Cellular Immunology 266 (2010) 61–66

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

TL1A induces the expression of TGF-β-inducible gene h3 (βig-h3) through PKC, PI3K, and ERK in THP-1 cells

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ARTICLE INFO

Article history:
Received 24 April 2010
Accepted 27 August 2010
Available online 21 September 2010

Keywords:
Macrophage
Extra cellular matrix
Inflammation
TNFSF
βig-h3

ABSTRACT

βig-h3, an extracellular matrix protein involved in various biological processes including cellular growth, differentiation, adhesion, migration, and angiogenesis, has been shown to be elevated in various inflammatory processes. Death receptor 3 (DR3), a member of the TNF-receptor superfamily that is expressed on T cells and macrophages, is involved in the regulation of inflammatory processes through interaction with its cognate ligand, TNF-like ligand 1A (TL1A). In order to find out whether the TL1A-induced inflammatory activation of macrophages is associated with the up-regulation of βig-h3 expression, the human acute monocytic leukemia cell line (THP-1) was stimulated with either recombinant human TL1A- or DR3-specific monoclonal antibodies. Stimulation of DR3 up-regulated the intracellular levels as well as the secretion of βig-h3. Utilization of various inhibitors and Western blot analysis revealed that activation of protein kinase C (PKC), extracellular signal-regulated kinase (ERK), phosphoinositide kinase-3 (PI3K), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is required for TL1A-induced βig-h3 expression. PKC appears to be the upstream regulator of PI3K since the presence of PKC inhibitor blocked the phosphorylation of AKT without affecting ERK phosphorylation. On the other hand, suppression of either PI3K or ERK activity resulted in the suppression of IκB phosphorylation. These findings indicate that TL1A can regulate the inflammatory processes through modulation of the βig-h3 expression through two separate pathways, one through PKC and PI3K and the other through ERK, which culminates at NF-κB activation.

1. Introduction

As a member of TNF-receptor superfamily, death receptor 3 (DR3, TNFRSF25, Apo-3, TRAMP, LARD, or WSL-1) contains a conserved death domain in its cytoplasmic region similar to tumor necrosis factor receptor-1 (TNFR-1) and CD95 (also called FAS or APO-1) [1]. DR3 was found to be expressed primarily in T lymphocytes and myeloid lineage cells [2–4]. The ligand for DR3 was identified to be TNF-like ligand 1A (TL1A, TNFSF15) [5]. TL1A regulates, through its interaction with DR3, the function of T and NK cells and the negative selection process during thymocyte development [2,4]. TL1A treatment or cross-linking DR3 with monoclonal antibodies (mAb) induced proliferation and/or cytokine/chemokine production in T cells and macrophages [3,6]. Accordingly, DR3 has been implicated in the pathogenesis of inflammatory diseases such as irritable bowel disease [7], atherosclerosis [3], and rheumatoid arthritis (RA) [2].

βig-h3 (TGFBI, MP78/70, RGD-CAP, keratoepithelin) was first identified in the human adenocarcinoma cell line A549 that had been treated with TGF-β [8–11]. βig-h3 has been shown to bind to extracellular matrix (ECM) proteins such as collagen type I, II, IV, and VI [9,12], and fibronectin [13] through integrins and modulate the adhesion, migration, and chemotactic potential of cells [14,15]. The over-expression of βig-h3 was first detected in inflammatory processes associated with atherosclerosis, wound healing, diabetic angiopathy, cyclosporine nephropathy, and RA [16–20]. These observations suggest that βig-h3 is involved with the modulation of inflammatory responses and regulation of its expression could be a potential target for the treatment of chronic inflammatory diseases. In this study, βig-h3 expression patterns were investigated in the human macrophage-like cell line, THP-1 after stimulation of DR3 using recombinant human TL1A (rhTL1A) or DR3-specific mAb. The signaling pathway involved in DR3-induced βig-h3 expression was also investigated.

2. Materials and methods

2.1. Antibodies, reagents, and cell culture

Human TL1A and mouse IgG isotype control were purchased from R&D System (Minneapolis, MN, USA). The anti-human

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doi:10.1016/j.cellimm.2010.08.013

βig-h3 mAb (clone 18B3) were generated as previously described [21]. Rottlerin, GÖ6976, Ro31-8425, SB203580, c-Jun N-terminal kinase (JNK) inhibitor I (JNK-I1), negative control for JNK inhibitor I and LY294002 were obtained from Calbiochem International Inc. (La Jolla, CA, USA). Mouse mAb against DR3 (clone 4E3) was purchased from ABGENT (San Diego, USA) or from Abnova (clone M07) (Taipei City, Taiwan). Both antibodies induced similar responses in the THP-1 cells. Rabbit polyclonal antibodies to ERK (p42/44 MAPK), phospho-ERK (Thr202/Tyr204), IκB-α, AKT, phospho-AKT (Ser473), mouse mAb to phospho-IκB-α (Ser32/36) (5A5), PD98059, and U0126 were purchased from Cell Signaling (Danvers, MA, USA). Rabbit polyclonal antibodies to p50 and actin were purchased from Santa Cruz (CA, USA). Ethyl pyruvate, sulfasalazine, bacterial lipopolysaccharide (LPS), saponin, and brefeldin-A were purchased from Sigma (St. Louis, MO, USA). The human monocytic cell line, THP-1, cells were cultured according to the supplier's instruction (American Type Culture Collection, Manassas, VA, USA). The THP-1 cells were grown in RPMI 1640 medium (WelGENE Inc., Daegu, Korea) which was supplemented with 10% FBS, 0.05 mM β-mercaptoethanol, glucose, and streptomycin–penicillin at 37 °C in 5% CO_2 incubators.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

