdown, and no less expression was seen with JAK1 or TYK2 knockdown (Fig. 2B and C). Additionally, pretreatment of cells with the JAK2 specific inhibitor AG490, also blocked PHLDA1 protein expression in a dose-dependent manner (Fig. 2D), consistent with JAK2 contributing to PHLDA1 induction after TLR2 stimulation. These results suggest that PHLDA1 expression by TLR2 stimulation in Raw 264.7 cells is mainly dependent upon JAK2 or JAK3.

We next probed the transcription factors responsible for JAK2-mediated PHLDA1 expression. We initially assumed that STAT3 was acting downstream of JAK2. Pam_3CSK_4-mediated TLR2 stimulation increased STAT3 phosphorylation at serine 727 (S727), although pretreatment with JAK inhibitor significantly attenuated this phosphorylation (Fig. 3A). Phosphorylation of this residue is critical to the ability of STAT3 to act as a transcriptional transactivator; therefore, we tested STAT3 involvement in Pam_3CSK_4-induced PHLDA1 expression by siRNA. Knockdown of the STAT3 transcript was successful 24 h after siRNA transfection. Expression of PHLDA1 upon Pam_3CSK_4-mediated TLR2 stimulation was significantly reduced in STAT3 siRNA-transfected cells compared to that in

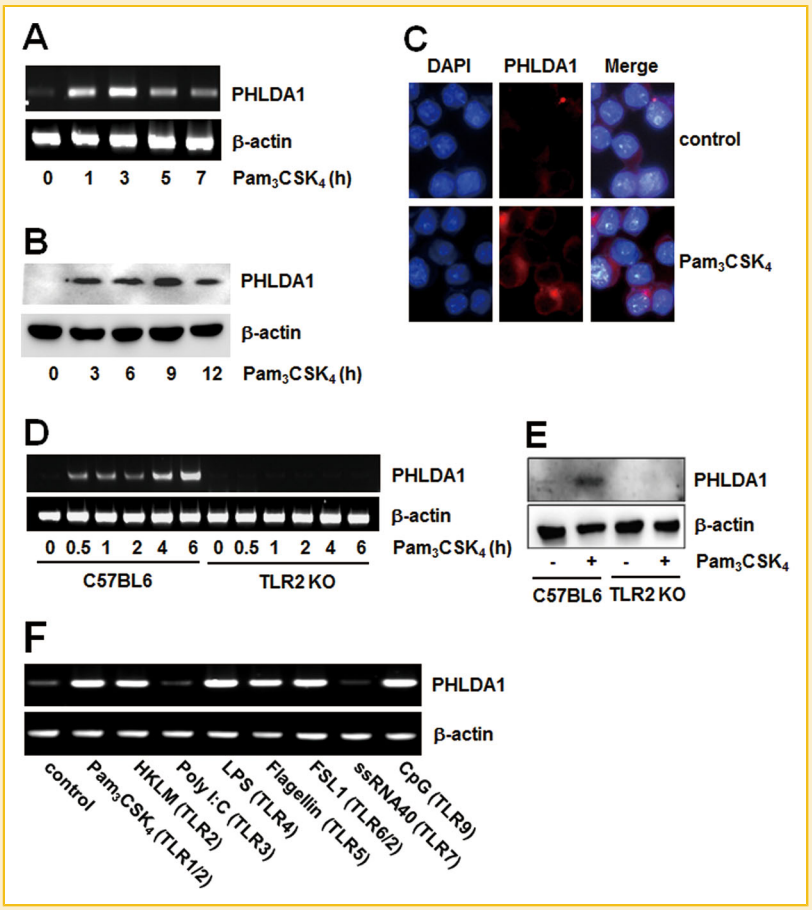

Fig. 1. Pam_3CSK_4 induces PHLDA1 expression via a TLR2-dependent pathway. A and B: Raw 264.7 cells were treated with Pam_3CSK_4 (100 ng/ml) for the indicated times. PHLDA1 mRNA and protein expression were determined by RT-PCR and Western blot, respectively. C: Raw 264.7 cells were treated with Pam_3CSK_4, fixed, and permeabilized. PHLDA1 (red) was immunostained with goat anti-PHLDA1 antibody followed by rhodamine-conjugated secondary antibody. DAPI (blue) was used as a nuclear marker. D and E: BMDMs were isolated from WT or TLR2 KO mouse bone marrow and treated with Pam_3CSK_4 (100 ng/ml). PHLDA1 mRNA and protein expression were determined by RT-PCR or Western blot, respectively. F: Raw 264.7 cells were treated with each TLR ligand. PHLDA1 mRNA was determined by RT-PCR. The representative experiments were repeated in triplicate with similar results.

control siRNA cells (Fig. 3B). These results suggest that STAT3 is an important signal transducer of TLR2-mediated expression of the PHLDA1.

