The Second-Generation mTOR Kinase Inhibitor INK128 Exhibits Anti-inflammatory Activity in Lipopolysaccharide-Activated RAW 264.7 Cells

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Abstract—Cross-talk between the mTOR (mechanistic target of rapamycin) and NF-κB (nuclear factor kappa-B) pathways has been reported to regulate macrophage responses to lipopolysaccharide (LPS). In this study, we aimed to explore the effect of INK128, a second-generation inhibitor of mTOR, on the inflammatory cytokine production in LPS-stimulated RAW 264.7 cells. Our data showed that INK128 strikingly inhibited the phosphorylation of p70S6K, 4E-BP1 and AKT_{Ser473} in both unstimulated and LPS-stimulated cells. Although it increased the phosphorylation levels of inhibitor kappa-B (IκB) in LPS-stimulated cells, INK128 did not significantly change the levels of NF-κB phosphorylation. In addition, LPS-induced expression of IL-1β and IL-6 was markedly suppressed by INK128 at both mRNA and protein levels. However, the expression of Tumor necrosis factor-alpha (TNF-α protein), but not its mRNA level, was suppressed by this reagent. Our results suggest that the mTOR inhibitor INK128 not only regulates the NF-κB signaling but also influences the inflammatory cytokine expression at both transcriptional and translational levels.

KEY WORDS: INK128; mechanistic target of rapamycin; nuclear factor of kappa-B; lipopolysaccharide; inflammatory cytokine.

INTRODUCTION

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved molecule that regulates many aspects of cellular metabolism [1, 2]. By binding with other proteins, mTOR forms two different large complexes, mTORC1 and mTORC2, which have different serine/threonine kinase activities. mTORC1 mediates the phosphorylation of 4E-BP1 and p70S6K, while mTORC2 controls the phosphorylation of AKT at the Ser473 residue [3]. In addition to regulating cellular metabolism, mTOR also regulates the differentiating and function of immune cells.

It has been reported that the proliferation and differentiation of T lymphocytes, as well as their recognition of antigens presented by dendritic cells, are dependent on the mTOR signaling. Loss of mTOR activity prevents T lymphocytes from differentiation into T helper (Th)1, Th2 and Th17 cells [4, 5]. Recent studies have demonstrated that mTOR also regulates the innate immunity [6]. Both mTOR and nuclear factor kappa-B (NF-κB) signaling can be activated by bacterial infection or lipopolysaccharide (LPS) stimulation [7]. NF-κB is a homo- or heterodimeric complex formed by the Rel-like domain-containing proteins RelA/p65, c-Rel, RelB, NF-κB1 (p105/p50) and NF-κB2 (p100/p52), but the heterodimeric p65-p50 complex appears to be the most abundant one [8]. In unstimulated cells, NF-κB is held in the cytoplasm by NF-κB inhibitor (IκB), primarily through the interaction with p65 [9, 10]. But when cells are stimulated by bacterial infection or LPS, IκB is phosphorylated by IκB kinases (IKKs), leading to its degradation via the ubiquitin-proteasome pathway. The active NF-κB complex is subsequently released and translocates

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into the nucleus, serving as a transcription factor for its targets including inflammatory cytokine genes [11, 12].

Suppression of mTOR activity may influence the activation and transcriptional activity of NF-κB through the cross-talk between the mTOR and NF-κB pathways. It has been reported that LPS may increase the lung injury and apoptosis upon mTOR inhibition [13]. Others have indicated that TSC-mediated mTOR signaling negatively regulates the NF-κB pathway and the production of pro-inflammatory cytokines, but positively regulates STAT3 signaling and the expression of anti-inflammatory interleukin (IL)-10 [6]. Mechanically, both tuberous sclerosis 1 (TSC1) and TSC2, two mTOR suppressors, are required for NF-κB activation, whereas NF-κB is suppressed in TSC1-/TSC2- cells [14]. Moreover, it is believed that AKT-induced activation of NF-κB needs the participation of the mTORC1-associated protein Raptor [15]. On the other hand, the role of mTORC2 in innate immunity regulation should be different from that of mTORC1, since mTORC2 upregulates NF-κB signaling through activation of protein kinase C [16, 17]. However, the differential activities of mTORC1 and mTORC2 on innate immunity regulation are still incompletely understood.