To quantify the βig-h3 mRNA expressions in the THP-1 cells, RT-PCR was performed. THP-1 cells (2×10^6 cells/well in 24 well plates containing 2 ml serum-free RPMI medium) were stimulated with 30 μg/ml immobilized anti-DR3 mAb for 12, 18, and 24 h. Total cellular RNA was extracted from whole cells and 3 μg of RNA was treated with RNase free DNase (Takara, Otsu Shiga, Japan) for the synthesis of first-strand cDNA using a RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Hanover, USA) according to the manufacturer's protocol. Primer sequences for the 660 bp-long βig-h3 product were 5′CCA TCA CCA ACA ACA TCC AG3′ (forward primer) and 5′GAG TTT CCA GGG TCT GTC CA3′ (reverse primer). For GAPDH (391 bp products), 5′ATC ACT GCC ACC CAG AAG AC3′ (forward primer) and 5′TGA GCT TGA CAA AGT GGT CG3′ (reverse primer) were used. A PCR was carried out under following conditions: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56.6 °C for 40 s and 72 °C for 40 s followed by one cycle of 72 °C for 7 min. PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

2.3. Western blotting

For the detection of βig-h3, ERK, phospho-ERK, AKT, phospho-AKT, IκB, and phospho-IκB, the THP-1 cells were incubated in a serum-free RPMI medium and stimulated with anti-DR3 mAb that had been immobilized at 2–20 μg/ml concentrations. Supernatants were collected for the analysis of secreted form of βig-h3 and cell lysates were collected to assess cell-associated βig-h3. For the cell lysis, NP-40 (IGEPAL CA-630) lysis buffer (150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0) with a protease inhibitor cocktail (Calbiochem) was used. The lysates were clarified by centrifugation (12,000 rpm for 15 min at 4 °C). Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Millipore, USA), the membrane was incubated in blocking solution (TBS–T: 5% nonfat dried milk in TBS containing 0.1% Tween-20), washed with TBS–T, incubated at 4 °C with primary antibodies in blocking solution for 1–13 h, washed with TBS–T, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 4 °C for 1 h and then washed. Bands were visualized by using enhanced chemiluminescence detection reagents (Pierce, Rockford, USA) and by exposure to X-ray films.

2.4. Intracellular staining and flow cytometry

The THP-1 cells were stimulated with anti-DR3 mAb or mouse IgG that had been immobilized at 20 μg/ml concentration for 24 h. Six hours after stimulation, cells were incubated with 3 μg/ml of brefeldin-A. The cells were washed once with ice-cold staining buffer (1 mM Sodium azide, 0.5% BSA in PBS) and resuspended in 100 μl PBS. The cells were then fixed by adding 100 μl of 4% paraformaldehyde, incubated at room temperature in dark for 20 min, and washed twice with ice-cold staining buffer. The fixed cells were then permeabilized with staining solution containing 0.5% saponin at room temperature (RT) for 10 min. The cells were stained with anti-βig-h3 mAb for 30 min in the dark, washed twice, and incubated with 0.5 μg of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Caltag-Medsystems, Buckingham, UK). For background level fluorescence, cells were stained with an isotype matching control antibody. Flow cytometry was performed on the FACS-calibur system (Becton Dickinson, Mountain View, CA). Fluorescence profiles of 1×10^4 cells were collected and analyzed.

2.5. Immunofluorescence assay

For the detection of subcellular location of NF-κB p50 subunit, THP-1 cells (1.5×10^5 cells/well in 96-well plates) were treated with anti-DR3 antibody, lipopolysaccharide (LPS), or mouse IgG (isotype control). After treatment, cells were washed with PBS, resuspended in 10 μl of 4% paraformaldehyde in PBS, and placed onto slide glass for 30 min that was followed by 5 min washing in PBS. The fixed cells were then stained with 1.43 μM 4′,6-diamidino-2-phenylindole (DAPI) (Molecular probe, Eugene, USA) for 10 min at 37 °C and then washed with PBS for 5 min. Cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at RT, washed with 0.02% Tween-20 in PBS for 5 min, and 0.02% Tween-20/1% BSA in PBS for 5 min. Cells were then treated with 20 μg/ml anti-p50 polyclonal antibody for 45 min at 37 °C in a humid chamber, and washed with 0.02% Tween-20/1% BSA in PBS for 5 min. Alexa Fluor 594-labeled goat anti-rabbit antibody (20 μg/ml) (Molecular probe, Eugene, USA) was then added for 45 min at 37 °C in a humid chamber, and then cells were sequentially washed with 0.02% Tween-20 in PBS for 5 min and PBS for 5 min. Finally, the cells were dried at RT and mounted with a mounting medium (Dako, Glostrup, Denmark). For the calculation of the percentage of cells with nuclear NF-κB, pictures of five random high-power fields were taken (with a total of about 100 cells in each sample) and cells were counted with bare eyes.

2.6. ELISA-based measurement of NF-κB binding activity

A previously described method [22] was followed for the measurement of NF-κB binding activity. Briefly, biotin labeled double-stranded oligonucleotides containing a consensus NF-κB binding site (5′cacagttgaggggactttcccaggc3′) was immobilized (0.02 nm/well) onto streptavidin-coated 96-well culture plates to obtain NF-κB oligo plates. Cells were stimulated with LPS or immobilized anti-DR3 mAb/mouse IgG for 15–90 min and total cell lysates were obtained using NP-40 lysis buffer. Cell lysates (10 μg/well) were then added into the NF-κB oligo plates and incubated at room temperature for 1 h with mild agitation. The plates were then incubated with mAb specific to NF-κB p65 subunit (clone F-6, Santa Cruz). The amounts of bound mAbs were then detected with sequential incubation with HPR-labeled goat anti-mouse IgG and tetramethylbenzidine (chromogen). Absorbance (450–540 nm) was then measured and the values were normalized by subtracting the background values which were obtained in wells treated with all the reagents except cell lysate. For the blocking, cell lysate were

pre-incubated with 0.2 nm/sample of double-stranded oligonucleotides containing wild type NF-κB binding sequence or a mutant sequence (5′cacagttgaggccactttcccaggc3′) before adding to the NF-κB oligo plates.