ERK1/2 IS AN INTERMEDIATOR BETWEEN JAK2 AND STAT3 FOR PHLDA1 EXPRESSION

ERK1/2 was previously reported to be an important kinase for PHLDA1 expression in cancer cells [Toyoshima et al., 2004; Oberst et al., 2008]. Therefore, we sought to determine the role of ERK1/2 and the relationship between ERK1/2 and JAK2-STAT3 pathway in TLR2-mediated up-regulation of PHLDA1. Surprisingly, pretreatment of Raw 264.7 cells with JAK inhibitor strongly blocked the phosphorylation of ERK1/2 by TLR2 stimulation (Fig. 4A). These results suggest that ERK1/2 is downstream molecule of JAK2 in the TLR2 signaling pathway. U0126 is often used as an inhibitor of ERK1/2 activity. Pretreatment of Raw 264.7 cells with U0126 blocked the phosphorylation of ERK1/2 as well as STAT3 by TLR2 stimulation (Fig. 4B). We also investigated the identity of ERK1/2 specific to PHLDA1 gene and protein expression using inhibitors of individual MAPKs in cells stimulated with Pam_3CSK_4. The induction of PHLDA1 by Pam_3CSK_4 was only inhibited by U0126 (U), but less effect was seen in response to p38 MAPK (SB) or JNK (SP) (Fig. 4C). These results suggest that ERK1/2 is important intermediator between JAK2 and STAT3 during PHLDA1 expression.

ANALYSIS OF MOUSE PHLDA1 PROMOTER

In silico analysis revealed that the PHLDA1 promoter contains putative STAT3/GAS binding sites at −136/−129 bp (TTCnnGAA), −504/−497 bp (TTAnnTAA), and −1,179/−1,172 bp (TTCnnGAA) (Fig. 5A). The location of the nuclear factor consensus sequences were counted relative to the ATG codon (+1). To identify the regions required for basic promoter activity, the promoter of mouse PHLDA1 gene, and the corresponding deletion mutants were cloned into pGL3 basic vector (C1: −1201, C2: −525, C3: −169, and C4: −7). The plasmids were transfected into cells, after which the activity of the luciferase reporter gene was analyzed as described above. The basic promoter activity of C1 was increased ~200-fold relative to that of the pGL3 basic vector. Maximal luciferase activity was observed by