Rapamycin, a well-known mTOR inhibitor, is currently used as an immunosuppressant in clinic because it inhibits the proliferation and functions of T and B lymphocytes and attenuates the differentiation, maturation and antigen-presentation of dendritic cells [3, 18–20]. It has also been reported that rapamycin increases LPS-induced production of tumor necrosis factor-alpha (TNF-α) and other pro-inflammatory cytokines [6, 21]. However, as an allosteric inhibitor of mTORC1, rapamycin is insensitive to 4E-BP1 and does not inhibit mTORC2 activity [22]. As a second-generation inhibitor of mTOR, INK128 inhibits both mTORC1 and mTORC2 signaling by competing for the ATP-binding site of the mTOR kinase [23], and it is currently undergoing clinical trial as an anticancer agent [24]. However, the effect of INK128 on LPS-induced inflammatory cytokine production has not been reported.

In this study, our aim was to evaluate the immunomodulatory effects of INK128 in a cellular model of LPS-stimulated mouse RAW 264.7 macrophages. The results showed that INK128 dose-dependently inhibited the production of TNF-α, IL-1β and IL-6 proteins, as evaluated by intracellular cytokine staining or cytometric bead assay for soluble cytokines. These results demonstrated that INK128, as a second-generation inhibitor of mTOR, has anti-inflammatory effects, which appeared different from that of rapamycin.

MATERIALS AND METHODS

Reagents

INK128 was obtained from Active Biochemicals (Hong Kong, China). Rapamycin, LPS (*Escherichia coli* 0111:B4), monensin, dimethyl sulfoxide (DMSO) and saponin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, L-glutamine, fetal bovine serum (FBS), and Trizol reagent were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Anti-TNF-α-PE and anti-pro-IL-1β-FITC were from eBioscience (San Jose, CA, USA). Both INK128 and rapamycin were prepared as a 2-mM stock solution in DMSO, and stored at −20 °C.

Cell Culture

Mouse RAW 264.7 macrophage cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), maintained in DMEM supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine at 37 °C in a humidified incubator with 5 % CO_2 and subcultured every 2–3 days.

Cell Proliferation Assay

Cells in log-phase were seeded in 96-well plates (1×10^4 cells/well) overnight. The cells were then pretreated with or without indicated concentrations of INK128, followed by LPS stimulation. The working concentration of DMSO never exceeded 0.2 %, which showed no cytotoxicity in RAW 264.7 cells in preliminary experiments. After 48 h, 10 μl of WST-1 reagent (Roche, Penzberg, Germany) was added to each well and the plates were incubated for less than 4 h at 37 °C. Relative proliferation was quantified by measuring the absorbance at 450 nm with a reference at 630 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA), and the 50 % inhibition concentration (IC_{50}) was determined from dose–response curves.

Intracellular Cytokine Staining

Analysis of intracellular cytokine expression was performed as described previously [25]. Briefly, cells were plated in six-well plates (5×10^5 cells/well), incubated in the presence or absence of INK128 at 37 °C for 1 h followed by treatment with LPS (0.1 μg/ml) and monensin (2 μM) for another 6 h. Cells were then fixed

and permeabilized followed by staining with anti-TNF-α-PE and anti-IL-1β-FITC. Data were acquired using CELLQuest software on a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA, USA).

Determination of Soluble Cytokines

Proinflammatory cytokine proteins in culture medium were determined by Cytometric Bead Array (CBA) mouse inflammation kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction.

Relative Quantitative RT-PCR

Total RNA samples were isolated from RAW 264.7 cells using Trizol reagent according to the manufacturer's instruction. The total RNA products were immediately transcribed into cDNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Specific PCR primers used in this study were listed in Table 1. PCR amplification was performed in a Chromo4 Four-Color Real-Time PCR Detection System (Bio-Rad) using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TaKaRa). The specificity of PCR was determined by a following melting curve analysis and agarose gel electrophoresis. The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Western blotting was performed as described previously [26]. Total proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred by electro-transfer to polyvinylidene difluoride (PVDF) membranes (Hybond-P; GE Healthcare Life Sciences, Piscataway, NJ, USA). After incubation in blocking buffer, the membranes were incubated overnight in the presence of the primary antibody against phospho(p)-p70S6K (Thr389), total p70S6K, p-4E-BP1 (Thr37/46), total 4E-BP1, p-AKT (Ser473), total AKT and β-tubulin