2.7. Statistical analysis

The statistical significance of differences was evaluated by means of two-sided Student's *t*-test assuming equal variances. The differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Induction of βig-h3 expression by the DR3 stimulation

A previous report demonstrated that THP-1 cells expressed high levels of DR3 and the stimulation of DR3 with rhTL1A or anti-DR3 mAb can induce the expression of inflammatory mediators such as cytokines and matrix degrading enzymes [3]. In order to analyze the expression of βig-h3 by the stimulation of DR3, THP-1 cells were stimulated with anti-DR3 mAb that had been immobilized at 2–20 μg/ml concentrations and the expression levels of βig-h3 were analyzed using Western blot analysis (Fig. 1A). Induction of βig-h3 was not observed in cells that were treated with isotype matching mouse IgG (Supplementary data #1). Low basal level expressions of βig-h3 were detected in cells without stimulation and it was up-regulated in a dose-dependent manner for both culture supernatants and cell lysates. RT-PCR analysis of the THP-1 cells further confirmed the increase of βig-h3 mRNA levels within 12 h post-stimulation (Fig. 1B). Furthermore, intracellular staining using βig-h3-specific mAb detected significant up-regulation of its expression levels in cells treated with anti-DR3 mAb (mean MFI ± SEM of three independent experiments were 25.3 ± 3.8 for control and 58.5 ± 10.6 for anti-DR3 treatment, $p < 0.01$), but not with isotype matching mouse IgG (23.8 ± 4.7) (Fig. 1C) indicating that the response resulted from a specific interaction. The viability of the cells was not affected by the treatment (Supplementary data #2).

3.2. PKC, ERK, and PI3K are required for DR3-induced βig-h3 expression

In order to identify the signaling molecules that are involved in DR3-induced βig-h3 expression, the THP-1 cells were stimulated through DR3 in the presence of various inhibitors of signaling. The THP-1 cells were pretreated with protein kinase C (PKC) inhibitors such as Rottlerin, GÖ6976, and Ro31-8425 for a period of 30 min and stimulated with anti-DR3 mAb. Western blot analysis of βig-h3 revealed that all PKC inhibitors blocked the up-regulation of the βig-h3 expression (Fig. 2A). The viability of the cells was not affected by the treatment (Supplementary data #2). Since the

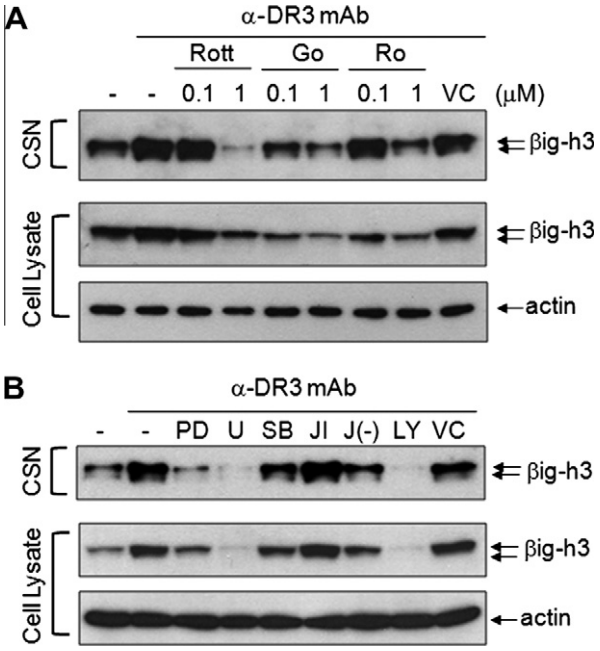

Fig. 2. Stimulation of DR3 induces βig-h3 expression through PKC, ERK, and PI3K. (A) THP-1 cells were pretreated with indicated concentration of Rottlerin (Rott), GÖ6976 (Go), and Ro31-8425 (Ro) for 30 min. Cells were then stimulated with immobilized anti-DR3 mAb (20 μg/ml) for 24 h. Western blot analysis of cell lysates and culture supernatant (CSN) were performed with antibodies against βig-h3 and actin. (B) Cells were pretreated with 10 μM of PD98059 (PD, Erk inhibitor), U0126 (U, Erk inhibitor), JNK inhibitor I (JI), negative control for JNK inhibitor I (J(−)) or LY294002 (LY, PI3K inhibitor) or 5 μM of SB203580 (SB, p38 inhibitor) for 30 min. Cells were then stimulated with immobilized anti-DR3 (20 μg/ml) mAb for 24 h for Western blot analysis of cell lysates and culture supernatants. As a vehicle control (VC), 0.1% DMSO was used which represent the highest concentration of it in the inhibitor treated samples. These experiments were repeated more than three times with essentially the same results.