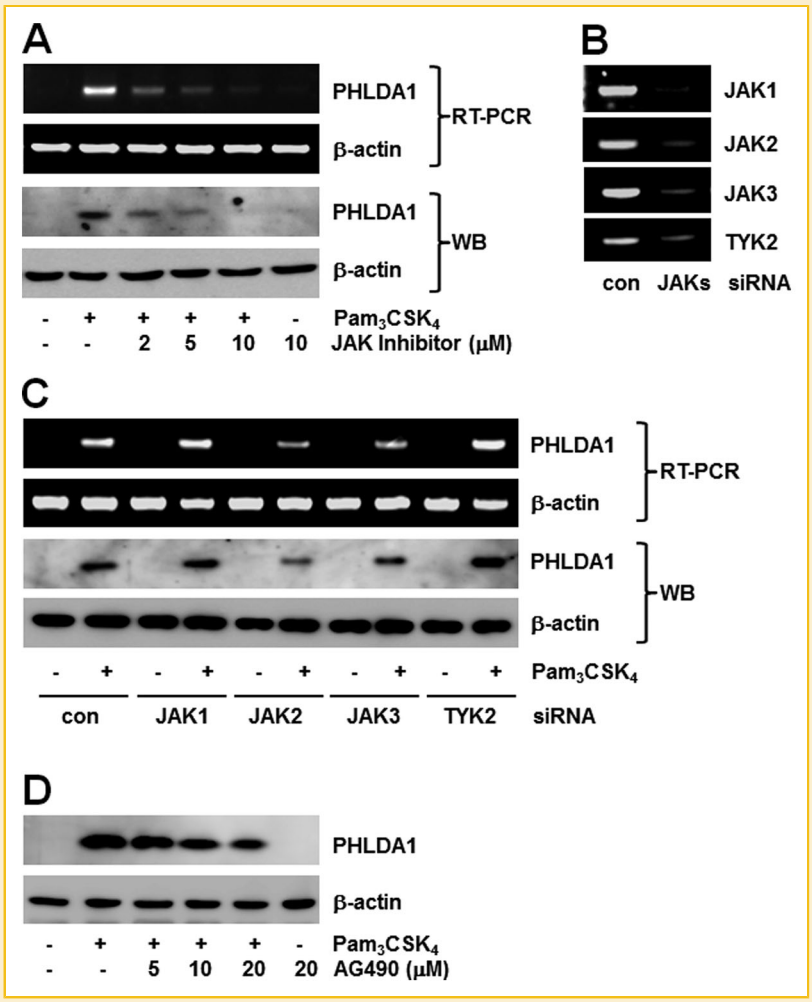

Fig. 2. JAK2 is important for TLR2-stimulated PHLDA1 expression. A: Raw 264.7 cells were pretreated with the indicated dose of JAK inhibitor and stimulated with Pam_3CSK_4 (100 ng/ml). Total RNA was isolated from half of the cells and PHLDA1 mRNA expression was determined. Cell lysates were prepared from the rest and PHLDA1 protein expression was determined. B and C: Raw 264.7 cells were transfected with JAK1, JAK2, JAK3, or TYK2 siRNA (150 pM) for 24 h. Cells were stimulated with vehicle or Pam_3CSK_4 for 4 h, after which PHLDA1 mRNA and protein expression were determined by RT-PCR or western blot. D: Raw 264.7 cells were pretreated with the indicated dose of AG490 (JAK2 inhibitor) and stimulated with Pam_3CSK_4 (100 ng/ml). PHLDA1 protein expression was determined by western blotting and normalized against β-actin. The representative experiments were repeated in triplicate with similar results.

the promoter sites of −525 bp (C2 mutant). Truncation analysis of the promoter region revealed the presence of a potential silencer element in the region spanning −1201 to −525, as loss of this ∼670 bp fragment led to a significant increase in promoter activity. A deletion of ∼350 bp fragment from −525 bp (C3 mutant) maintained the promoter activity up to ∼70% of C2. A further deletion of 162 bp fragment from −169 bp (C4 mutant) significantly decreased the luciferase level to approximately 88% of the maximal level indicating the loss of essential elements including GAS (Fig. 5B). To confirm that the PHLDA1 promoter activity is regulated by JAK, the inhibitor was used again. The promoter activity of PHLDA1 by Pam_3CSK_4 was partially inhibited by pretreatment JAK inhibitor. A similar effect as observed in the JAK inhibitory experiment was observed when ERK1/2 was suppressed (Fig. 5C). Moreover, STAT3 knock-down also decreased PHLDA1 promoter activity containing the putative GAS element sites (C2) (Fig. 5D).

DISCUSSION

PHLDA1 contains pleckstrin homology-like domain and mediates the connection between proteins. Microarray analysis revealed that PHLDA1 expression increased in response to TLR2 agonist of macrophages and confirmed the involvement of JAK2-ERK1/2-STAT3 pathway for regulation of PHLDA1 expression.