(Cell Signaling Technology, Danvers, MA, USA), followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. The bands were revealed with an enhanced chemiluminescence kit (BeyoECL Plus; Beyotime, Haimen, China) and recorded on X-ray films (Kodak, Xiamen, China). The densitometry of each band was quantified by FluorChem 8000 (AlphaInnotech, San Leandro, CA, USA).

Statistical Analysis

All experiments were performed in triplicate, with one representative of three separated experiments shown. Data were expressed as mean±SD. Statistical analysis was performed using Graphpad Prism 5.0 (GraphPad Software Inc., San Diego, CA). One-way ANOVA, followed by Newman–Keuls' multiple comparison tests, was used to analyze the statistical significance among multiple groups. P values <0.05 were considered statistically significant.

RESULTS

INK128 Had Low Cytotoxicity on RAW 264.7 Cells

To determine the appropriate concentrations of INK128 for this study, the inhibitory effect of INK128 on cell proliferation was tested in RAW 264.7 cells. The results showed that INK128 suppressed the growth of RAW 264.7 cells in a dose-dependent manner with an IC_{50} value of~3,270 nM (Fig. 1a). After the addition of LPS, the IC_{50} value was increased to~6,310 nM (Fig. 1b), suggesting that a concentration of INK128 below 1,000 nM had low cytotoxicity on LPS-stimulated macrophages. Thus, two doses (30 and 300 nM) of INK128 were used for most of the subsequent experiments as low and high doses, respectively.

Table 1. Semi-quantitative RT-PCR Primers

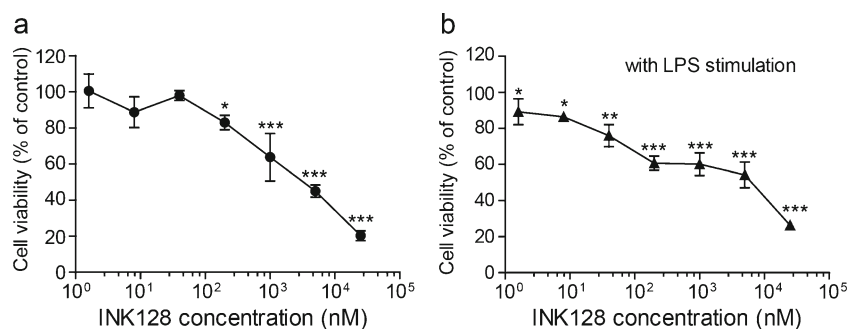|	Forward (5′→3′)	Reverse (5′→3′)
TNF-α	CAGAAAGCATGATCCGCGAC	TTGAGATCCATGCCGTTGGC
IL-1β	AACCTTTGACCTGGGCTGTC	GTCGTTGCTTGGTTCTCCTTG
IL-6	CCACTTCACAAGTCGGAGGC	GCCACTCCTTCTGTGACTCC
β-Actin	TGGTACCACCATGTACCCAG	AAGGGTGTAAAACGCAGCTC

Fig. 1. The inhibitory effect of INK128 on the proliferation of RAW 264.7 cells with or without LPS stimulation. Cells were seeded in 96-well plates overnight and then treated with indicated doses of INK128 with or without LPS (0.1 μg/ml) for 48 h. Cell viability was measured by WST-1 assay as described in Materials and Methods. Values are shown as mean±SD (n=3). *P<0.05, **P<0.01, ***P<0.001, as compared to control (vehicle).