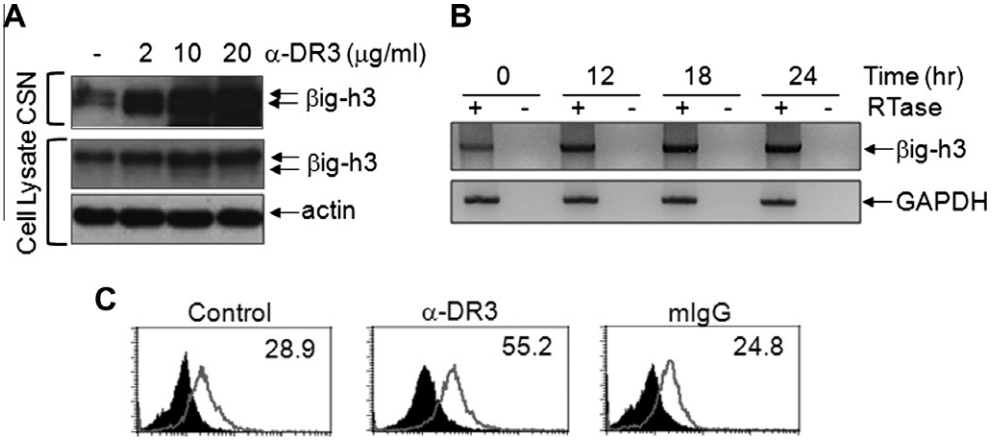

Fig. 1. βig-h3 expression is induced by DR3 stimulation. (A) THP-1 cells were stimulated with immobilized anti-DR3 mAb (2, 10, and 20 μg/ml) for 24 h in serum-free condition. Western blot analyses of cell lysates and culture supernatant (CSN) were performed using anti-βig-h3 mAb and anti-actin antibody. (B) THP-1 cells were stimulated with immobilized anti-DR3 mAb (20 μg/ml). Total cellular RNA was isolated at indicated time points for RT-PCR analysis using βig-h3 or GAPDH specific primers. (C) THP-1 cells were stimulated with immobilized anti-DR3 mAb or mouse IgG (20 μg/ml) for 24 h. Brefeldin-A was added at 3 μg/ml concentration 6 h after stimulation. The cells were then permeabilized and stained with anti-βig-h3 mAb. Histograms from specific staining (open area) and background staining (filled area, stained with mouse IgG) were compared. Numbers indicate mean fluorescence intensity (MFI) values of βig-h3 staining.

common targets of these inhibitors are conventional PKC isotypes, it appears that PKCα and/or PKCβ mediate the DR3-induced signaling event.

Inhibitors of mitogen-activated protein kinase (MAPK) pathways were then tested in DR3-induced βig-h3 expression. As shown in Fig. 2B, ERK inhibitors such as PD98059 and U0126 blocked the expression of βig-h3 while SB203580 (p38 inhibitor) and JNK-I1 (JNK inhibitor) failed to inhibit βig-h3 expression (Fig. 2B). Interestingly, JNK inhibitor slightly increased βig-h3 expression. This is more evident when the βig-h3 expression levels were compared with samples treated with JNK inhibitor negative control (the lane marked with J(−)). Similar observations have been reported in cases of glucocorticoid-induced TNF-receptor ligand (GITRL)-mediated expression of IL-8 in THP-1 cells. In that case, suppression of one form of MAPK (p38 or JNK) enhanced its activation potential, in both basal and upon stimulation, of another MAPK (ERK) [23] resulting in the increased expression of IL-8. These observations indicate that the activation status of MAPKs is interrelated.

In order to identify whether phosphoinositide-3 kinase (PI3K) is required for the DR3-induced expression of βig-h3, LY294002 was used as a PI3K specific inhibitor. LY294002 blocked the up-regulation of βig-h3 expression levels (Fig. 2B), suggesting that the PI3K pathway is also involved in the DR3-induced βig-h3 expression. The viability of the cells was not affected by the treatment (Supplementary data #2).

3.3. DR3-mediated increase in βig-h3 expression requires the activation of NF-κB

The nuclear factor (NF)-κB is a critical regulator of innate and adaptive immunity. Before cell activation, it is present in the cytosol in an inactive state and is complexed with an inhibitory protein (IκB). Activation signal induces phosphorylation and subsequent degradation of the IκB. Free NF-κB dimers (p50/p65) then translocate into the nucleus and activate transcription of various pro-inflammatory mediators such as cytokine, adhesion molecules, and matrix degrading enzymes. In order to determine whether DR3-induced βig-h3 expression requires NF-κB activation, the THP-1 cells were pretreated with ethyl pyruvate [24], a well known NF-κB inhibitor, before treatment with immobilized anti-DR3 mAb. Ethyl pyruvate dose-dependently blocked DR3-induced βig-h3 expression (Fig. 3A). Accordingly, Western blot analyses detected phosphorylation and degradation of IκB in the THP-1 cells that had been stimulated with anti-DR3 mAb (Fig. 3B and Supplementary data #3). Stimulation of the THP-1 cells with isotype matching mouse antibody failed to induce phosphorylation and degradation of the IκB.

Next, the THP-1 cells were stimulated through DR3 and the nuclear translocation of NF-κB was tested though immunofluorescence. Stimulation of DR3-induced nuclear translocation of the NF-κB p50 subunit. Isotype matching control antibodies, however, failed to induce nuclear translocation of the NF-κB p50 subunit (Supplementary data #4). Quantification of the cells with the nuclear NF-κB p50 subunit further confirmed the activation of NF-κB after stimulation of DR3 (Fig. 3C). In order to detect the functional activation of NF-κB, an ELISA-based DNA binding assay was performed using a consensus NF-κB binding site. As shown in Fig. 3D, DNA binding activity of NF-κB p65 subunit was enhanced in cells stimulated with anti-DR3 but not in cells treated with mouse IgG. Preincubation of cell lysate with the consensus NF-κB oligonucleotide, but not with mutant oligonucleotide, blocked the NF-κB binding activity, indicating that the interaction was specific. These observations demonstrated that the activation of NF-κB is required for the DR3-induced βig-h3 expression and both p65 and p50 subunits are activated. According to a recent report, reverse signaling mediated by cell surface BAFF (a member of TNF superfamily) also induced the expression of βig-h3 through signaling pathway that requires the activation of NF-κB [25]. This further supports that the activation of NF-κB is required for the induction of βig-h3 expression. However, it is not possible to speculate whether NF-κB is recruited directly to the promoter or it works

