A Tubulin Inhibitor, N-(5-Benzyl-1,3-thiazol-2-yl)-3-(furan-2-yl)prop-2-enamide, Induces Anti-inflammatory Innate Immune Responses to Attenuate LPS-mediated Septic Shock

Hyun Jung Park,†,‡* Sung Won Lee,†,‡* Hwangseo Park,† Se-Ho Park,‡* and Seokmann Hong,†‡*

†Department of Bioscience and Biotechnology, Institute of Bioscience, Sejong University, Seoul 143-747, Korea
‡Department of Life Sciences, Korea University, Seoul 136-701, Korea. *E-mail: shong@sejong.ac.kr

The anti-inflammatory effect of a tubulin inhibitor, N-(5-benzyl-1,3-thiazol-2-yl)-3-(furan-2-yl)prop-2-enamide (1), on innate immune responses remains unclear. Thus, we investigated the effect of 1 on the immune responses mediated by lipopolysaccharide (LPS). The in vitro addition of 1 to dendritic cells and macrophages dose-dependently reduced tumor necrosis factor alpha production elicited by LPS stimulation. Additionally, the stimulation of natural killer (NK) and natural killer T (NKT) cells with 1 resulted in the decrease of interferon gamma (IFNγ) induced by LPS treatment. Moreover, 1 substantially reduced interleukin 12 in dendritic cells (DC) as well as IFNγ in NKDCs induced by LPS in vitro. Furthermore, the in vivo administration of 1 ameliorated LPS/D-galactosamine-induced endotoxic lethality in mice. Taken together, our results demonstrate for the first time that 1 possesses anti-inflammatory properties, most notably by modulating LPS-induced innate immune responses. Therefore, 1 might have therapeutic potential for the treatment of inflammation-mediated diseases such as sepsis.

Key Words: Tubulin inhibitor, N-(5-Benzyl-1,3-thiazol-2-yl)-3-(furan-2-yl)prop-2-enamide, Anticancer, Anti-inflammatory, Sepsis

Introduction

The innate immune system plays an important role in the first line of defense against foreign pathogens, such as Gram negative bacteria. Toll-like receptors (TLR), a type of pattern recognition receptor expressed on innate immune cells, such as dendritic cells (DCs), recognize extracellular or endosomal pathogen-associated molecular patterns (PAMPs) that are conserved in pathogens. The recognition of lipopolysaccharide (LPS) by its receptor TLR4 initiates pro-inflammatory innate immune pathways that link to adaptive immune responses. Exposure to LPS has stimulatory effects on a variety of innate immune cells. For example, LPS stimulates macrophages and DCs to produce pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin 12 (IL12). LPS can also activate natural killer (NK) and natural killer T (NKT) cells to secrete IFNγ. Recently, a novel DC subset, natural killer dendritic cell (NKDC), has been identified that has hybrid characteristics between DCs and NK cells; NKDCs express both NK1.1, an NK cell surface marker, and CD11c, a DC-specific surface marker molecule. We previously showed that NKDCs are a potent producer of IFNγ in response to LPS, while NK1.1 conventional DCs are a main producer of IL12 upon stimulation with LPS.

Sepsis caused by bacterial infection accompanies systemic inflammation. Septic patients could die by rapidly exacerbated inflammatory responses within a few days. Large amounts of pro-inflammatory cytokines, such as TNFα and IFNγ, produced by these innate immune cells upon bacterial infection or LPS treatment are key factors in the pathogenesis and lethality of endotoxic shock.

