14-3-3ε and 14-3-3σ Inhibit Toll-like Receptor (TLR)-mediated Proinflammatory Cytokine Induction

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Background: TLRs are key sensors of viral and bacterial components and lead to the production of proinflammatory cytokines.

Results: 14-3-3ε and 14-3-3σ proteins curtail TLR2-, TLR3-, TLR4-, TLR7/8-, and TLR9-mediated proinflammatory cytokine induction.

Conclusion: 14-3-3ε and 14-3-3σ play a critical, hitherto underappreciated role in modulating TLR functionality.

Significance: Learning how TLRs are modulated is crucial for understanding innate immunity.

Toll-like receptors (TLRs) are a group of pattern recognition receptors that play a crucial role in the induction of the innate immune response against bacterial and viral infections. TLR3 has emerged as a key sensor of viral double-stranded RNA. Thus, a clearer understanding of the biological processes that mediate TLR3 signaling is essential. Limited studies have applied proteomics toward understanding the dynamics of TLR signaling. Herein, a proteomics approach identified 14-3-3ε and 14-3-3σ proteins as new members of the TLR signaling complex. Toward the functional characterization of 14-3-3ε and 14-3-3σ in TLR signaling, we have shown that these proteins impair TLR2, TLR3, TLR4, TLR5, TLR6, TLR7/8, and TLR9-mediated RANTES production, 14-3-3 proteins augment NF-κB signaling. Herein, a proteomics approach identified 14-3-3ε and 14-3-3σ proteins as new members of the TLR signaling complex. Toward the functional characterization of 14-3-3ε and 14-3-3σ in TLR signaling, we have shown that these proteins impair TLR2, TLR3, TLR4, TLR5, TLR6, TLR7/8, and TLR9-mediated RANTES production, 14-3-3 proteins augment NF-κB signaling.

The human innate immune system provides the first line of defense against various infectious agents, such as bacteria, viruses, parasites, and helminths. Toward the effective functioning of innate immunity, various classes of protein-based receptors serve to sense various danger-associated molecular patterns and pathogen-associated molecular patterns (PAMPs). These different classes of pathogen recognition receptors include TLRs, C-type lectin-like receptors, scavenger receptors, mannose receptors, and inhibitory pattern recognition receptors. Among the latter class of pathogen recognition receptors, the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family plays a critical role in innate immunity.

The abbreviations used are: PAMP, pathogen-associated molecular pattern; BMDM, bone marrow-derived macrophage; iBMDM, immortalized BMDM; TIR, Toll/interleukin-1 receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor-inducing interferon-β (IFN-β); RLR, RIG-I-like receptor; esiRNA, endoribonuclease-prepared siRNA; ANOVA, analysis of variance; IPG, immobilized pH gradient.
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6). TRIF mediates TLR3 and TLR4 signaling, and TRAM serves as a bridging adaptor between TRIF and TLR4 and leads to activation of interferon regulatory factor 3 (IRF3) (3, 7). Upon recruitment of the activation adaptor proteins to the TLR via homotypic TIR domain interactions, a series of TLR signaling cascades are elicited, which culminates in the production of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β, and IL-12. In addition, activation of TLR3, -7, -8, and -9 induces the production of antiviral IFN-β and IFN-α (2).

TLR3 plays a critical role in innate immunity because it serves to recognize viral dsRNA originating from dsRNA viruses, such as reovirus (4). TLR3 also recognizes dsRNA produced during the replication of many viruses, including ssRNA viruses, such as West Nile virus, respiratory syncytial virus, and encephalomyocarditis virus. In addition, TLR3 also recognizes a synthetic analog of dsRNA known as polyriboinosinic:polyrribocytidylic acid (poly(I:C)) (3). It has been shown that activation of TLR3 using poly(I:C) inhibits HIV infection by increasing the expression of type I IFN antiviral factors, thus restricting HIV expression and replication (8). Also, poly(I:C) has proven beneficial as a mucosal adjuvant for an influenza virus vaccine in a murine infection model (4). Activation of TLR3 signaling has also been linked to the inhibition of tumor cell growth (9) and to amplification of inflammation following the sensing of microbial septic peritonitis and ischemic gut injury (4). Activation of interferon regulatory factor 3 (IRF3) (3, 7). Upon stimulation of cells with the corresponding ligands, Pam3CSK4, poly(I:C), LPS, R848, and CpG, respectively. We show that 14-3-3ε and 14-3-3σ inhibit TLR2+, TLR3+, TLR4+, TLR7/8+, and TLR9-mediated signaling. In contrast, 14-3-3ε and 14-3-3σ enhance TLR2+, TLR4+, TLR7/8+, and TLR9-mediated RANTES production and inhibit TLR3-mediated RANTES production.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Wild-type (WT), MAVS−/−, TRIF−/−, and Myd88−/− immortalized BMDMs (iBMDMs), HEK293, HEK293-TLR2, HEK293-TLR3, HEK293-TLR4, HEK293-TLR7/8, and HEK293-TLR9 were gifts from Professor Katherine Fitzgerald (University of Massachusetts Medical School). HEK293-Blue IFN-α/β, HEK293-Blue IFN-γ, and HEK293-Blue IFN-λ1 cells were purchased from InvivoGen and maintained with GlutaMAX (Invitrogen) and 100 μg/ml gentamycin (250 μg/ml). The HEK293-Blue IFN-α/β culture medium was supplemented with zeocin (100 μg/ml) and blasticidin (100 μg/ml). HEK293-Blue IFN-γ, HEK293-Blue IFN-λ1, and HEK293-Blue IFN-γ cells were cultured in RPMI 1640 with GlutaMAX (Invitrogen) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% Fungizone, and 250 μg/ml hygromycin B and 100 μg/ml G418. Human fibroblast-like synoviocytes were purchased from Cell Applications and were cultured in synoviocyte growth medium (Cell Applications) maintained at 37 °C in a humidified atmosphere of 5% CO2, 95% air. HEK293-Blue IFN-α/β, HEK293-Blue IFN-γ, and HEK293-Blue IFN-λ1 cells were cultured in RPMI 1640 with GlutaMAX (Invitrogen) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% Fungizone, and 250 μg/ml hygromycin B and 100 μg/ml G418. Escherichia coli strain 011:B4 (Alexis), R848 (InvivoGen), CLO97 (InvivoGen), and CpG (InvivoGen) were used for all treatments. 14-3-3ε and 14-3-3σ endoribonuclease-prepared siRNAs (esiRNAs) were purchased from Sigma-Aldrich. Lamin A/C-negative control was from Ambion.