Our results are interesting in that they demonstrate JAK2-STAT3 pathway control of TLR2-mediated PHLDA1 expression because it is assumed that this pathway participates in PHLDA1 expression via a direct mechanism. Although, JAK-STAT pathway plays a role in TLR signaling, little is known about direct involvement of this pathway. There have been many reports of the indirect participation of JAK or STAT in TLR signaling [Liljeroos et al., 2008; Samavati et al., 2009; Greenhill et al., 2011]. For example, the indirect participation of STAT1/3 in the TLR2-induced interferon-α production via interferon

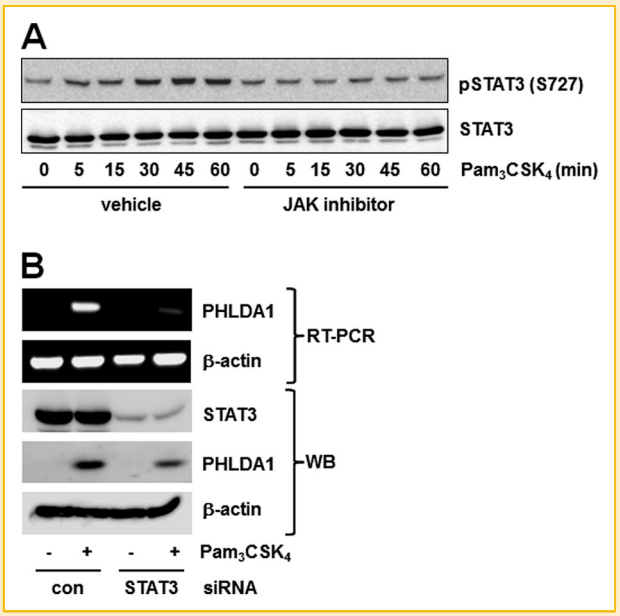

Fig. 3. STAT3 is a key factor in TLR2-mediated PHLDA1 expression. A: Raw 264.7 cells were stimulated with Pam_3CSK_4 (100 ng/ml) for the times indicated. STAT3 phosphorylation was determined by western blotting using α-phospho-STAT3 antibody (Ser 727) and normalized to STAT3 total protein. B: Raw 264.7 cells were transfected with either control or STAT3 siRNA (150 pM) for 24 h. Cells were stimulated with vehicle or Pam_3CSK_4 for 4 h. STAT3 and PHLDA1 mRNA expression were determined by RT-PCR and normalized against β-actin control. STAT3 and PHLDA1 protein expression were determined by western blotting and normalized against β-actin. The representative experiments were repeated in triplicate with similar results.

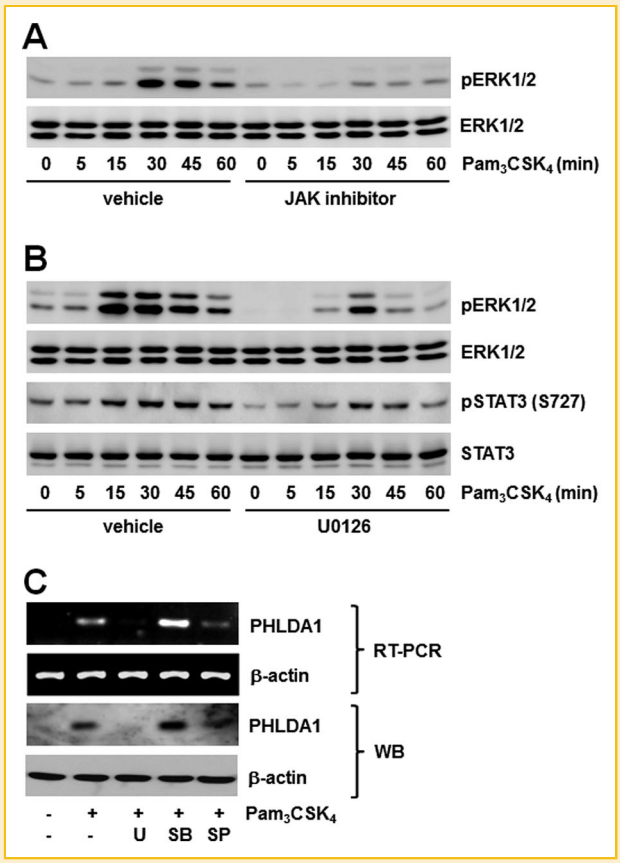

Fig. 4. ERK1/2 is an intermediator between JAK2 and STAT3 in TLR2-stimulated PHLDA1 expression. A: Raw 264.7 cells were pretreated with vehicle or JAK inhibitor before treated with Pam_3CSK_4 (100 ng/ml) for the times indicated. ERK1/2 phosphorylation was determined by western blotting using α-phospho-ERK1/2 antibody and normalized to ERK1/2 total protein. B: Raw 264.7 cells were pretreated with vehicle or U0126 before treated with Pam_3CSK_4 for the times indicated. ERK1/2 or STAT3 phosphorylation was determined by Western blotting using α-phospho-specific antibodies and normalized against total protein. C: Raw 264.7 cells were pretreated with vehicle or various MAPK inhibitors (U: U0126, SB: SB203580, and SP: SP600125) before treatment with Pam_3CSK_4 and PHLDA1 expression was determined by RT-PCR or Western blotting. The representative experiments were repeated three times.