Anti-proliferation agents commonly used in cancer chemotherapeutics can be classified into several groups based on their acting mechanisms. Some reagents inhibit the proliferation of cancer cells by acting on microtubule formation. For example, colchicine and vinblastine inhibit the polymerization of tubulin. Furthermore, paclitaxel (so-called Taxol) inhibits the depolymerization of polymerized tubulin. Moreover, nocaspine induces spindle multipolarity via centrosome amplification and declustering. These agents possess properties such as anti-inflammatory activities in addition to anti-proliferative function. Colchicine and vinblastine have been shown to down-regulate TNFα secretion in macrophages. In particular, colchicine and its analogs inhibit LPS-induced GM-CSF expression in murine macrophages, the neutrophil adhesion molecules, and carrageenan-induced footpad edema in rats. Paclitaxel reduces the production of pro-inflammatory cytokines, such as IL12 and TNFα, in DCs but not endotoxin-induced inflammation. Moreover, nocaspine analogs inhibit the production of cytokines, such as TNFα, IL8, and nitric oxide, and chemokines in macrophage cell lines upon stimulation with various TLR and non–TLR ligands.
Some years ago, Mikhail et al. showed that the derivatives of 2-amino-5-arylmethyl-1,3-thiazole, including N-(5-benzyl-1,3-thiazol-2-yl)-3-(furan-2-yl)prop-2-enamide (1, Figure 1), had significant anti-proliferative activity against a human prostate carcinoma cell line.23 Because many anti-proliferation agents may possess anti-inflammatory activities as aforementioned, here we decided to examine the anti-inflammatory effect of 1 on LPS-induced inflammatory immune responses. We evaluated the in vitro effects of 1 on the LPS-induced production of pro-inflammatory cytokines from innate immune cells, including DCs, macrophages and NK cells. Furthermore, we confirmed that 1 had a suppressive effect on an in vivo model of murine septic shock using LPS/D-galactosamine. Our results demonstrate that 1 exerts anti-inflammatory capacities via the down-regulation of pro-inflammatory cytokine production from innate immune cells.

Experimental Methods

Mice and Reagents. C57BL/6 (B6) mice were purchased from Jung Ang Lab Animal Inc. (Seoul, Korea). Mice were maintained at Sejong University and were used at 6-12 weeks of age. The animal experiments were approved by the Institutional Animal Care and Use Committee at Sejong University (SJ-20100401009). 1 was purchased from InterBioScreen Ltd. (http://www.ibscreen.com). LPS derived from E. coli (serotype 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS was used at a final concentration of 1 μg/mL.

Cell Isolation by Magnetic Activated Cell Sorting (MACS) and Culture. A single-cell suspension of splenocytes was prepared and resuspended in RPMI complete medium consisting of RPMI 1640 (Gibco BRL, USA) media supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 units/mL penicillin-streptomycin, and 5 mM 2-mercaptoethanol. CD11c+ splenic DCs were enriched using CD11c MACS beads (Miltenyi Biotec, Germany), and the DC population was > 97% after MACS.

Flow Cytometry. Cells were stained with fluorescence-conjugated monoclonal antibodies (mAbs) and washed with FACS buffer (PBS containing 1% FBS). The following mAbs from BD Biosciences (San Jose, USA) were used: fluorescence-conjugated anti-iCD11b (clone M1/70), anti-iCD11c (clone HL3), anti-iCD3e (clone 145-2C11), anti-iF4/80 (clone BM8), anti-iNK1.1 (clone PK-136), anti-iIL12p40 (clone C15.6), anti-iTNFα (clone MP6-XT22), and IgG1, κ as the isotype control (clone R3-34). Fluorescence-conjugated anti-iIFNγ (clone XMG1.2) was purchased from eBioscience (San Diego, CA, USA). All flow cytometric data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Intracellular Cytokine Staining. Splenocytes or purified CD11c+ cells were stimulated with LPS (1 μg/mL), 1 (50 μM, 100 μM) or media for 16 h in vitro. To prevent cytokine secretion, brefeldin A (10 μg/mL) was added for the last 2 h of incubation. The cells were stained for cell surface markers, fixed with 4% paraformaldehyde, washed once with cold FACS buffer, and permeabilized with 0.5% saponin. The cells were then incubated with anti-iIL12p40, anti-iIFNγ, anti-iTNFα, or the appropriate isotype control for an additional 30 min at 4 °C.