Expression Vectors/Recombinant Plasmids—HA-tagged pcDNA3–14-3-3ε, 14-3-3σ, and 14-3-3ε and 14-3-3σ were kind gifts from Dr. Christian Ottmann (Chemical Genomics Center of the Max Planck Society, Dortmund, Germany). The plasmids pcDNA3: MyD88-cmyc and pcDNA3:p38-FLAG were kind gifts from Professor Luke O’Neill (Trinity College Dublin). The plasmids pcDNA3: TRAF3-FLAG, pcDNA3:TRAF3-FLAG, pcDNA3: TRAF3-FLAG, pcDNA3:TRAF6-FLAG, pcDNA3:IRF7-FLAG, and pcDNA3:MAL-FLAG were kind gifts from Professor Paul Moynagh (National University of Ireland Maynooth). The IFN-β and CCL5 reporter gene construct, FLAG-IKKε, FLAG-
IRF3, and plasmids were as described (20). The NF-κB luciferase reporter construct was as described (21).

Two-dimensional Gel Electrophoresis—Whole cell lysates were extracted from WT and MAVS<sup>−/−</sup> bMDMs following stimulation with poly(I:C) for various times (0, 1.5, 8, and 24 h). The proteins were precipitated using the acetone precipitation method followed by incubation in lysis buffer (7 M urea, 4% CHAPS, 2 M thiourea, 100 mM DTT, 5% ampholytes, and one protease inhibitor mixture tablet (PICS)/50 ml of lysis buffer). Protein separation by two-dimensional gel electrophoresis was performed by isoelectric focusing using 24-cm pH 4–7 IPG strips (GE Healthcare) and in the second dimension by SDS-PAGE. Rehydration of IPG strips, isoelectric focusing, equilibration of focused strips, and SDS-PAGE second dimensional separation was carried out as described previously (22). The separated proteins were visualized by silver staining (23), and high resolution gel images were scanned and analyzed using a Typhoon Trio variable mode imager from GE Healthcare. Comparative and statistical analysis of two-dimensional gels was performed with the Progenesis software program from Non-Linear Dynamics (Newcastle, Tyne, UK).

Mass Spectrometry Analysis (Progenesis, MS, Mascot)—Differentially expressed proteins were subjected to in-gel trypsin digestion, and the resulting peptides were analyzed by peptide mass fingerprinting using an Ion Trap LC/MS apparatus from Agilent Technologies (model 6430). Excision, washing, trypsin digestion, and peptide recovery were as described previously (22). Peptides were eluted using nanoflow Agilent 1200 series system (300SB C18 5-μm, 4-mm, 40-μl trap column; C18 5-μm, 43 mm × 75 μm, 3-μl analytical reversed phase column). Mobile phase A, and 5% ampholytes, 100 mM DTT, 5% ampholytes were used as mobile phase B. The flow rate was 50 μl/min onto the enrichment columns and the temperature of the dryer was at 300 °C, respectively. Database searches were performed using Mascot MS/MS ion search (Matrix Science). All pI values and molecular masses of the identified proteins were compared with the relative position of their corresponding two-dimensional spots on analytical slabs.

First Strand cDNA Synthesis—Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Thereafter, 1 μg of total RNA was mixed with 1 μl of random hexamers (500 μg/ml), and water was added to a final volume of 17 μl and incubated for 5 min at 70 °C. The mixture was then briefly centrifuged and chilled on ice for 2 min. Thereafter, the other reaction components were added in the following order: 5 μl of 5× RT buffer, 1.3 μl of 10 mM dNTP, 0.7 μl of RNasin (Promega), 1 μl of Moloney murine leukemia virus reverse transcriptase (Promega), and nuclease-free water to a total volume of 25 μl. Reactions were incubated at 37 °C for 40 min followed by 42 °C for 40 min and heating to 80 °C for 5 min. The first strand cDNA was stored at −20 °C for up to 1 month.