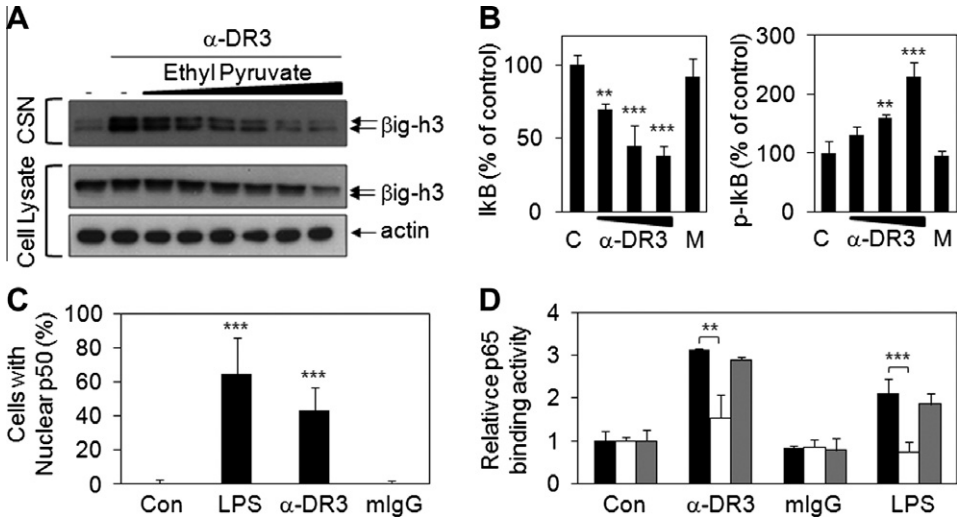

Fig. 3. Anti-DR3 antibody induces βig-h3 expression through NF-κB. (A) THP-1 cells were pretreated with of 5, 6, 7, 8, 9, and 10 mM of ethyl pyruvate and then stimulated with immobilized anti-DR3 mAb (20 μg/ml) for 24 h. Western blot analyses of cell lysates and culture supernatants (CSN) were performed with anti-βig-h3 mAb and anti-actin antibody. (B) THP-1 cells were stimulated with immobilized anti-DR3 antibody (60, 90, and 120 min) and mouse IgG (120 min) at 20 μg/ml concentration and the levels of IκB, and phospho-IκB and actin were measured in whole cell extract using Western blot analysis. The band intensities were measured using densitometer and normalized with corresponding actin band intensities. Data points are represented as mean ± SEM for three independent experiments. (C) THP-1 cells were stimulated with 1 μg/ml LPS or 20 μg/ml of immobilized anti-DR3 mAb or mouse IgG. Ninety-minute after stimulation, the subcellular location of the NF-κB p50 subunit was detected using an immunofluorescence assay and the percentage of cells with nuclear NF-κB p50 subunit was counted. LPS was used as positive control for NF-κB activation, mouse IgG was used as an isotype control. Data points are represented as mean ± SD for triplicate measurements. (D) Cells were stimulated as in C and whole cell lysates were used for the measurement of NF-κB binding activity in ELISA-based assay (black bars). For blocking, cell lysate were pre-incubated with 10-fold excess of wild type NF-κB binding site (white bars) or mutant DNA (grey bars). Data points are represented as mean ± SD for triplicate measurements. These experiments were repeated more than twice with essentially the same results.

indirectly through interaction with other transcription factors, since the promoter for big-h3 has not been defined.

3.4. Inhibition of PKC activity blocks activation PI3K of but not ERK

In order to explore the inter-relationship between PKC, ERK, and PI3K in DR3-induced βig-h3 expression, the THP-1 cells were stimulated in the presence of GÖ6976 and the phosphorylation levels of ERK and AKT (a down-stream indicator of PI3K activation) were tested using Western blot analysis. In the absence of PKC inhibitor, stimulation of DR3-induced phosphorylation of both ERK and AKT as early as 4 min after stimulation. The addition of PKC inhibitor completely blocked the phosphorylation of AKT, while ERK phosphorylation was not affected (Fig. 4A). These data indicate that stimulation of DR3 activates two separate pathways: one of which is mediated through PKC and PI3K and the other is mediated through ERK. Stimulation of DR3 in the presence of PI3K inhibitor failed to block the phosphorylation of ERK (Fig. 4B). This further confirms that the activation of ERK and PI3K are separate events in cells that have been stimulated through DR3.