regulatory factor 1/2 has been reported. The above results assumed a secondary response of the produced cytokines or mediators. Nevertheless, some reports have suggested direct involvement of JAK or STAT in TLR signaling. One example is the importance of JAK2 activity in the LPS-stimulated IL-1β production, despite the proposed role of phosphoinositide 3-kinase instead of STAT [Okugawa et al., 2003]. Wang et al. highlighted the role of JAK3 in regulation of TLR-mediated inflammatory cytokine production [Wang et al., 2013]. Our previous report also showed the importance of JAK2-STAT3 pathway in TLR2-mediated tissue factor expression [Park et al., 2013]. Another example is that STAT1 plays a role in TLR signaling and inflammatory response [Luu et al., 2014]. In addition, some reports have shown crosstalk between the TLR and JAK-STAT pathways [Hu et al., 2007; Zgheib et al., 2013]. Therefore, the observed direct participation of JAK2-STAT3 pathway in TLR2 signaling is an important finding that will be useful to future studies.

Our results clearly demonstrated direct engagement of JAK2-STAT3 pathway in the TLR2-mediated PHLDA1 expression. However, we failed to detect JAK2 phosphorylation, even though we attempted to capture JAK2 phosphorylation by Pam_3CSK_4 treatment using several commercial antibodies. Nevertheless, we conclude that JAK2 participated in the TLR2-mediated PHLDA1 expression owing to the reduction of PHLDA1 expression by JAK2 siRNA, and of the effects of the JAK2 inhibitor, AG490. We believe that PHLDA1 expression may be controlled by the JAK3 with JAK2. Although the effects of JAK3 are weaker than JAK2, PHLDA1 expression is reduced by JAK3 siRNA. These results suggest that cooperative action of the two JAKs regulates TLR2-mediated PHLDA1 expression.

There is little information available regarding the signal transduction pathway for PHLDA1 expression, although the importance of ERK1/2 is already known [Toyoshima et al., 2004; Oberst et al., 2008]. Our results also demonstrated the role of ERK1/2 in PHLDA1 expression. However, the pattern observed was different from the conventional pathway of ERK1/2. Specifically, ERK1/2 plays a role of connecting kinase JAK2, and STAT3. This was totally unexpected because STAT activation is typically enabled by the direct action of the JAK family [Villarino et al., 2015]. Although, MAPK participation in the activation process of STAT3 is very rare, some cases have recently reported. For example, ERK activation by Ras-MEK signaling pathway increases serine phosphorylation of STAT3 in the