Quantitative Real-time PCR—First strand cDNA was used as starting material for real-time RT-PCR quantitation with 2× SensiMix™ SYBR No-ROX Mastermix (Bioline) on a real-time PCR system (DNA Engine Opticon system, MJ Research). For amplification of specific genes, the following primers were used: hIFNB, forward (5′-AACGTCAACCTTTCCAGG-3′) and reverse (5′-TGTCGCCTACTACCTGTGTC-3′); hTNFa, forward (5′-CACCACCTGGAACCTGGGA-3′) and reverse (5′-CACTCTACGTGCAGGCCAC-3′); hCLL5, forward (5′-TGCCCTTTCTGTGCTGCTG-3′) and reverse (5′-TGTTGGCGATCTCCTCGTAGG-3′); hILP-10, forward (5′-ATTATCCTGAGACCAAATTGGTG-3′) and reverse (5′-TCCACCCCTTTTTTCTATGGAGA-3′); hIL-6, forward (5′-AAATCTCGGTAACATCCTCGAGGCA-3′) and reverse (5′-GTTCCTTTCTGTGCTGCTG-3′) and reverse (5′-GTCCCTTTCTGTGCTGCTG-3′) and reverse (5′-ACCCTGAGACCAAATTGGTG-3′) and reverse (5′-TCCACCCCTTTTTTCTATGGAGA-3′); h14-3-3<sup>−</sup>b, forward (5′-GACCTCGCCATATTT-3′) and reverse (5′-GCCTTTCTCAGTACAGGCAGAAT-3′). Real-time PCR data were analyzed using the comparative CT method.

14-3-3 Inhibits TLR-mediated Cytokine Induction—Human U373-CD14 cells were transfected with 14-3-3<sup>−</sup>b, 14-3-3<sup>−</sup>c, and negative control, respectively. In a 6-well plate, 20 nM esiRNA was transfected into cells using Dreamfect Gold (OZ Biosciences) as described by the manufacturer. After 24 and 48 h, efficiency of 14-3-3<sup>−</sup>b and 14-3-3<sup>−</sup>c knockdown was assessed by RT-PCR using 14-3-3<sup>−</sup>b and 14-3-3<sup>−</sup>c forward and reverse primers.
Cytokine Analysis—14-3-3ε, 14-3-3σ, and control esiRNA-transfected U373-CD14 cells were stimulated with Pam3CSK4, poly(I:C), LPS, R848, and CpG as indicated. After 24 h, cell-free supernatants were removed and analyzed for IL-6, TNFα, and CCL5 cytokine release as described by the manufacturer (Peprotech). Regarding IFN measurements, Pam3CSK4, poly(I:C), LPS, R848, and CpG-induced human type I IFNs were measured using HEK293-Blue IFN-α/β cells (InvivoGen), essentially as described by the manufacturer.

Extraction of Cellular Nuclear Fraction—14-3-3ε, 14-3-3σ, and control esiRNA-transfected U373-CD14 cells were stimulated with Pam3CSK4, poly(I:C), LPS, R848, and CpG for the indicated times. After ligand stimulations, the cells were collected, and nuclear extracts were prepared using the nuclear extraction kit as described by the manufacturer (Cayman Chemical).

p65, p38, and ERK1/2 Immunoblot Analysis—14-3-3ε, 14-3-3σ, and control esiRNA-transfected U373-CD14 cells were stimulated with Pam3CSK4, poly(I:C), LPS, R848, and CpG as described, and whole cell lysates were subjected to SDS-PAGE followed by immunoblot analysis with an anti-phospho-p65 antibody (Cell Signaling Technology), anti-phospho-p38 antibody (Cell Signaling Technology), and anti-phospho-ERK1/2 antibody (Cell Signaling Technology).

Transfection and Co-immunoprecipitation—HEK293, HEK293TLR3, and U373-CD14 cells (1 × 10^6 cells/well; 6-well plate)
were transfected with varying amount of DNA (3 μg) and transfection buffer (50 μM HEPES, pH 8.0, 1% Nonidet P-40, 0.5% saponin, 1 mM PMSF, 1 mM DTT, 1 tablet/10 ml of PICS) and left at 4 °C for 20 min. The lysates were then subjected to in-gel trypsin digestion followed by an assay of changes in protein expression using two-dimensional gel electrophoresis for each stimulation time point were performed in triplicate as independent experiments (mean ± S.E.).

