Cellular Signalling 43 (2018) 32–39

Contents lists available at ScienceDirect

Cellular Signalling

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

AKT1 distinctively suppresses MyD88-depenedent and TRIF-dependent Toll-like receptor signaling in a kinase activity-independent manner

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ARTICLE INFO

Keywords:
AKT1
Toll-like receptor
NF-κB
IRF3

ABSTRACT

We found that AKT1, a primary effector molecule of PI3K-AKT signaling, distinctively suppressed Toll-like receptor (TLR)-mediated MyD88-dependent and Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF)-dependent signaling by inhibiting NF-κB activation and IRF3 activity independently of its kinase activity. In *AKT1* knockout RAW264.7 cells, lipopolysaccharide (LPS)-induced transcription and protein production of cytokines including IL-1β and TNF-α (regulated by the MyD88-dependent pathway), as well as IFN-β and RANTES (C-C motif chemokine ligand 5: CCL-5; regulated by the TRIF-dependent pathways) was enhanced compared to wild type cells. In response to LPS stimulation, *AKT1* knockout cells also exhibited enhanced NF-κB and IFN-β promoter activities, which were reduced to a level comparable to that in wild type cells by complementation with either AKT1 or its kinase-dead mutant (AKT1-KD). Expression of AKT1 or AKT1-KD similarly suppressed NF-κB and IFN-β promoter activities induced by LPS and other TLR ligands in wild type cells. Analysis of NF-κB activation caused by transient expression of proteins involved in the MyD88-dependent pathway in TLR signaling revealed that AKT1 suppressed signaling that occurs between activation of IKKβ and that of NF-κB. In contrast, AKT1 appeared to suppress the IFN-β promoter through inhibition of IRF3 activity itself. These results demonstrate a novel, non-kinase function of AKT1 that inhibits TLR signaling, and suggest the multifunctional nature of AKT1.

1. Introduction

Toll-like receptor (TLR) signaling plays a fundamental role in host defense systems that protect against infection, and can induce innate immune responses as well as activate or augment adaptive immunity upon recognition of various pathogen-associated molecular patterns [1]. TLR signaling can largely be divided into two downstream signaling pathways according to the adaptor proteins that are recruited by TLRs. All TLRs except for TLR3 can trigger rapid sequential activation of a signaling cascade involving MyD88, IL-1R-associated kinase (IRAK) family proteins, TRAF6, and the IKK family kinases (MyD88-dependent pathway), which ultimately results in nuclear translocation and activation of NF-κB, the master transcription factor for a number of pro-inflammatory genes, including cytokines such as IL-1β [2]. On the other hand, activated TLR3 and TLR4 recruit the adaptor protein Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF), which mainly activates the IKK related kinases TBK1 and IKKε for subsequent activation of the IRF3 transcription factor that promotes IFN-β production (TRIF-dependent pathway). IFN-β induces the expression of several interferon stimulated genes (ISGs) that encode antiviral effector molecules and regulators of innate immune responses. Notably, only TLR4 is known to activate both the MyD88- and TRIF-dependent pathways. Although innate immune responses elicited by TLR signaling promote pathogen removal, a continued state of activation can be harmful, and, in some situations, such as in sepsis, can even be fatal to the host by damaging host tissues and circulatory systems. Thus, TLR signal transduction is tightly controlled by multi-stage regulatory mechanisms that include positive and negative interactions with other signaling pathways [3].

Several studies suggest that stimulation of TLRs such as TLR2 [4,5], TLR3 [6], TLR4 [7,8,9], TLR5 [10], and TLR9 [11] could also concurrently activate the PI3K-AKT signaling pathway, which is primarily known to regulate diverse cell functions, including growth, proliferation, and survival in response to a range of stimuli, such as growth factors [12]. Activated PI3K catalyzes phosphatidylinositol (3,4) bisphosphate phosphorylation in the plasma membrane to produce phosphatidylinositol (3,4,5) trisphosphate (PIP3), which then recruits