Cytokine Assays. The quantity of TNFα and IFNγ in the culture supernatant was determined using a sandwich ELISA according to the manufacturer’s instructions (BD PharMingen, USA). The optical density was measured using an ImmunoReader (Bio-Tek ELX-800, USA).

Induction of Septic Shock. For the induction of septic shock, mice were i.p. injected with LPS (2 μg/mouse)/2-amino-2-deoxy-D-galactose (D-GalN) (25 mg/mouse) plus vehicle or 1. All animals were continuously monitored for LPS-induced lethality for 24 h after LPS challenge.

Statistical Analysis. Statistical significance was determined using Excel (Microsoft, USA). To compare two groups, Student’s t-test was performed. *P < 0.05, **P < 0.01, ***P < 0.001 was considered significant.

Results and Discussion

Suppression of TNFα Production in LPS-activated DCs and Macrophages. Because many anti-mitotic or anti-proliferation agents are known to exert anti-inflammatory effects, as reported previously, we investigated whether the anti-proliferative activity of 1 could inhibit LPS-mediated innate immune activation in vitro. Total splenocytes were treated with LPS and 1 for 16 h, and the amount of TNFα in the culture supernatant was measured by ELISA. Treatment with 1 suppressed LPS-induced TNFα production in a dose-dependent manner in total splenocytes (Figure 2(a)). Because macrophages and DCs are the major source of TNFα in total splenocytes in response to LPS treatment, we examined whether stimulation with 1 could restrain LPS-induced TNFα production in macrophages and DCs. Treatment with 1 repressed TNFα production of macrophages and DCs in a dose-dependent manner in total splenocytes (Figure 2(b) and 2(c)). Our results clearly show that 1 could cause a dramatic reduction of LPS-induced TNFα production in the spleen through modulating macrophages and DCs. Because LPS induced the depolymerization of microtubules quite quickly in primary rat cells24 and the modification of microtubule networks directly affected LPS-triggered inflammatory response in macrophages,25 it is of note that some modification of microtubules in macrophages and DCs by 1 might affect the level of TNFα production in response to LPS stimulation.

Suppression of IFNγ Production in LPS-activated NK and NKT Cells. Because the cytokine IFNγ has been known to be another potent mediator of LPS-induced acute inflam-
matory responses, we investigated whether \( I \) could inhibit the induction of IFN\( \gamma \) production elicited by LPS. Total splenocytes were treated with LPS and \( I \), and 16 h later, the amount of IFN\( \gamma \) in the culture supernatant was measured by ELISA. Treatment with \( I \) suppressed LPS-induced IFN\( \gamma \) production in a dose-dependent manner in total splenocytes (Figure 3(a)). Because NK and NKT cells are the cellular source of IFN\( \gamma \) following LPS stimulation, we investigated whether \( I \) stimulation could restrain LPS-induced IFN\( \gamma \) production in NK and NKT cells. Treatment with \( I \) repressed IFN\( \gamma \) production by NK and NKT cells in a dose-dependent manner (Figure 3(b) and 3(c)). Thus, our results show that \( I \) inhibits IFN\( \gamma \) production from NK and NKT cells after LPS treatment, which has implications for its potential therapeutic ability against systemic inflammatory diseases, such as sepsis.