The pathway responsible for NF-κB activation was then analyzed. Phosphorylation/degradation of IκB started at 30 min after stimulation (Fig. 3B) and nuclear translocation of NF-κB was detected at 90 min after activation (Fig. 3D). These events were observed at later time points then the activation of ERK and PI3K that was detected as early as 4 min after activation indicating that these molecules are upstream of IκB and NF-κB. ERK, one of the MAPKs, is a well-known mediator of inflammation. The stimulation of THP-1 cells with agonistic antibodies against the membrane bound form of GITRL, a member of the tumor necrosis factor superfamily (TNFSF), induced activation of matrix metallopeptidase 9 (MMP-9) and IL-8 through activation of NF-κB which, in turn, was dependent on the ERK activation [23]. Furthermore, Requena et al. demonstrated that bovine glycomacropeptide-induced expression of IL-8, TNF, and IL-1β was dependent on MAPK-mediated activation of NF-κB in the THP-1 cells [26]. Cyclophilin A-induced expression of MMP-9 in THP-1 cells was also dependent on ERK which then regulated NF-κB activity [27]. On the other hand, a couple of reports demonstrated that ERK and PI3K separately activate NF-κB. Song et al. demonstrated that the serum amyloid A-induced activation of NF-κB was mediated by the separate actions of ERK and PI3K in peripheral blood mononuclear cells (PBMCs) and THP-1 cells [28]. Furthermore, angiocidin-mediated activation of NF-κB was shown to be mediated by two pathways each involving either ERK or PI3K in THP-1 cells [29]. In order to confirm the operation of these two pathways at the upstream of NF-κB, IκB phosphorylation levels were tested with anti-DR3 treated cells in the presence of either ERK or PI3K inhibitor. As shown in Fig. 4C, each of these inhibitors decreased phosphorylation levels of IκB. These data in combination with previous reports indicate that the DR3-mediated activation of NF-κB occurs via two separate pathways, one involving ERK and the other involving PKC/PI3K.

3.5. Stimulation of THP-1 cells with rhTL1A also induced βig-h3 expression that was mediated by PKC, ERK, PI3K, and NF-κB

Since DR3 is the receptor for TL1A, the THP-1 cells were stimulated with rhTL1A. As shown in Fig. 5, rhTL1A up-regulated the βig-h3 expression levels and it was blocked by the inhibitors of PKC, ERK, PI3K, and NF-κB. This confirms that the observations made using anti-DR3 mAb recapitulate the signaling pathway induced by the binding of TL1A to DR3 on the cell surface.

βig-h3 is a recently identified extracellular matrix protein expressed ubiquitously and known to be involved in the inflammatory processes as well as cellular growth, differentiation, adhesion, migration, and angiogenesis. It has a YH18 peptide within the fas-1 domain that can mediate interactions with several cell types through different integrins, including α3β1, αvβ3, and αvβ5 [15]. Furthermore, its expression levels are elevated in inflammatory conditions during both normal and abnormal conditions [16–20]. This indicates that the identification of the cell type and the stimuli

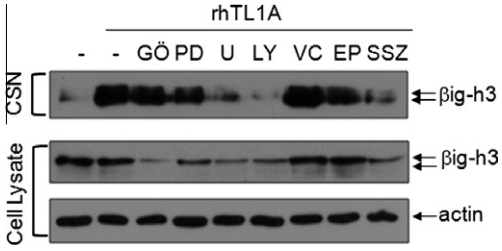

Fig. 5. Treatment with rhTL1A up-regulated βig-h3 expression levels. THP-1 cells were pretreated with 1 μM of GÖ6976 (GÖ), 10 μM of PD98059 (PD), U0126 (U) or LY294002 (LY), 10 mM of ethyl pyruvate (EP) or 0.5 mM of sulfasalazine (SSZ) for 30 min. Cells were then stimulated with rhTL1A (100 ng/ml) for 24 h. Western blot analysis of cell lysates and culture supernatants were performed with antibodies against βig-h3 and actin. As a vehicle control (VC), 0.1% DMSO was used which represent the highest concentration of it in the inhibitor treated samples. The experiment was repeated twice with essentially the same results.

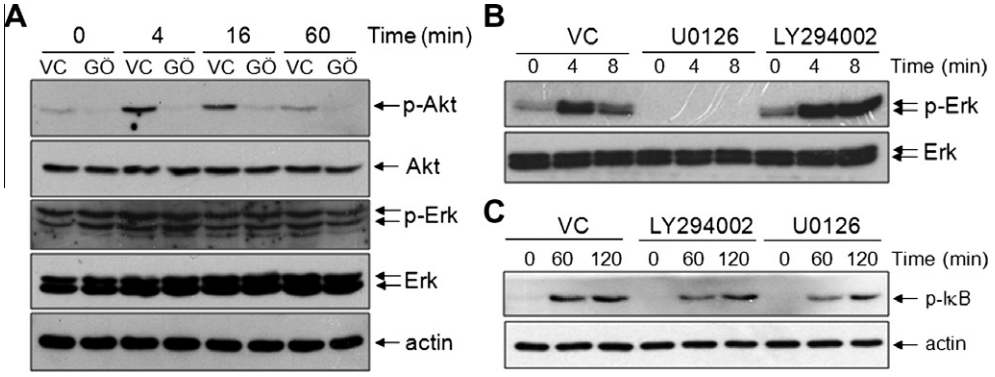

Fig. 4. Phosphorylation of IκB is mediated through PKC/PI3K- or ERK-mediated signaling pathways that operate in separate. (A) THP-1 cells were pretreated for 1 h with 1 μM of GÖ6976 (GÖ) or 0.1% DMSO (VC, vehicle control) in serum-free RPMI. Cells were then stimulated with immobilized anti-DR3 mAb (20 μg/ml) and the levels of AKT, phospho-AKT, ERK, phospho-ERK, and actin were measured in whole cell extract using Western blot analysis at indicated time points. (B) THP-1 cells were pretreated with 10 μM of U0126 and stimulated as in A. Western blot analysis of cell lysates was performed with anti-phospho-ERK and anti-ERK. (C) THP-1 cells were pretreated with 10 μM of LY294002 or U0126 and stimulated as in A and the levels of phosphor-IκB and actin were analyzed using Western blot. As a vehicle control (VC), 0.1% DMSO was used which represent the highest concentration of it in the inhibitor treated samples. These experiments were repeated three times with essentially the same results.