Abbreviations: TLR, Toll-like receptor; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β; LPS, lipopolysaccharide; KD, kinase-dead; IRAK, IL-1R-associated kinase; ISG, interferon stimulated gene; PIP3, phosphatidylinositol (3,4,5) trisphosphate; PH, pleckstrin homology; KO, knock out; Pam_3CSK_4, tripalmitoyl-Cys-Ser-Lys-Lys-Lys-Lys; MALP-2, macrophage activating lipopeptide-2; ODN, oligodeoxynucleotide

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https://doi.org/10.1016/j.cellsig.2017.12.002
Received 12 September 2017; Received in revised form 29 November 2017; Accepted 10 December 2017
Available online 11 December 2017
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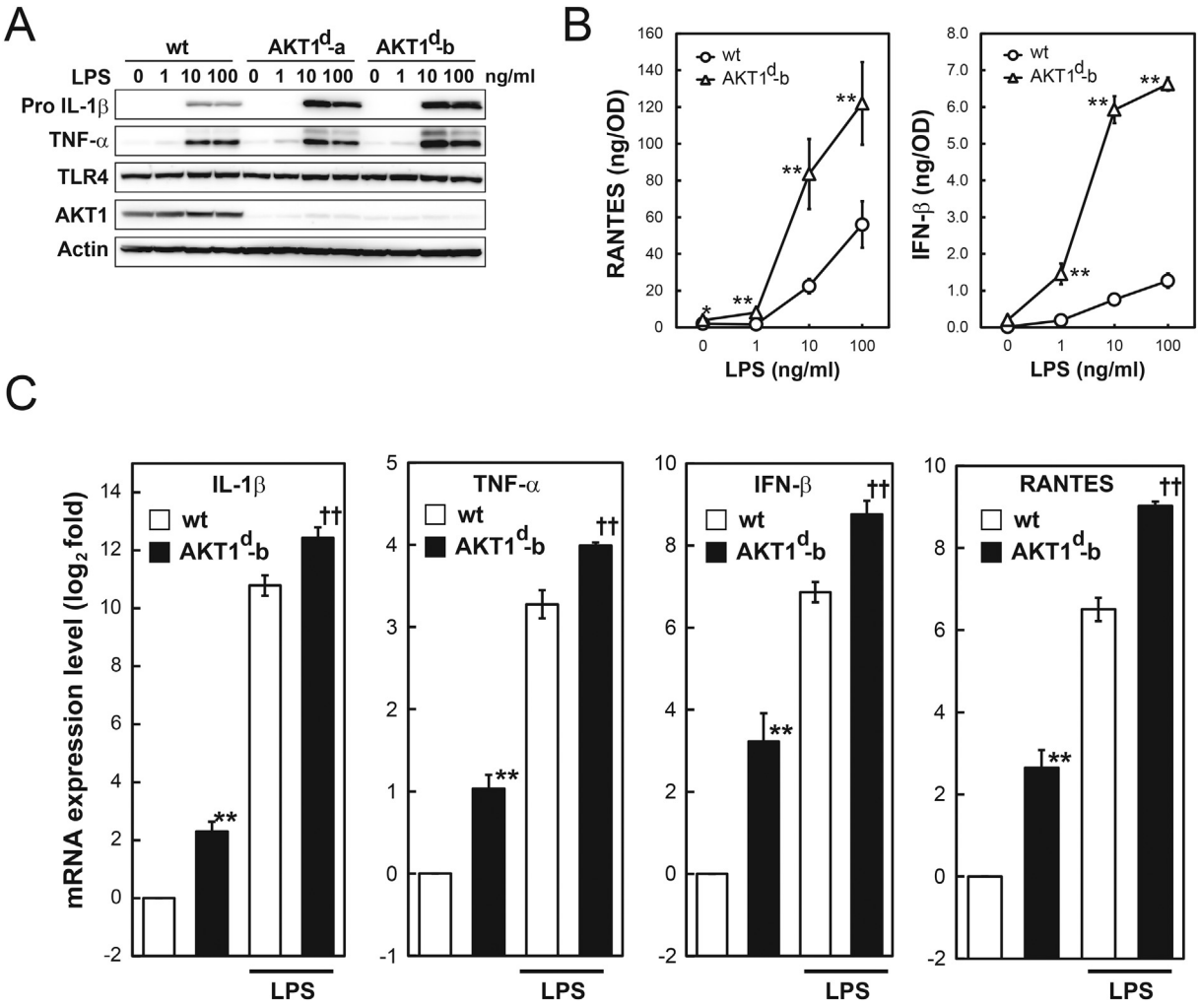