**Inhibition of the Production of IFN\( \gamma \) from NK1.1\(^+\) DCs Following LPS Stimulation.** Among heterogeneous DC populations, NKDCs have been reported as a DC subset with cytotoxic functions. These cells are known to play an important role in initiating early inflammatory immune responses due to their rapid production of IFN\( \gamma \) upon stimulation. Thus, we decided to examine whether \( I \) has an inhibitory influence on the cytokine production of NKDCs. For this purpose, total CD11c\(^-\) DCs isolated using MACS were stimulated with LPS and at the same time were treated with \( I \) at different concentrations. While LPS-stimulated NK1.1\(^-\) conventional DCs showed significantly reduced levels of IL12p40 secretion in a concentration-dependent manner when treated with \( I \) (Figure 4(a)), \( I \) dramatically reduced IFN\( \gamma \) production from NK1.1\(^-\) DCs following LPS stimulation (Figure 4(b)). Our results clearly demonstrated that \( I \) modulates the secretion of pro-inflammatory cytokines in NK1.1\(^-\) NKDCs as well as NK1.1\(^-\) conventional DCs following LPS treatment. Considering our recent report that potential crosstalk occurs between NKDCs and NKT cells to enhance adaptive immune responses,\(^{26}\) we speculate that the reduction of IFN\( \gamma \) signaling by \( I \) in LPS-stimulated NKDCs might contribute to the decreased activation of NKT cells, ultimately resulting in the suppression of cytokine

![Figure 2. Suppression of TNF\( \alpha \) production in LPS-activated DCs and macrophages by \( I \). Splenocytes were isolated from B6 mice and cultured in the absence or in the presence of LPS (1 \( \mu \)g/mL)/\( I \) (0, 50, or 100 \( \mu \)M) for 16 h. (a) TNF\( \alpha \) concentrations were determined by ELISA from supernatants harvested 16 h after stimulation. The mean value \( \pm \) SD is shown (n = 3, *\( P < 0.05 \), **\( P < 0.01 \)). (b) Intracellular TNF\( \alpha \) production was analyzed in DCs (CD11c\(^-\)) and macrophages (M\( \phi \), CD11c\(^-\)CD11b\(^+\)F4/80\(^+\)). Representative results from three independent experiments are shown. (c) The graph represents mean percentage \( \pm \) SD for the proportion of TNF\( \alpha \)^\( +\) in DCs and macrophages (n = 3, *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \)).
production.

Amelioration of LPS/D-GalN-induced Endotoxic Shock in Mice by the in vivo Administration of 1. LPS can cause septic shock through excessive inflammatory immune responses. Because our in vitro experiments clearly demonstrated that 1 has suppressive effects on pro-inflammatory cytokine production by various innate immune cells, we investigated whether its anti-inflammatory effects can be confirmed in an in vivo experimental setting, such as an LPS/D-GalN-induced septic shock mouse model. We monitored acute death rates caused by LPS/D-GalN-induced sepsis in either the presence or absence of 1. The survival rates in the LPS/D-GalN-only control group dramatically dropped to 14.2% by 24 h post-LPS injection, whereas 57.1% of the experimental group treated with 1 were still alive 24 h after LPS injection (Figure 5). These findings indicate that 1 played a protective role in LPS-induced septic shock.

Because both TNFα from macrophages and DCs and IFNγ from NK1.1+ cells are central to lethality in experimental endotoxic shock, a higher survival rate from LPS/D-GalN-induced sepsis following the injection of 1 may be correlated with the reduced production of TNFα and IFNγ by innate immune cells. Thus, the effective blocking by 1 of acute inflammation during sepsis may be attributed to inhibiting inflammatory immune responses elicited by a complex interaction of innate immune cells. In conclusion, 1 could be useful as an anti-inflammatory agent to protect the pathogenesis of LPS-induced septic shock.

Conclusions

Our findings suggest a novel mechanism by which 1 suppresses the innate immune responses mediated by LPS stimulation. Upon subsequent exposure to LPS, the treatment of 1 reduced the production of TNFα in macrophages and DCs in vitro. In addition, the frequency of IFNγ production by NK and NKT cells was also reduced by the treatment of 1 after LPS stimulation in vitro. Moreover, mice treated with 1 were resistant to LPS/D-GalN-induced septic shock...
The combined evidence of immunological studies indicates that 1 can orchestrate the activated inflammatory response mediated by a complex network of various innate immune cells. Taken together, our results can provide a new therapeutic strategy to rescue patients with severe sepsis from endotoxic lethality. Further studies should uncover the complete action mechanism and signaling pathway of 1 to innate immune cells.

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References