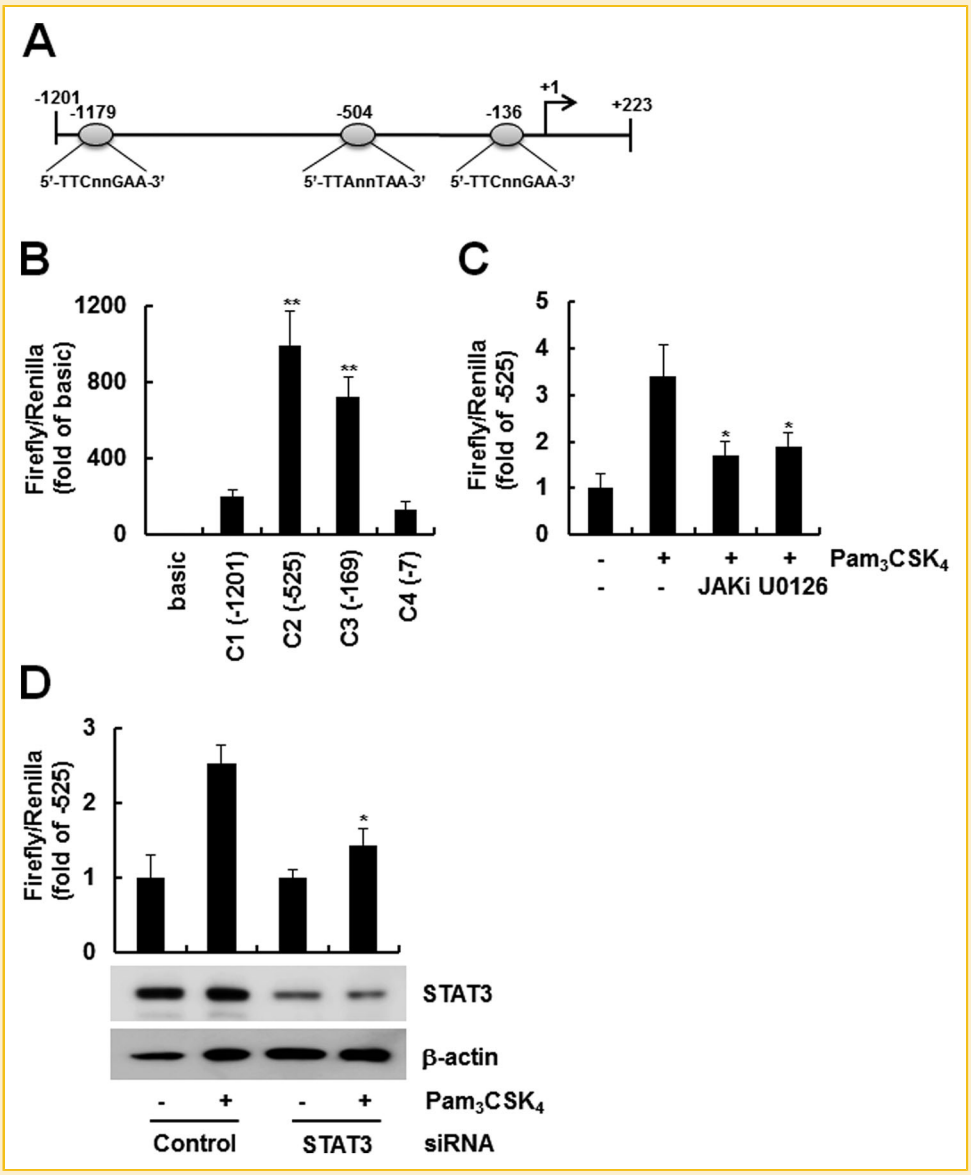

Fig. 5. GAS element sites of the PHLDA1 promoter are involved in the JAK2-ERK1/2-STAT3 pathway. A: Diagrammatic representation of the mouse PHLDA1 promoter from −1201 to +223 bp. The GAS consensus sites (TTCnnGAA or TTAnnTAA), which may potentially be bound by STAT3 proteins, are located at −1179, −504, and −136 bp. The location of PHLDA1 truncation is also shown. B: Raw 264.7 cells were transiently cotransfected either basic, PHLDA1, or various promoter-luciferase truncation constructs and pRL-TK, and assayed for dual luciferase activity. C: The cells were cotransfected with PHLDA1 construct (C2, −525 portion) and pRL-TK and then preincubated with JAK inhibitor (JAKi, 10 μM) or ERK1/2 inhibitor (U0126, 1 μM), and treated with Pam_3CSK_4, after which the activity was detected by dual luciferase activity. D: after the cells were cotransfected with PHLDA1 construct (C2, −525 portion) and either control or STAT3 siRNA, treated with Pam_3CSK_4, and then, luciferase activity was determined. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control. The representative experiments were repeated three times.

mitochondria [Gough et al., 2013]. Another example is that ERK1/2 activation by Raf1-MEK1/2 signaling pathway stimulates STAT1/3 during ischemic preconditioning [Xuan et al., 2005]. The above two reports also emphasize that phosphorylation of serine residue is important for STAT3 signaling. Our results are similar to the above reports, even though the stimulants are different. Therefore, we conclude that TLR2 activation by Pam_3CSK_4 stimulates JAK2-STAT3 pathway via ERK1/2, and eventually increases PHLDA1 expression. It is important to note that the mechanism through which TLR2 activates JAK2 is still unclear.