INK128 Robustly Suppressed mTOR Activity in LPS-Activated RAW 264.7 Cells

Rapamycin is a well-known inhibitor of mTOR, which forms a complex with the immunophilin FKBP12 [27, 28] and interrupts the interaction of mTOR and the regulatory associated protein of mTOR (Raptor) [29]. Since rapamycin does not bind mTOR protein directly, it allosterically inhibits the activity of mTORC1 rather than mTORC2 [30, 31]. Unlike rapamycin, INK128 directly competes for the ATP site of the mTOR kinase and inhibits both mTORC1 and mTORC2 activities, thus representing the second-generation inhibitor of mTOR kinases [23]. Consistent with these reports, our results showed that INK128 strongly inhibited the phosphorylation of p70S6K, 4E-BP1 (indicating mTORC1 activity) and AKT_{Ser473} (indicative of mTORC2 activity). The phosphorylation of 4E-BP1 was suppressed by INK128 in a time- and dose-dependent manner, though it was completely inhibited by INK128 only at a high dose (300 nM) and a later stage (2 h) as compared with the inhibition of p70S6K and AKT_{Ser473} phosphorylation (Fig. 2a). However, rapamycin had limited effects on inhibiting the 4E-BP1 phosphorylation and LPS-induced AKT_{Ser473} phosphorylation (Fig. 2b). Thus, INK128 strongly suppressed the kinase activities of mTORC1 and mTORC2 in RAW 264.7 cells in response to LPS stimulation.

INK128 Inhibited Inflammatory Cytokine Protein Expression

We subsequently determined the effect of INK128 on intracellular protein expression of inflammatory cytokines. When RAW 264.7 cells were stimulated with LPS for 6 h, the intracellular protein levels of TNF-α and pro-IL-1β were greatly increased as measured by intracellular cytokine staining together with flow cytometry. INK128 at both 30 nM and 300 nM significantly suppressed the production of TNF-α while it attenuated the expression of pro-IL-1β only at 300 nM (Fig. 3).

Next, we further detected the effect of INK128 on the inflammatory cytokine levels in the culture medium of LPS-stimulated RAW 264.7 cells by using a cytometric bead assay (CBA). As shown in Fig. 4, INK128 dose-dependently inhibited the production of IL-6 and TNF-α, which was consistent with the result of the intracellular cytokine staining assay except that soluble IL-1β was under the detectable limit by CBA. Therefore, INK128 showed dose-dependent effects on the production of different inflammatory cytokines.