Fig. 1. Enhancement of LPS-induced cytokine production in *AKT1* KO RAW264.7 cells. Wild type (wt) RAW264.7 cells and two different *AKT1* KO clones (AKT1^{d}-a and AKT1^{d}-b) were stimulated with elevated LPS concentrations for 6 h. (A) pro IL-1β, TNF-α, TLR4, AKT1, and β-actin levels were detected by immunoblotting after resolution by SDS-PAGE. A representative image is shown. (B) IFN-β and RANTES production in cell culture supernatants was measured by ELISA as described in the Materials and methods. Each data point represents the mean ± S.E. of 5 (RANTES) and 4 (IFN-β) independent experiments. Statistical analysis was performed using a two-tailed Student's *t*-test (*, $p < 0.05$; **, $p < 0.01$ compared with wt cells). (C) The mRNA expression level of IL-1β, TNF-α, IFN-β, and RANTES was quantified by quantitative real-time RT-PCR analysis of wt and AKT1^{d}-b cells stimulated with 100 ng/ml LPS for 6 h. Each data point represents the mean ± S.E. of 5 independent experiments. Statistical analysis was performed using a two-tailed Student's *t*-test (**, $p < 0.01$ compared with unstimulated wt cells; ††, $p < 0.01$, compared with LPS-stimulated wt cells).

the serine/threonine kinase AKT, a primary effector molecule of PI3K-AKT signaling, to the plasma membrane, where AKT is phosphorylated and activated by multiple kinases. The AKT isoforms AKT1, AKT2, and AKT3 are each encoded by different genes but share ~80% amino acid sequence similarity. Whereas AKT1 is ubiquitously expressed to high levels in the cytoplasm, expression of the other two isoforms is localized to certain tissues and subcellular loci [13], suggesting the relatively global role of AKT1 in regulating cell function. The structures of AKT1 and the other two isoforms share three common domains: the N-terminal pleckstrin homology (PH) domain, kinase domain, and the C-terminal regulatory domain [14,15]. The PH domain interacts with PIP3 and receives upstream signals. Activation of AKT1 requires a lysine residue at 179 (K179), which serves as an ATP binding site in the kinase domain. Phosphorylation of the threonine at amino acid 308 (T308) and serine 473 (S473) in the C-terminal regulatory region is also required for full activation of AKT1 [16,17], although the function of the C-terminal regulatory region remains largely undefined.

Although several studies reported that activated PI3K-AKT signaling could participate in regulating pro-inflammatory responses in TLR signaling, activation of PI3K-AKT signaling exerts both negative and positive effects on NF-κB activation and its activity depending on experimental conditions [18,19]. As such, the precise role of PI3K-AKT signaling and function of AKT1 in TLR signaling remains poorly defined. Moreover, the role of PI3K-AKT signaling in regulation of the TRIF-dependent pathway and subsequent IRF3-induced gene induction is unclear. In this study, we examined the effect of AKT1 on TLR signaling induced by LPS and other TLR ligands. Results from experiments involving *AKT1* knock out (KO) cells showed that AKT1 negatively regulates cytokines produced through both the Myd88- and TRIF-dependent pathways in TLR signaling. Further examination suggested that AKT1-dependent suppression of either NF-κB or IRF3 could be associated with this negative regulation, wherein AKT1 inhibits signaling that occurs between IKKβ activation and NF-κB activation in the MyD88-dependent pathway while concurrently inhibiting IRF3 activity itself in the TRIF-dependent pathway. We found that these effects were independent of AKT1 kinase activity.

2. Materials and methods

2.1. Cell culture and reagents

RAW264.7 and HEK293 cells (both from ATCC) were cultured in

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