**RESULTS**

14-3-3 Proteins Are Novel Components of the TLR Signaling Pathway—In order to explore the molecular mechanisms that modulate TLR3 functionality following viral sensing, iBMDMs were stimulated with the synthetic TLR3 ligand, poly(I:C), followed by an assay of changes in protein expression using two-dimensional gel electrophoresis. Although poly(I:C) is sensed by the RLRs RIG-1 and Mda-5, and the signaling adaptor MAVS (24); thus, MAVS−/− iBMDMs were used in the study as a control to preclude the effects induced through the cytosolic RLRs. Following stimulation of WT and MAVS−/− iBMDMs with poly(I:C), there was a 24 h and two-dimensional gel electrophoresis, differentially expressed proteins were selected using Progenesis software and were then subjected to in-gel trypsin digestion followed by nano-LC-MS/MS (Fig. 1A). A number of proteins were found to be differentially expressed (up-/down-regulated) at all time points following poly(I:C) stimulation of WT iBMDMs when compared with control (0 h) iBMDMs (Table 1). Importantly, we found that several members of the 14-3-3 protein family (14-3-3 protein ζ, δ, σ, β, α, ε, and θ) were suppressed upon stimulation with poly(I:C) for 1.5 and 8 h. Similarly, suppression of 14-3-3 expression was also observed in iBMDMs when compared with control (0 h) iBMDMs (Table 1).

**TABLE 1**

| Accession no. | Protein name | No. of peptide matches | Mascot Score | Coverage | pI | Mw | Subcellular localization | Gene ontology | Average -fold change upon poly(I:C) stimulation |
|---------------|--------------|------------------------|--------------|----------|----|-----|--------------------------|--------------|-----------------------------------------------|
| LMNA/162287370 | Prelamin-A/C | 2                      | 87           | 3%       | 6.54 | 74,483 | Nucleus | Intermediate filament | 2 | 0.01 |
| PEBP1/84794552 | Phosphatidylethanolamine-binding protein 1 | 6 | 220 | 56 | 5.19 | 20,991 | Cytoplasm | ATP binding | -2.6 | 0.01 |
| PRDX2/14874558 | Peroxiredoxin-2 | 2 | 42 | 14 | 5.2 | 21,939 | Cytoplasm | Cell redox homeostasis, anti-apoptosis | -2.6 | 0.01 |
| HSP7C/13242237 | Heat shock cognate 71-kDa protein | 3 | 75 | 7 | 5.37 | 71,059 | Cytoplasm | Stress response, transcription regulation | -2.3 | 0.011 |
| TCPA/110625624 | T-complex protein 1 subunit α | 4 | 143 | 16 | 5.82 | 60,875 | Cytoplasm | Protein folding | -5.4 | 0.012 |
| 1405933 | M2-type pyruvate kinase | 6 | 191 | 19 | 7.18 | 58,458 | Cytoplasm, nucleus | Glycolysis, programmed cell death | -3.1 | 0.014 |
| 1433Z/6756041 | 14-3-3 protein ζ | 3 | 222 | 15 | 4.73 | 27,928 | Cytoplasm | Numerous biological processes and pathways | -5.3 | 0.016 |
| 1433S/134023662 | 14-3-3 protein σ | 4 | 163 | 12 | 4.72 | 27,803 | Cytoplasm | Numerous biological processes and pathways | -5.3 | 0.016 |
| 1433B/31543974 | 14-3-3 protein β/α | 7 | 314 | 24 | 4.77 | 28,183 | Cytoplasm | Numerous biological processes and pathways | -5.3 | 0.016 |
| 1433E/5803225 | 14-3-3 protein ε | 3 | 149 | 11 | 4.63 | 29,329 | Cytoplasm | Numerous biological processes and pathways | -5.3 | 0.016 |
| 1433T/6756039 | 14-3-3 protein θ | 3 | 95 | 5 | 4.69 | 28,051 | Cytoplasm | Numerous biological processes and pathways | -5.3 | 0.016 |
| ACTB/4501885 | Actin, cytoplasmic 1 | 2 | 47 | 7 | 5.29 | 42,059 | Cytoplasm | Numerous biological processes and pathways | -10.7 | 0.019 |
| PLSL/31542113 | Plastin-2 | 11 | 239 | 25 | 5.47 | 29,304 | Cytoplasm | Numerous biological processes and pathways | -8.3 | 0.025 |
| EWS/2500583 | RNA-binding protein EWS | 4 | 71 | 8 | 5.31 | 26,238 | Cytoplasm | Numerous biological processes and pathways | -8.3 | 0.025 |
| PLSI/85986777 | Plastin-1 | 2 | 51 | 8 | 5.07 | 24,238 | Cytoplasm | Numerous biological processes and pathways | 0.025 | 0.043 |
| TCPE/6671702 | T-complex protein 1 subunit β | 8 | 51 | 8 | 5.07 | 24,238 | Cytoplasm | Numerous biological processes and pathways | 0.025 | 0.043 |