INK128 Differentially Influenced the mRNA Levels of Inflammatory Cytokines

We next sought to explore whether INK128 influenced the transcript levels of inflammatory cytokines. Quantitative RT-PCR (qPCR) analysis showed that LPS stimulation increased the mRNA levels of TNF-α, IL-1β and IL-6 in RAW 264.7 cells. Incubation with INK128 did not influence the mRNA levels of TNF-α, but significantly suppressed those of IL-6 and IL-1β in cells stimulated with LPS. These results suggested that INK128 suppressed IL-6 and pro-IL-1β protein expression by decreasing their mRNA levels whereas the reduction of TNF-α protein expression was likely due to a post-transcriptional regulation (Fig. 5). Thus, INK128 differentially regulated the transcription of inflammatory cytokines in LPS-stimulated RAW 264.7 cells.
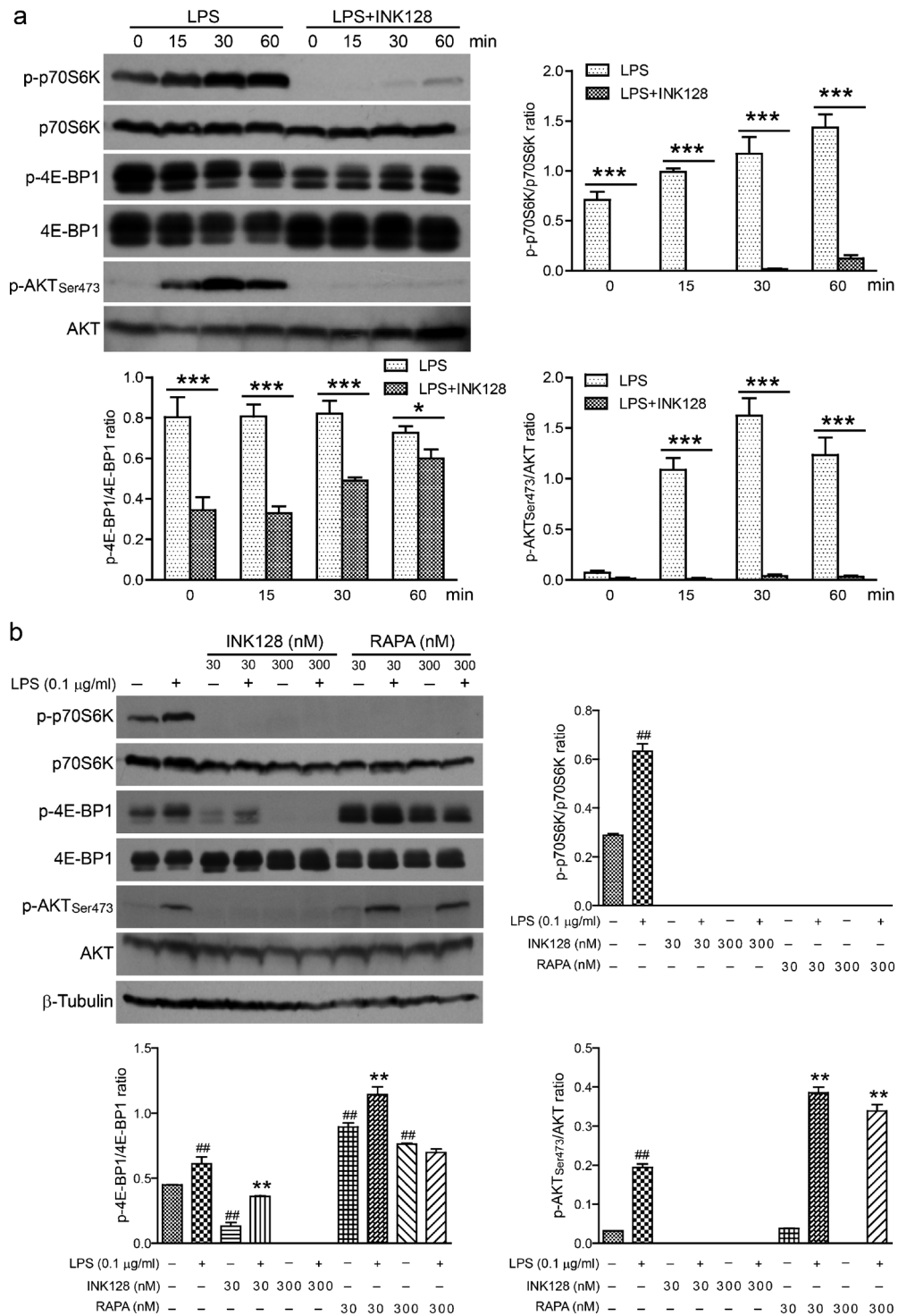
Fig. 2. Effect of INK128 on the phosphorylation of mTOR signaling proteins in LPS-activated RAW 264.7 cells. **a** RAW 264.7 cells were incubated with or without 30 nM of INK128 for 1 h followed by incubation with LPS (0.1 μg/ml) for indicated time lengths. **b** Cells were incubated with or without indicated concentrations of INK128 for 1 h followed by incubation with LPS (0.1 μg/ml) for 2 h. Whole-cell lysates were analyzed by immunoblotting with the indicated antibodies. Quantitative analysis of Western blot is shown as the ratio of densities of p-p70S6K to p70S6K, p-4E-BP1 to 4E-BP1, and p-AKT$_{Ser473}$ to AKT, relative to unstimulated control cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus LPS group ; ##$P < 0.01$ versus control (untreated) group.
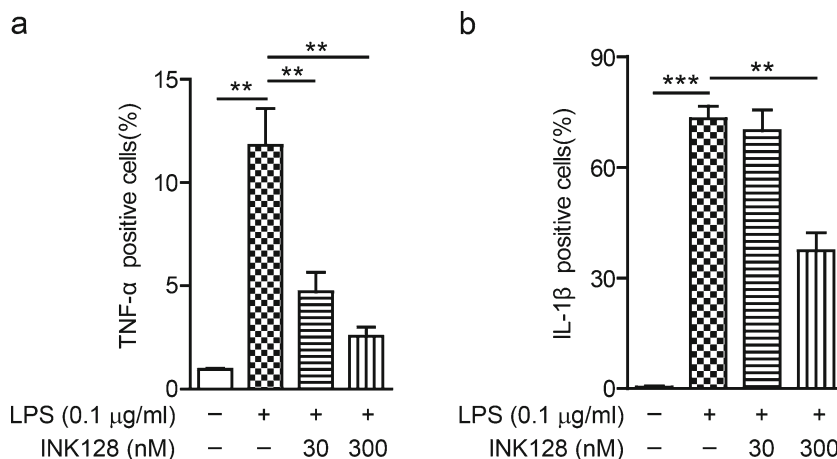
Fig. 3. Effect of INK128 on intracellular TNF-α and pro-IL-1β expression in RAW 264.7 cells in response to LPS stimulation. Cells were pretreated with INK128 for 1 h followed by exposure to LPS (0.1 μg/ml) for 6 h. TNF-α (**a**) and pro-IL-1β (**b**) expression was determined by intracellular cytokine staining. Data are shown as mean$\pm$SD ($n=3$). **$P<0.01$ and ***$P<0.001$.