The importance of STAT3 to PHLDA1 expression was confirmed by promoter analysis. Although the promoter region of PHLDA1 shows an imperfect palindrome for one AP-1 and three SP-1 binding sites [Marchiori et al., 2008], less is known about transcription factor binding sites, especially STAT3. The activity of a PHLDA1 promoter was significantly enhanced by Pam_3CSK_4 in Raw 264.7 cells. To establish whether STAT3 is involved in the regulation of transcription we conducted computer analysis of the PHLDA1 promoter. Computational analysis revealed that the PHLDA1 promoter contains three putative STAT3 GAS binding

sites. Of particular interest is that two putative GAS elements are located in the proximity of ATG site, suggesting an essential role of JAK2-STAT3 signaling in the up-regulation of PHLDA1 in response to TLR2 stimuli.

To our knowledge, this is first report providing evidence of regulation of PHLDA1 protein, highlighting the mechanism through which the JAK2-ERK1/2-STAT3 pathway is involved in modulation of PHLDA1 expression.

ACKNOWLEDGMENTS

This work was supported by 2012 Yeungnam University Research Grant (212A345025). J. H. L: Conception and design, development of methodology, acquisition of data, analysis, and interpretation, writing/review of manuscript. B. H.: Conception and design, development of methodology, acquisition of data, analysis, and interpretation, writing/review of manuscript. D. W. P.: Development of methodology, acquisition of data. S. H. B.: Conception and design, analysis, and interpretation, writing/review of manuscript, study supervision.

REFERENCES

Basseri S, Lhotak S, Fullerton MD, Palanivel R, Jiang H, Lynn EG, Ford RJ, Maclean KN, Steinberg GR, Austin RC. 2013. Loss of TDAG51 results in mature-onset obesity, hepatic steatosis, and insulin resistance by regulating lipogenesis. Diabetes 62:158–169.

Bowie A, O'Neill LA. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: Signal generators for pro-inflammatory interleukins and microbial products. J Leukoc Biol 67:508–514.

Gough DJ, Koetz L, Levy DE. 2013. The MEK-ERK pathway is necessary for serine phosphorylation of mitochondrial STAT3 and Ras-mediated transformation. PLoS ONE 8:e83395.

Greenhill CJ, Rose-John S, Lissilaa R, Ferlin W, Ernst M, Hertzog PJ, Mansell A, Jenkins BJ. 2011. IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3. J Immunol 186:1199–1208.

Hayashida N, Inouye S, Fujimoto M, Tanaka Y, Izu H, Takaki E, Ichikawa H, Rho J, Nakai A. 2006. A novel HSF1-mediated death pathway that is suppressed by heat shock proteins. EMBO J 25:4773–4783.

Hossain GS, Lynn EG, Maclean KN, Zhou J, Dickhout JG, Lhotak S, Trigatti B, Capone J, Rho J, Tang D, McCulloch CA, Al-Bondokji I, Malloy MJ, Pullinger CR, Kane JP, Li Y, Shiffman D, Austin RC. 2013. Deficiency of TDAG51 protects against atherosclerosis by modulating apoptosis, cholesterol efflux, and peroxiredoxin-1 expression. J Am Heart Assoc 2:e000134.

Hossain GS, van Thienen JV, Werstuck GH, Zhou J, Sood SK, Dickhout JG, de Koning AB, Tang D, Wu D, Falk E, Poddar R, Jacobsen DW, Zhang K, Kaufman RJ, Austin RC. 2003. TDAG51 is induced by homocysteine, promotes detachment-mediated programmed cell death, and contributes to the cevelopment of atherosclerosis in hyperhomocysteinemia. J Biol Chem 278:30317–30327.

Hu X, Chen J, Wang L, Ivashkiv LB. 2007. Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. J Leukoc Biol 82:237–243.

Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO. 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 130:1071–1082.

Johnson EO, Chang KH, de Pablo Y, Ghosh S, Mehta R, Badve S, Shah K. 2011. PHLDA1 is a crucial negative regulator and effector of Aurora A kinase in breast cancer. J Cell Sci 124:2711–2722.

Liljeroos M, Vuolteenaho R, Rounioja S, Henriques-Normark B, Hallman M, Ojaniemi M. 2008. Bacterial ligand of TLR2 signals Stat activation via induction of IRF1/2 and interferon-alpha production. Cell Signal 20:1873–1881.