MAVS inhibited TLR-mediated cytokine induction through TLR3 rather than through the RLRs (Table 2). Although a limited number of studies have described a role for 14-3-3 proteins in TLR signaling (18, 25), their functional characterization and role in TLR signaling require further clarification.
In WT BMDMs, using quantitative RT-PCR, we show that stimulation with Pam3CSK4, poly(I:C), LPS, R848, and CpG (Fig. 1, mRNA expression (3–8 h) profiles were observed upon stimulation, whereupon comparable suppression (0.5–3 h) and enhanced mRNA expression (3–8 h) profiles were observed upon stimulation with Pam3CSK4, poly(I:C), LPS, R848, and CpG (Fig. 1, J–N). Similar to iBMDMs (Fig. 1, E–I) and U373-CD14 cells (Fig. 1, J–N), it was found that 14-3-3ε and 14-3-3σ mRNA expression was suppressed upon stimulation of synoviocytes with Pam3CSK4, poly(I:C), LPS, CLO97, and CpG for 0.5–3 h (Fig. 1, O–S). Thereafter, significant induction of 14-3-3ε and 14-3-3σ mRNA was observed following stimulation of the gels using Progenesis software showed comparable suppression of 14-3-3ε and 14-3-3σ mRNA expression in both WT and iBMDMs. An absence of MAVS expression does not significantly affect the modulation of 14-3-3ε and 14-3-3σ mRNA induction following stimulation with poly(I:C) for 1.5, 3, 6, and 16 h (Fig. 1, O–S). Thus, suppression is initially observed and then significantly enhanced following poly(I:C) stimulation but suppressed following Pam3CSK4, poly(I:C), LPS, R848, and CpG stimulation (Fig. 2F). Next, we sought to investigate the role of 14-3-3 proteins in the translational regulation of IL-6, TNFα, IFN-β, and RANTES. To this end,
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A–F, U373-CD14 cells were pretreated with Pam3CSK4 (1 μg/ml; P3C), poly(I:C) (20 μg/ml; P(I:C)), LPS (1 μg/ml), R848 (1 μg/ml), and CpG (5 μg/ml) for 3 h, and total RNA was isolated, converted to first strand DNA, and used as a template for quantitative real-time RT-PCR as described under “Experimental Procedures” to assay the expression levels of IL-6 (B), TNF-α (C), IFN-β (D), IP-10 (E), and CCL5 (F) or basal 14-3-3 expression in unstimulated cells (A). GAPDH was used to normalize all samples, and fold changes were determined relative to the respective unstimulated control. The data presented are representative of at least three independent experiments performed in triplicate (mean ± S.E. (error bars)). Statistical analysis was performed using unpaired Student’s $t$ test and two-way ANOVA tests comparing the test samples with their respective controls. NS, non-stimulated.
in the production of RANTES via different TLRs, we examined the ability of 14-3-3 to inhibit TRIF- and TRAM-mediated activation of NF-κB and IFN-β, and CCL5 reporter genes (Fig. 5, A–J). Taken together, these data suggest that both 14-3-3 and 14-3-3α drive TLR2, TLR3, TLR4, TLR7/8, and TLR9-mediated CCL5 reporter gene activity, through a mechanism that involves MyD88 and Mal but not TRIF and TRAM. Together, these data provide evidence that 14-3-3α and 14-3-3β differentially modulate TLR-mediated RANTES production.

Pam3CSK4, LPS, R848, and CpG-mediated 14-3-3 α Expression Is Controlled by MyD88; however, Poly(I:C)-mediated 14-3-3 α Expression Is Controlled by TRIF—Upon observing the differential role of TRIF and MyD88 in TLR-mediated RANTES production, we sought to further explore the critical role of the adaptors TRIF and MyD88 in 14-3-3-dependent TLR activation of NF-κB, IFN-β, and CCL5 reporter gene, 14-3-3 α and 14-3-3 β inhibited MyD88-, Mal-, TRIF-, and TRAM-mediated activation of 14-3-3 and 14-3-3α mRNA levels relative to WT iBMDMs (Fig. 6, B and G). As expected, the expression of 14-3-3 mRNA was unaltered upon stimulation of TRIF−/− iBMDMs with poly(I:C) (Fig. 6, B and G). In contrast to TRIF−/− iBMDMs, it was observed that stimulation of MyD88−/− iBMDMs with poly(I:C) induced comparable modulation of 14-3-3 and 14-3-3α mRNAs relative to WT iBMDMs (Fig. 6, B and G). As expected, the expression of 14-3-3 and 14-3-3α mRNAs remained unaltered upon stimulation of MyD88−/− iBMDMs with Pam3CSK4, LPS, R848, and CpG (Fig. 6, A–J). Taken together, these data suggest that whereas TLR3-mediated 14-3-3α and 14-3-3β expression is controlled by TRIF, TLR2, TLR4, TLR7/8, and TLR9-mediated 14-3-3α and 14-3-3β expression is controlled by MyD88.

*Suppression of 14-3-3α and 14-3-3β Enhances TLR2, TLR3, TLR4, TLR7/8, and TLR9-Mediated Phosphorylation of p65, IRF3, p38, and ERK1/2*—We have demonstrated that 14-3-3α and 14-3-3β impairs Pam3CSK4, poly(I:C), LPS, R848, and CpG-induced activation of the NF-κB and IFN-β reporter genes and differentially affects CCL5 reporter gene activity. To support our data, we opted to investigate the ability of 14-3-3α and 14-3-3β to modulate the activity of the transcription factors, NF-κB and IRF3, and MAPK signaling. We found that suppression of 14-3-3α and 14-3-3β in U373-CD14 cells aug-