INK128 Did Not Suppress LPS-Induced NF-κB Activation

As INK128 reduced the expression of IL-1β and IL-6 mRNAs, we tested whether INK128 modulated the signaling pathway of NF-κB, a critical transcription factor regulating cytokine gene expression. Western blot analysis showed that INK128 (30 nM) did not influence the levels of LPS-induced NF-κB(p65) phosphorylation, although it time-dependently upregulated the phosphorylation of IκB (Fig. 6). Since NF-κB regulates the expression of inflammatory cytokines at transcriptional levels, these results suggested that the reduction of TNF-α, IL-1β and IL-6 proteins was not due to the regulatory effect of INK128 on NF-κB signaling. However, it was still elusive how

INK128 treatment led to a reduction of IL-1β and IL-6 mRNA levels in LPS-treated RAW 264.7 cells.

DISCUSSION

Macrophages play a prominent role in the innate immune response to pathogens. They recognize the pathogenic microorganisms via the interaction of their pattern recognition receptors (such as TLR4) with pathogen-associated molecular patterns (such as LPS), which consequently activates inflammatory pathways leading to the secretion of pro-inflammatory cytokines and chemokines, resulting in the recruitment of other inflammatory cells into
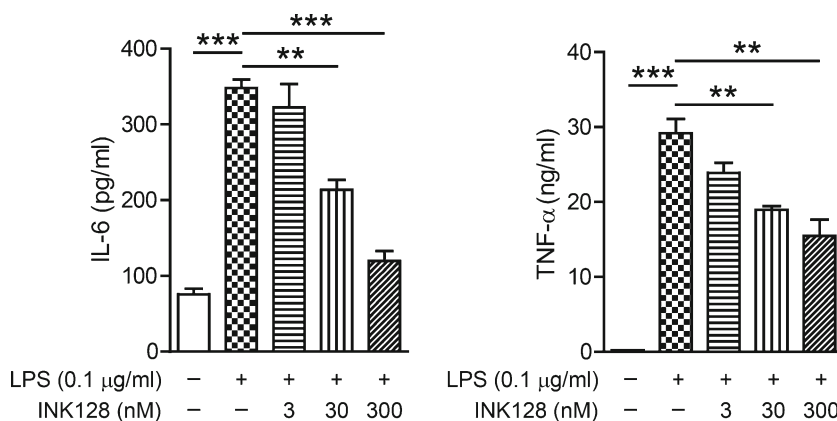