that are responsible for its expression are important for the regulation of pathologic conditions where βig-h3 is involved. Macrophages play important roles in the pathogenesis of inflammatory diseases such as atherosclerosis and RA. They are involved in inflammation via production of matrix degrading enzymes, pro-inflammatory cytokines, cell adhesion molecules, and nitric oxide [30]. Members of the TNF-receptor superfamily and their ligands are expressed on macrophages. Both TL1A and DR3 has been shown to be expressed in atherosclerotic plaques and TL1A-mediated activation of DR3 is believed to play a role in atherogenesis through stimulation of the production of cytokines and matrix degrading enzymes, such as MMP-1, 9, and 13 [3,31]. Our data provide the first demonstration of TL1A-induced signaling pathways in cells of monocyte/macrophage lineage. These signaling pathways are likely to be in operation for the expression of not only βig-h3 but also other pro-inflammatory mediators that are induced by TL1A. Macrophages are crucial mediators in the inflammatory responses and express DR3 in pathologic conditions [2,3,7]. TL1A-induced activation of macrophages may enhance the expression of βig-h3 as well as other pro-inflammatory mediators and these, in turn, will mediate pathological changes associated with inflammatory diseases such as atherosclerosis and RA.

Acknowledgment

This work was supported by the Grant of the Korean Ministry of Education, Science and Technology (The Regional Core Research Program/Anti-aging and Well-being Research Center).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellimm.2010.08.013.