Figure 3. Suppression of 14-3-3α and 14-3-3β augments TLR2-, TLR3-, TLR4-, and TLR7/8-induced CCL5 reporter gene activity. A–D, U373-CD14 cells were pretreated with either control lamin or 14-3-3α and 14-3-3β (1 μg/ml; P3C), poly(I:C) (20 μg/ml; P(I:C)), LPS (1 μg/ml), R848 (1 μg/ml), and CpG (5 μg/ml; P3C) for 24 h as indicated. Thereafter, IL-6 (A), TNF-α (B), Type-1 IFN (C), and Rantes (D) were measured by ELISA as described under "Experimental Procedures." The data presented are representative of at least three independent experiments performed in triplicate (mean ± S.E. (error bars)). Statistical analysis was performed using unpaired Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. **
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Figure 4. 14-3-3ε and 14-3-3σ inhibit TLR2-, TLR3-, TLR4-, TLR7/8-, and TLR9-mediated NF-κB and IFN-β reporter gene activity but enhance TLR2-, TLR4-, TLR7/8-, and TLR9-mediated CCL5 reporter gene activity. HEK293-TLR2 (A–C), HEK293-TLR3 (D–F), HEK293-TLR4 (G–I), HEK293-TLR7 (J–L), and HEK293-TLR9 cells (M–O) were co-transfected with a reporter gene for the IFN-β, NF-κB, or CCL5 promoter (80 ng) and either empty vector (40 ng) or increasing amounts of an expression vector encoding 14-3-3ε or 14-3-3σ (0, 1, 5, 10, 20, 30, or 40 ng), as indicated. A total of 40 ng/well pRR-Luciferase reporter gene was co-transfected simultaneously to normalize data for transfection efficiency. After 24 h, cells were stimulated with Pam3CSK4 (1 μg/ml; P3C) (A–C), poly(I:C) (20 μg/ml; P(I:C)) (D–F), LPS (1 μg/ml) (G–I), R848 (1 μg/ml) (J–L), and CpG (5 μg/ml) (M–O) for 24 h followed by harvesting of cell lysates and assessment of luciferase reporter gene activity. The data presented are representative of at least three independent experiments performed in triplicate (mean ± S.E. (error bars)). Statistical analysis was performed using an unpaired Student’s t test and two-way ANOVA tests comparing the test samples with their respective controls.
tor functionality rather than through modulation of transcription factor activity.

14-3-3/H9280 and 14-3-3/H9268 Bind to the TLR Adaptors, TRAF3, and TRAF6—Given our results and the fact that MyD88 has previously been shown to interact with 14-3-3/H9256 following IL-1/H9252 and Pam3CSK4 stimulation (19), we sought to further explore the molecular mechanisms utilized by 14-3-3 proteins to impair TLR signaling. To do this, co-immunoprecipitation studies were performed in HEK-293 cells. It was found that MyD88, Mal, TRIF, and TRAM co-immunoprecipitated with 14-3-3/H9280 and 14-3-3/H9268 (Fig. 8, A and B) in a ligand-independent manner. Moreover, 14-3-3/H9280 co-immunoprecipitated with TRAF3 (Fig. 8C, lane 2) and TRAF6 (lane 7) but not with IKK (lane 3), IRF3 (lane 4), IRF7 (lane 5), or p38 (lane 6). Comparable co-immunoprecipitation results were obtained using 14-3-3/H9268 (data not shown). To preclude the possibility of cell type-dependent differences in the protein interactions, co-immunoprecipitation of 14-3-3/H9280 and 14-3-3/H9268 with MyD88, Mal, TRIF, TRAM, TRAF3, and TRAF6 was also investigated in human astrocytoma U373-CD14 cells. Correlating with our HEK293 data, we found that MyD88, Mal, TRIF, TRAM, TRAF3, and TRAF6 co-immunoprecipitated with 14-3-3/H9280 and 14-3-3/H9268 (Fig. 8D and data not shown, respectively). Together, these data support the hypothesis that both 14-3-3/H9280 and 14-3-3/H9268 and their interacting partners play a regulatory role in biological processes, such as cytokine production and the inflammatory response (Fig. 9A).

Common Biological Processes Modulated by 14-3-3/H9280 and 14-3-3/H9268 in TLR Signaling Networks—Upon identification and validation of MyD88, Mal, TRIF, TRAM, TRAF3, and TRAF6 as interacting partners of 14-3-3/H9280 and 14-3-3/H9268, we opted to investigate whether 14-3-3/H9280 and 14-3-3/H9268 and their interacting proteins, MyD88, Mal, TRIF, TRAM, TRAF3, and TRAF6, are involved in the co-regulation of similar biological processes and diseases. To do this, Pathway Studio Software (Ariadne Genomics) was used to investigate and integrate the search for biological processes and diseases co-associated with 14-3-3 proteins and their interacting partners. Assimilated data demonstrated that both 14-3-3/H9280 and 14-3-3/H9268 and their interacting partners play a regulatory role in biological processes, such as cytokine production and the inflammatory response (Fig. 9A).
Also, the 14-3-3 proteins and their interacting partners co-modulate pathological processes, such as atherosclerosis and rheumatoid arthritis (Fig. 9B). Given our data showing that 14-3-3/H9280 and 14-3-3/H9268 interact with TLR signaling molecules, including MyD88, Mal, TRIF, TRAM, TRAF3, and TRAF6, and concomitantly modulate TLR signaling, we can postulate that 14-3-3 proteins may play a role in TLR-mediated cellular processes, such as immune and inflammatory responses, cytokine production, and responses to viral and bacterial infections (Fig. 9).