Fig. 4. Differential effects of INK128 on inflammatory cytokine levels in the culture medium of RAW 264.7 cells stimulated with LPS. Cells were pretreated with INK128 for 1 h followed by exposure to LPS (0.1 μg/ml) for 6 h. The levels of inflammatory cytokines in the culture medium were determined by CBA assays. Data are shown as mean$\pm$SD ($n=3$). *$P<0.05$, **$P<0.01$, ***$P<0.001$.
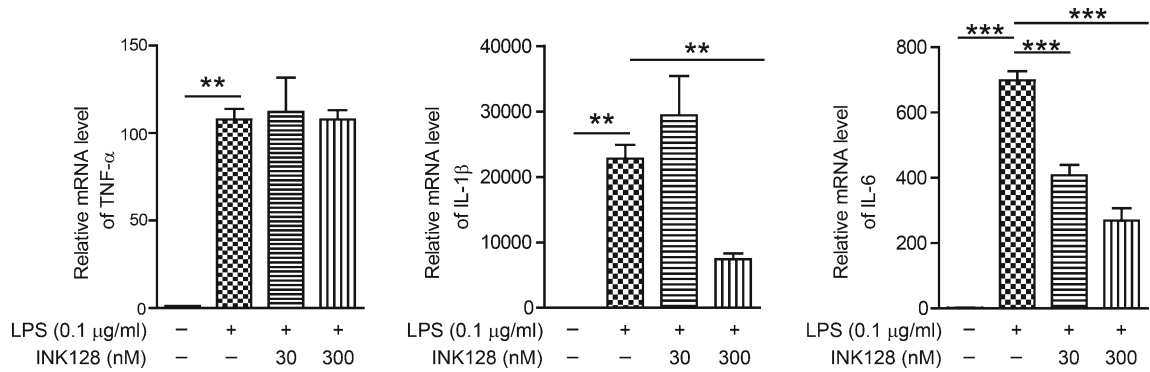
Fig. 5. INK128 differentially regulated LPS-induced inflammatory cytokine mRNA expression in RAW 264.7 cells. Cells were pretreated with INK128 for 1 h and then stimulated with LPS (0.1 μg/ml) for 3 h. Total RNA was extracted and the inflammatory cytokine mRNA levels were evaluated by quantitative real-time RT-PCR (qPCR). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the infected tissues [32]. During this process, the critical metabolic regulator mTOR is also activated by the pathogen recognition receptor triggering [33]. In this study, we utilized LPS-stimulated murine RAW 264.7 macrophages as a model to explore the effects of mTOR inhibitor INK128 on the inflammatory response. Our data demonstrated that INK128 inhibited LPS-induced mTOR signaling, the production of IL-6 and pro-IL-1β at both mRNA and protein

levels, and the production of TNF-α at the protein level but not at its mRNA level.

Consistent with previously published reports [23, 34–36], we demonstrated that both rapamycin and INK128 inhibited the phosphorylation of p70S6K, but the two mTOR inhibitors showed differential activities on 4E-BP1 and AKT$_{Ser473}$ phosphorylation; rapamycin increased the phosphorylation of 4E-BP1 but had little effect on the
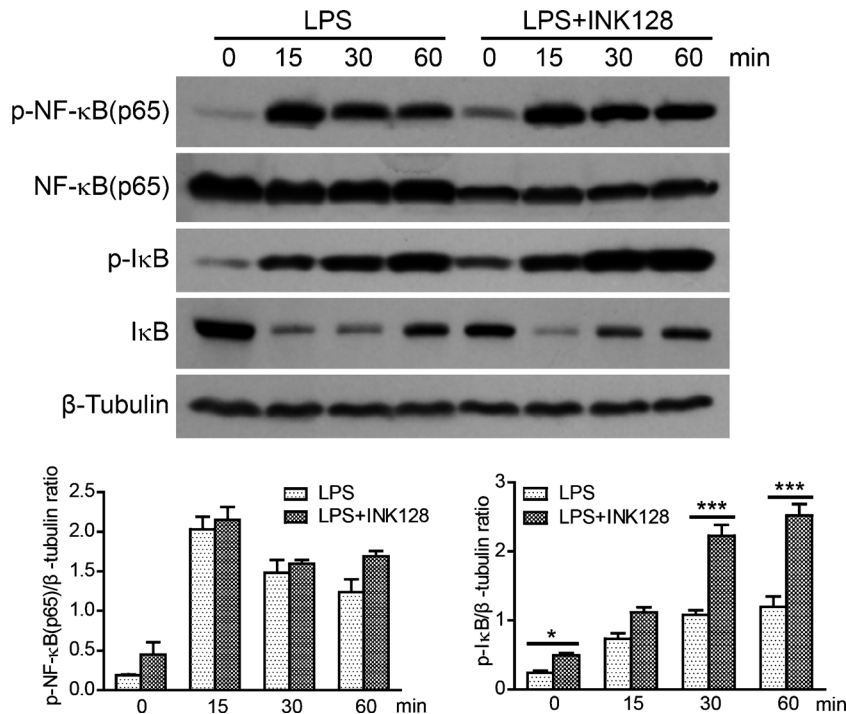