References

[1] A.M. Chinnaiyan, K. O'Rourke, G.L. Yu, R.H. Lyons, M. Garg, D.R. Duan, L. Xing, R. Gentz, J. Ni, V.M. Dixit, Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95, Science 274 (1996) 990–992.
[2] M.J. Bull, A.S. Williams, Z. Mecklenburgh, C.J. Calder, J.P. Twohig, C. Elford, B.A. Evans, T.F. Rowley, T.J. Slebioda, V.Y. Taraban, A. Al-Shamkhani, E.C. Wang, The death receptor 3-TNF-like protein 1A pathway drives adverse bone pathology in inflammatory arthritis, J. Exp. Med. 205 (2008) 2457–2464.
[3] Y.J. Kang, W.J. Kim, H.U. Bae, D.I. Kim, Y.B. Park, J.E. Park, B.S. Kwon, W.H. Lee, Involvement of TL1A and DR3 in induction of pro-inflammatory cytokines and matrix metalloproteinase-9 in atherogenesis, Cytokine 29 (2005) 229–235.
[4] E.C. Wang, A. Thern, A. Denzel, J. Kitson, S.N. Farrow, M.J. Owen, DR3 regulates negative selection during thymocyte development, Mol. Cell. Biol. 21 (2001) 3451–3461.
[5] T.S. Migone, J. Zhang, X. Luo, L. Zhuang, C. Chen, B. Hu, J.S. Hong, J.W. Perry, S.F. Chen, J.X. Zhou, Y.H. Cho, S. Ullrich, P. Kanakaraj, J. Carrell, E. Boyd, H.S. Olsen, G. Hu, L. Pukac, D. Liu, J. Ni, S. Kim, R. Gentz, P. Feng, P.A. Moore, S.M. Ruben, P. Wei, TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator, Immunity 16 (2002) 479–492.
[6] F. Meylan, T.S. Davidson, E. Kahle, M. Kinder, K. Acharya, D. Jankovic, V. Bundoc, M. Hodges, E.M. Shevach, A. Keane-Myers, E.C. Wang, R.M. Siegel, The TNF-family receptor DR3 is essential for diverse T cell-mediated inflammatory diseases, Immunity 29 (2008) 79–89.
[7] G. Bamias, C. Martin 3rd, M. Marini, S. Hoang, M. Mishina, W.G. Ross, M.A. Sachedina, C.M. Friel, J. Mize, S.J. Bickston, T.T. Pizarro, P. Wei, F. Cominelli, Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease, J. Immunol. 171 (2003) 4868–4874.
[8] M.A. Gibson, J.S. Kumaratilake, E.G. Cleary, Immunohistochemical and ultrastructural localization of MP78/70 (βig-h3) in extracellular matrix of developing and mature bovine tissues, J. Histochem. Cytochem. 45 (1997) 1683–1696.
[9] K. Hashimoto, M. Noshiro, S. Ohno, T. Kawamoto, H. Satakeda, Y. Akagawa, K. Nakashima, A. Okimura, H. Ishida, T. Okamoto, H. Pan, M. Shen, W. Yan, Y. Kato, Characterization of a cartilage-derived 66-kDa protein (RGD-CAP/βig-h3) that binds to collagen, Biochim. Biophys. Acta 1355 (1997) 303–314.
[10] F.L. Munier, E. Korvatska, A. Djemai, D. Le Paslier, L. Zografos, G. Pescia, D.F. Schorderet, Kerato-epithelin mutations in four 5q31-linked corneal dystrophies, Nat. Genet. 15 (1997) 247–251.
[11] J. Skonier, M. Neubauer, L. Madisen, K. Bennett, G.D. Plowman, A.F. Purchio, CDNA cloning and sequence analysis of βig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-β, DNA Cell Biol. 11 (1992) 511–522.
[12] E. Hanssen, B. Reinboth, M.A. Gibson, Covalent and non-covalent interactions of βig-h3 with collagen VI. βig-h3 is covalently attached to the amino-terminal region of collagen VI in tissue microfibrils, J. Biol. Chem. 278 (2003) 24334–24341.
[13] P.C. Billings, J.C. Whitbeck, C.S. Adams, W.R. Abrams, A.J. Cohen, B.N. Engelsberg, P.S. Howard, J. Rosenbloom, The transforming growth factor-β-inducible matrix protein (β)ig-h3 interacts with fibronectin, J. Biol. Chem. 277 (2002) 28003–28009.
[14] H.J. Kim, I.S. Kim, Transforming growth factor-β-induced gene product, as a novel ligand of integrin αMβ2, promotes monocytes adhesion, migration and chemotaxis, Int. J. Biochem. Cell Biol. 40 (2008) 991–1004.
[15] N. Thapa, B.H. Lee, I.S. Kim, TGFBIp/βig-h3 protein: a versatile matrix molecule induced by TGF-β, Int. J. Biochem. Cell Biol. 39 (2007) 2183–2194.
[16] S.W. Ha, J.S. Bae, H.J. Yeo, S.H. Lee, J.Y. Choi, Y.K. Sohn, J.G. Kim, I.S. Kim, B.W. Kim, TGF-β-induced protein βig-h3 is upregulated by high glucose in vascular smooth muscle cells, J. Cell. Biochem. 88 (2003) 774–782.
[17] C. Li, S.W. Lim, B.S. Choi, S.H. Lee, J.H. Cha, I.S. Kim, J. Kim, C.W. Yang, Inhibitory effect of pravastatin on transforming growth factor β1-inducible gene h3 expression in a rat model of chronic cyclosporine nephropathy, Am. J. Nephrol. 25 (2005) 611–620.
[18] E.J. Nam, K.H. Sa, D.W. You, J.H. Cho, J.S. Seo, S.W. Han, J.Y. Park, S.I. Kim, H.S. Kyung, I.S. Kim, Y.M. Kang, Up-regulated transforming growth factor β-inducible gene h3 in rheumatoid arthritis mediates adhesion and migration of synoviocytes through α v β3 integrin: regulation by cytokines, Arthritis Rheum. 54 (2006) 2734–2744.
[19] E.R. O'Brien, K.L. Bennett, M.R. Garvin, T.W. Zderic, T. Hinohara, J.B. Simpson, T. Kimura, M. Nobuyoshi, H. Mizgala, A. Purchio, S.M. Schwartz, βig-h3, a transforming growth factor-β-inducible gene, is overexpressed in atherosclerotic and restenotic human vascular lesions, Arterioscler. Thromb. Vasc. Biol. 16 (1996) 576–584.
[20] S.J. Yun, M.O. Kim, S.O. Kim, J. Park, Y.K. Kwon, I.S. Kim, E.H. Lee, Induction of TGF-β-inducible gene-h3 (βig-h3) by TGF-β1 in astrocytes: implications for astrocyte response to brain injury, Brain Res. Mol. Brain Res. 107 (2002) 57–64.
[21] S.H. Lee, J.S. Bae, S.H. Park, B.H. Lee, R.W. Park, J.Y. Choi, J.Y. Park, S.W. Ha, Y.L. Kim, T.H. Kwon, I.S. Kim, Expression of TGF-β-induced matrix protein βig-h3 is up-regulated in the diabetic rat kidney and human proximal tubular epithelial cells treated with high glucose, Kidney Int. 64 (2003) 1012–1021.
[22] C. Rosenau, D. Emery, B. Kaboord, M.W. Qoronfleh, Development of a high-throughput plate-based chemiluminescent transcription factor assay, J. Biomol. Screen. 9 (2004) 334–342.
[23] E.M. Bae, W.J. Kim, K. Suk, Y.M. Kang, J.E. Park, W.Y. Kim, E.M. Choi, B.K. Choi, B.S. Kwon, W.H. Lee, Reverse signaling initiated from GITRL induces NF-κB activation through ERK in the inflammatory activation of macrophages, Mol. Immunol. 45 (2008) 523–533.
[24] Y. Han, J.A. Englert, R. Yang, R.L. Delude, M.P. Fink, Ethyl pyruvate inhibits nuclear factor-κB-dependent signaling by directly targeting p65, J. Pharmacol. Exp. Ther. 312 (2005) 1097–1105.
[25] S.T. Jeon, W.J. Kim, S.M. Lee, M.Y. Lee, S.B. Park, S.H. Lee, I.S. Kim, K. Suk, B.K. Choi, E.M. Choi, B.S. Kwon, W.H. Lee, Reverse signaling through BAFF differentially regulates the expression of inflammatory mediators and cytoskeletal movements in THP-1 cells, Immunol. Cell Biol. 88 (2010) 148–156.
[26] P. Requena, A. Daddaoua, E. Guadix, A. Zarzuelo, M.D. Suarez, F. Sanchez de Medina, O. Martinez-Augustin, Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-κB signal transduction pathways, Br. J. Pharmacol. 157 (2009) 1232–1240.
[27] Y. Yang, N. Lu, J. Zhou, Z.N. Chen, P. Zhu, Cyclophilin A up-regulates MMP-9 expression and adhesion of monocytes/macrophages via CD147 signalling pathway in rheumatoid arthritis, Rheumatology (Oxford) 47 (2008) 1299–1310.
[28] C. Song, K. Hsu, E. Yamen, W. Yan, J. Fock, P.K. Witting, C.L. Geczy, S.B. Freedman, Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes, Atherosclerosis 207 (2009) 374–383.
[29] A. Gaurnier-Hausser, V.L. Rothman, S. Dimitrov, G.P. Tuszynski, The novel angiogenic inhibitor, angiocidin, induces differentiation of monocytes to macrophages, Cancer Res. 68 (2008) 5905–5914.
[30] R. Ross, Atherosclerosis – an inflammatory disease, N. Engl. J. Med. 340 (1999) 115–126.
[31] S.H. Kim, W.H. Lee, B.S. Kwon, G.T. Oh, Y.H. Choi, J.E. Park, Tumor necrosis factor receptor superfamily 12 may destabilize atherosclerotic plaques by inducing matrix metalloproteinases, Jpn. Circ. J. 65 (2001) 136–138.