DISCUSSION

TLRs are critically involved in mediating a normal physiological response to invading pathogens toward their elimination, and it is becoming increasingly appreciated that TLRs are involved in inflammatory pathologies (26–28). The innate immune system has evolved in such a way to provide many strategies to limit TLR functionality with a view to curtailing unwanted inflammatory responses (7, 11). Given the central role played by TLRs toward the sensing of PAMPs and danger-associated molecular patterns and emanation of the inflammatory response, albeit inappropriate in certain cases, we sought to investigate novel mechanisms that may have evolved toward the modulation of TLR functionality. Given that dsRNA is one of the most important viral PAMPs, we opted to investigate the immune responses that are instigated upon exposure of iBMDMs to synthetic dsRNA, namely poly(I:C). Using an integrated quantitative proteomics approach, we have identified 14-3-3e and 14-3-3σ proteins as critical regulators of the

FIGURE 6. Pam3CSK4-, LPS-, R848-, and CpG-mediated 14-3-3 expression is MyD88-dependent, whereas poly(I:C)-mediated 14-3-3 expression is TRIF-dependent. A–J, WT, TRIF−/−, and MyD88−/− iBMDMs (C and H), R848 (1 μg/ml) (D and I), and CpG (5 μg/ml) (E and J) were stimulated with Pam3CSK4 (1 μg/ml) (A and F), poly(I:C) (20 μg/ml) (B and G), LPS (1 μg/ml) (C and H), R848 (1 μg/ml) (D and I), and CpG (5 μg/ml) (E and J) for the indicated times (0, 0.5, 1.5, 3, and 8 h). Then total RNA was isolated, converted to first strand DNA, and used as a template for quantitative real-time RT-PCR as described under “Experimental Procedures” to assay the expression levels of 14-3-3/H9280 (A–E) and 14-3-3/H9268 (F–J). The data presented are representative of at least three independent experiments performed in triplicate (mean ± S.E. (error bars)). Statistical analysis was performed using unpaired Student’s t test and two-way ANOVA tests comparing the test samples with their respective controls.
reporter gene activity (19). However, it must be noted that differences in functionality exist between the various 14-3-3 isoforms and may account for the observed experimental discrepancies (25). Regarding the modulation of TLR2-, TLR3-, TLR4-, TLR7/8-, and TLR9-driven RANTES production, we provide evidence that, similar to NF-κB/H9260 and IFN-α/H9252 induction, 14-3-3 proteins serve to inhibit poly(I:C)-mediated CCL5 mRNA and protein production. Interestingly, 14-3-3 proteins are required for Pam3CSK4-, LPS-, R848-, and CpG-mediated production of RANTES in a Mal/MyD88-dependent manner. Given these findings, we propose that the differential effects of Pam3CSK4, poly(I:C), LPS, R848, and CpG on RANTES production may be facilitated through the differential activation of the other key CCL5 transcriptional activators, such as AP-1, SP-1, IRF-1-RE, and STAT1. Thus, it is plausible to speculate that PAMPs that drive MyD88-dependent TRIF signaling may, in conjunction with 14-3-3, induce the expression of specific transcriptional activators to drive CCL5 transcription. In contrast, PAMPs that drive MyD88-independent TRIF signaling, in conjunction with 14-3-3, may induce the expression of inhibitory elements or suppress the expression of transcriptional activators toward the inhibition of CCL5 transcription. Interestingly, we have previously shown
that differences exist in terms of the regulation of RANTES production by poly(I:C). Specifically, we have shown that although MyD88 inhibits poly(I:C)-mediated RANTES production, Mal does not (7, 11). Therefore, our work correlates with other studies showing that CCL5 induction is a complex cell type- and ligand-dependant process in vivo (32, 33).

Recently, the role of 14-3-3 in TLR signaling has been investigated (18). Following LPS stimulation of cells, PKCe is recruited to TLR4, followed by phosphorylation of PKCe and subsequent association with 14-3-3β in a MyD88-dependent manner (18). Binding of 14-3-3β to PKCe has been shown to lock PKCe in an open conformation, thus regulating its lipid binding activity (34). Another study has shown that dsRNA induces 14-3-3-mediated signaling pathways in human keratinocytes (29).

However, the exact role played by 14-3-3 proteins in TLR signaling requires further clarification. Herein, we employed co-immunoprecipitation experiments and have shown that 14-3-3ε and 14-3-3α bind to the TLR adaptors, TRAF3 and TRAF6. A and B, HEK293-TLR3 cells were co-transfected with vectors encoding MyD88-c-myc, Mal-FLAG, TRIF-FLAG, TRAM-FLAG, or empty vector (EV) and with either 14-3-3ε-HA (A) or 14-3-3α-HA (B). After 24 h, cells were stimulated with poly(I:C) (20 µg/ml) for 30 min as indicated. Thereafter, immunoprecipitation (IP) of 14-3-3ε (A) and 14-3-3α (B) was performed using an anti-HA antibody as described under “Experimental Procedures.” C, HEK293 cells were cotransfected with vectors encoding TRAF3-FLAG, IKKe-FLAG, IRF3-FLAG, IRF7-FLAG, p38-FLAG, TRAF6, 14-3-3ε-HA, or empty vector as indicated. After 24 h, immunoprecipitation was performed using an anti-HA antibody as described under “Experimental Procedures.” D, U373-CD14 cells were co-transfected with vectors encoding MyD88-FLAG, Mal-FLAG, TRIF-FLAG, TRAM-FLAG, or empty vector and with 14-3-3ε-HA. After 24 h, immunoprecipitation was performed using an anti-FLAG antibody as described under “Experimental Procedures.” The data presented are representative of two independent experiments. IB, immunoblot; WCL, whole cell lysate.