Fig. 6. INK128 regulated LPS-induced NF-κB signaling in RAW 264.7 cells. Cells were pretreated with 30 nM INK128 for 1 h and then treated with 0.1 μg/ml LPS for indicated time lengths. Whole-cell lysates were analyzed by immunoblotting with the indicated antibodies. Quantitative analysis Western blot is shown as the relative ratio of densities of p-NF-κB to β-tubulin, and p-IκB to β-tubulin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

phosphorylation of AKT_{Ser473}, whereas INK128 suppressed both 4E-BP1 and AKT_{Ser473} phosphorylation indicating a more robust inhibition of both mTORC1 and mTORC2 activity. Emerging evidence has indicated that the mTOR pathway regulates not only the adaptive immunity but also the innate immunity [37–39]. Some of these studies have indicated that both mTOR and NF-κB signaling contribute to macrophage responses to LPS stimulation [40]. These pathways may have a cross-talk in regulating the expression of inflammatory cytokines. It has been reported that TSC-mTOR signaling negatively regulates inflammatory response by inhibiting the NF-κB pathway and pro-inflammatory cytokine production [6]. In addition, mTOR suppresses caspase-1-mediated IL-1β production [41]. Therefore, the application of mTOR inhibitors to LPS-stimulated macrophages is supposed to increase the activation of NF-κB and promote the production of inflammatory cytokines. Indeed, inhibition of mTOR activity by rapamycin increases the expression of TNF-α in LPS-stimulated peritoneal macrophages [21] and augments LPS-induced lung injury and apoptosis [13]. Consistent with these studies, INK128 treatment could slightly enhance LPS-induced NF-κB signaling. However, we observed that INK128 suppressed the protein production of TNF-α in LPS-stimulated RAW 264.7 cells, without influencing its mRNA levels. Moreover, INK128 inhibited the pro-IL-1β and IL-6 expression at both mRNA and protein levels. Thus, INK128-induced differential regulation of the cytokine expression in response to LPS stimulation may involve the protein translational regulation pathways and other signaling mechanism(s) than NF-κB pathway.

A major function of the mTOR pathway is to regulate protein translation with the underlying mechanism becoming clear recently. For example, 4E-BP1 inhibits the translation of mTOR-responsive mRNAs by binding with cap-binding eIF4E, a rate-limiting translation initiation factor. Once 4E-BP1 is phosphorylated by mTORC1, it dissociates from eIF4E, allowing it to bind mTOR-regulating mRNAs. Several recent studies have indicated that the mTOR-regulating motifs of those mRNAs exist in their 5′-untranslational regions (5′-UTR). Most of these 5′-UTRs have a 5′-terminal oligopyrimidine tract (5′-TOP) [42] or/and a 5′-pyrimidine-rich translational element (5′-PRTE) [43]. It seems that there is a PRTE-like motif in the 5′-UTRs of TNF-α mRNAs (GeneBank accession no. NM_013693.2). This might explain why the protein levels of TNF-α were significantly suppressed by INK128 in LPS-stimulated RAW 264.7 cells even though its mRNA levels were not influenced.

In addition to regulating protein expression, mTOR has also been reported to regulate gene expression at transcriptional levels [44]. For example, *cyclin D1* and *c-myc*, two cell cycle-related genes, were negatively regulated by AKT activity; rapamycin suppressed *cyclin D1* and *c-myc* expression at both translational and transcriptional levels when intracellular activity of AKT was high, but increased their translation when AKT activity was low [45, 46]. Yet, further research is warranted to explore the mechanism underlying INK128-induced reduction of the inflammatory cytokine expression at translational and/or transcriptional levels in LPS-stimulated macrophages.

In summary, INK128, a second-generation mTOR inhibitor, differentially regulated the expression of LPS-induced pro-inflammatory cytokines. Although more research works are needed to reveal the details of a cross-talk between the mTOR and NF-κB pathways, this study implicates that INK128 has potential clinical application as an anti-inflammatory agent.

ACKNOWLEDGMENTS

Conflict of Interest. The authors declare no conflicts of interest.

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