Luu K, Greenhill CJ, Majoros A, Decker T, Jenkins BJ, Mansell A. 2014. STAT1 plays a role in TLR signal transduction and inflammatory responses. Immunol Cell Biol 92:761–769.

Manetsch M, Seidel P, Heintz U, Che W, Hughes JM, Ge Q, Sukkar MB, Ammit AJ. 2012. TLR2 ligand engagement upregulates airway smooth muscle TNFalpha-induced cytokine production. Am J Physiol Lung Cell Mol Physiol 302:L838–L845.

Marchiori AC, Casolari DA, Nagai MA. 2008. Transcriptional up-regulation of PHLDA1 by 17beta-estradiol in MCF-7 breast cancer cells. Braz J Med Biol Res 41:579–582.

O'Shea JJ, Plenge R. 2012. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. Immunity 36:542–550.

Oberg HH, Sipos B, Kalthoff H, Janssen O, Kabelitz D. 2004. Regulation of T-cell death-associated gene 51 (TDAG51) expression in human T-cells. Cell Death Differ 11:674–684.

Oberst MD, Beberman SJ, Zhao L, Yin JJ, Ward Y, Kelly K. 2008. TDAG51 is an ERK signaling target that opposes ERK-mediated HME16C mammary epithelial cell transformation. BMC Cancer 8:189.

Okugawa S, Ota Y, Kitazawa T, Nakayama K, Yanagimoto S, Tsukada K, Kawada M, Kimura S. 2003. Janus kinase 2 is involved in lipopolysaccharide-induced activation of macrophages. Am J Physiol Cell Physiol 285: C399–C408.

Park CG, Lee SY, Kandala G, Lee SY, Choi Y. 1996. A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. Immunity 4:583–591.

Park DW, Lyu JH, Kim JS, Chin H, Bae YS, Baek SH. 2013. Role of JAK2-STAT3 in TLR2-mediated tissue factor expression. J Cell Biochem 114:1315–1321.

Samavati L, Rastogi R, Du W, Huttemann M, Fite A, Franchi L. 2009. STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. Mol Immunol 46:1867–1877.

Sellheyer K, Krahl D. 2011. PHLDA1 (TDAG51) is a follicular stem cell marker and differentiates between morphoeic basal cell carcinoma and desmoplastic trichoepithelioma. Br J Dermatol 164:141–147.

Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. Annu Rev Immunol 21:335–376.

Toyoshima Y, Karas M, Yakar S, Dupont J, Lee H, LeRoith D. 2004. TDAG51 mediates the effects of insulin-like growth factor I (IGF-I) on cell survival. J Biol Chem 279:25898–25904.

Villarino AV, Kanno Y, Ferdinand JR, O'Shea JJ. 2015. Mechanisms of Jak/STAT signaling in immunity and disease. J Immunol 194:21–27.

Wang H, Brown J, Gao S, Liang S, Jotwani R, Zhou H, Suttles J, Scott DA, Lamont RJ. 2013. The role of JAK-3 in regulating TLR-mediated inflammatory cytokine production in innate immune cells. J Immunol 191:1164–1174.

Wu S, Walenkamp MJ, Lankester A, Bidlingmaier M, Wit JM, De Luca F. 2010. Growth hormone and insulin-like growth factor I insensitivity of fibroblasts isolated from a patient with an I{kappa}B{alpha} mutation. J Clin Endocrinol Metab 95:1220–1228.

Xuan YT, Guo Y, Zhu Y, Wang OL, Rokosh G, Messing RO, Bolli R. 2005. Role of the protein kinase C-epsilon-Raf-1-MEK-1/2-p44/42 MAPK signaling cascade in the activation of signal transducers and activators of transcription 1 and 3 and induction of cyclooxygenase-2 after ischemic preconditioning. Circulation 112:1971–1978.

Zgheib A, Pelletier-Bonnier E, Levros LC, Jr., Annabi B. 2013. SelectiveJAK/STAT3 signalling regulates transcription of colony stimulating factor −2 and −3 in Concanavalin-A-activated mesenchymal stromal cells. Cytokine 63:187–193.