FIGURE 8. 14-3-3ε and 14-3-3α bind to the TLR adaptors, TRAF3 and TRAF6. A and B, HEK293-TLR3 cells were co-transfected with vectors encoding MyD88-c-myc, Mal-FLAG, TRIF-FLAG, TRAM-FLAG, or empty vector (EV) and with either 14-3-3ε-HA (A) or 14-3-3α-HA (B). After 24 h, cells were stimulated with poly(I:C) (20 µg/ml) for 30 min as indicated. Thereafter, immunoprecipitation (IP) of 14-3-3ε (A) and 14-3-3α (B) was performed using an anti-HA antibody as described under “Experimental Procedures.” C, HEK293 cells were cotransfected with vectors encoding TRAF3-FLAG, IKKe-FLAG, IRF3-FLAG, IRF7-FLAG, p38-FLAG, TRAF6, 14-3-3ε-HA, or empty vector as indicated. After 24 h, immunoprecipitation was performed using an anti-HA antibody as described under “Experimental Procedures.” D, U373-CD14 cells were co-transfected with vectors encoding MyD88-FLAG, Mal-FLAG, TRIF-FLAG, TRAM-FLAG, or empty vector and with 14-3-3ε-HA. After 24 h, immunoprecipitation was performed using an anti-FLAG antibody as described under “Experimental Procedures.” The data presented are representative of two independent experiments. IB, immunoblot; WCL, whole cell lysate.

FIGURE 9. Cellular processes and diseases associated with 14-3-3ε and 14-3-3α protein interacting partners. The newly identified and verified 14-3-3ε and 14-3-3α and interacting partners, including MyD88, MAL, TRIF, TRAM, TRAF3, TRAF6 were uploaded onto Pathway Studio software (Ariadne Genomics). A and B, 14-3-3ε and 14-3-3α and interacting partners were analyzed using Pathway Studio software for the cellular process network (A) and common diseases (B) shared between them. The dark highlighted circular/oval-shaped entities represent the 14-3-3 proteins and interacting partners. The rectangular entities indicate the cellular processes (A) and diseases (B) that are co-regulated by 14-3-3ε and 14-3-3α and interacting partners.
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and components of the TLR signaling pathway, namely TAB2, TAB1, and TAK1 (TRAF family member–associated NF-κB activator–binding kinase 1) (1, 25, 35). Together, these data suggest that 14-3-3ε and 14-3-3σ mediate their cellular effects through the TLR adaptors and TRAF3 and TRAF6.

The 14-3-3 proteins are a family of conserved, ubiquitously expressed, acidic proteins, consisting of seven known mammalian isoforms (β, γ, ε, σ, ζ, τ, and η), which are differentially expressed in mammalian cells and are subject to phosphorylation (13–15). 14-3-3 proteins have at least 200 interacting partners, and in most cases, binding is dependent on the phosphorylation of the target protein at either serine or threonine motifs (15). Binding partners include a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors, which allows 14-3-3 proteins to play a vital role in a variety of regulatory processes, such as metabolism, apoptosis, cell proliferation, growth, differentiation, and intracellular signaling (16). It is becoming increasingly appreciated that abnormal 14-3-3 expression or dysregulation of 14-3-3/target protein interactions contribute to many diseases (e.g., cancer, joint inflammation, and bacterial/viral infections) (17). More specifically, 14-3-3σ is purported to be a tumor suppressor and is silenced in breast cancers (17). In contrast, 14-3-3ε expression is enhanced in lung cancers (36). Because TLR activation and dysregulation have also been linked with several pathologies (1, 25, 30, 37), it is tempting to speculate that coordinated dysregulation of TLRs and 14-3-3 proteins may occur in these diseases. Herein, we propose that upon stimulation of these transmembrane receptors, which allows 14-3-3 proteins to play a vital role in a variety of regulatory processes, such as metabolism, apoptosis, cell proliferation, growth, differentiation, and intracellular signaling (16). It is becoming increasingly appreciated that abnormal 14-3-3 expression or dysregulation of 14-3-3/target protein interactions contribute to many diseases (e.g., cancer, joint inflammation, and bacterial/viral infections) (17).

More specifically, 14-3-3 proteins respond to changes in the phosphorylation status of target proteins within the cell, so they respond to the dynamic changes that occur within a cell upon sensing of invading pathogens.

In conclusion, 14-3-3ε and 14-3-3σ play a major regulatory role in balancing the host inflammatory response to viral and bacterial infections. Thus, monitoring and manipulation of 14-3-3 proteins may represent novel diagnostic and therapeutic targets for inflammatory conditions and infections.

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