http://dx.doi.org/10.5012/bkcs.2014.35.11.3307

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

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Received June 26, 2014, Accepted July 31, 2014

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.[1,2] The recognition of lipopolysaccharide (LPS) by its receptor TLR4 initiates pro-inflammatory innate immune pathways that link to adaptive immune responses.[2] 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).[3,4] LPS can also activate natural killer (NK) and natural killer T (NKT) cells to secrete IFNγ.[5] 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.[6] 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.[7]

Sepsis caused by bacterial infection accompanies systemic inflammation. Septic patients could die by rapidly exacerbated inflammatory responses within a few days.[8,9] 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.[9-11]

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.[12,13] Furthermore, paclitaxel (so-called Taxol) inhibits the depolymerization of polymerized tubulin.[14] Moreover, noscapine induces spindle multipolarity *via* centrosome amplification and declustering.[15] 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.[16] 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.[17-19] Paclitaxel reduces the production of pro-inflammatory cytokines, such as IL12 and TNFα, in DCs[20] but not endotoxin-induced inflammation.[21] Moreover, noscapine 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.[22]

[a]These authors contributed equally to this work.

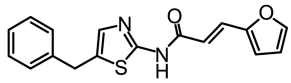

Figure 1. Chemical structure of *N*-(5-benzyl-1,3-thiazol-2-yl)-3-(furan-2-yl)prop-2-enamide.

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 Inter-BioScreen 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-CD11b (clone M1/70), anti-CD11c (clone HL3), anti-CD3e (clone 145-2C11), anti-F4/80 (clone BM8), anti-NK1.1 (clone PK-136), anti-IL12p40 (clone C15.6), anti-TNFα (clone MP6-XT22), and IgG1, κ as the isotype control (clone R3-34). Fluorescence-conjugated anti-IFNγ (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-IL12p40, anti-IFNγ, anti-TNFα, 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 cells[24] 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-

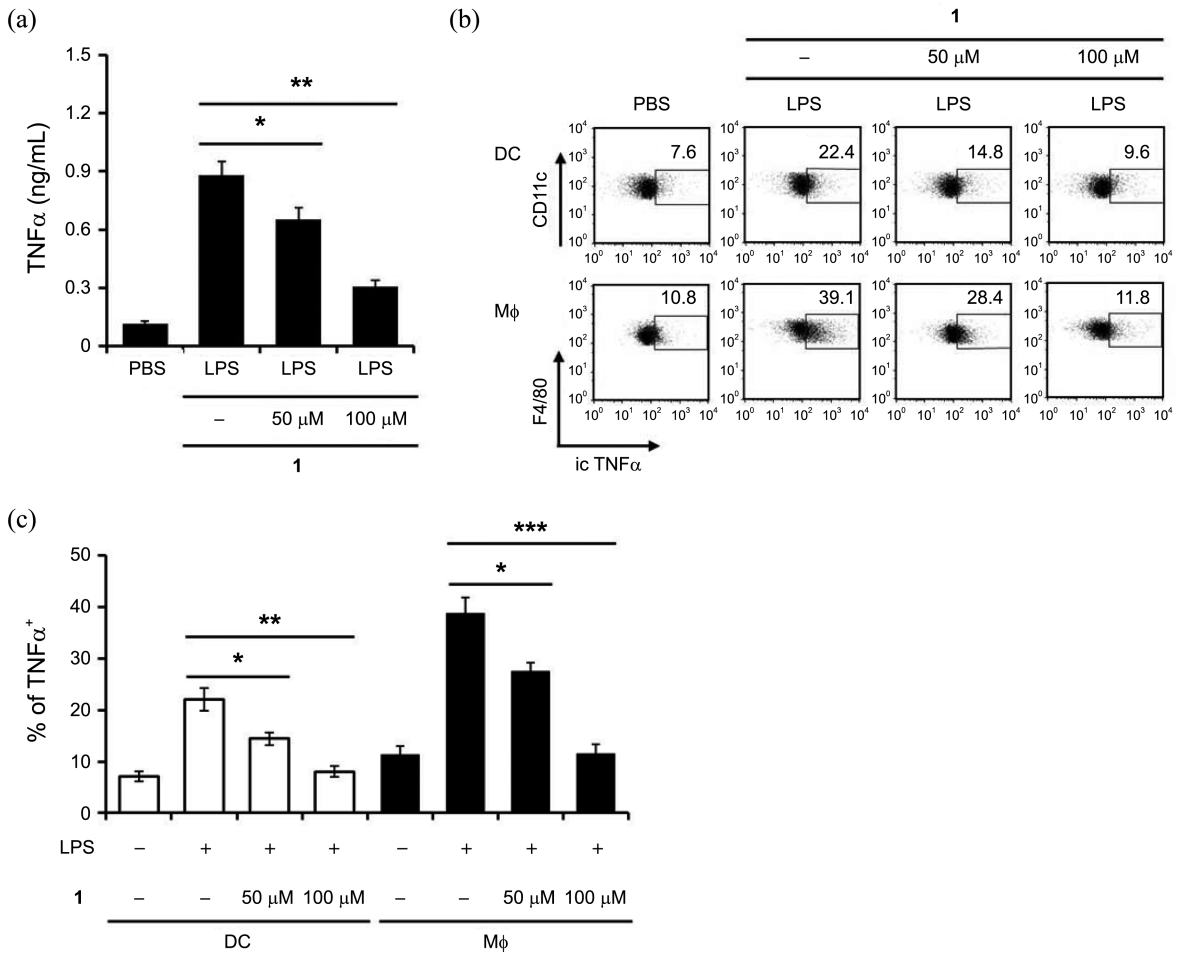

Figure 2. Suppression of TNFα production in LPS-activated DCs and macrophages by **1**. Splenocytes were isolated from B6 mice and cultured in the absence or in the presence of LPS (1 μg/mL)/**1** (0, 50, or 100 μM) for 16 h. (a) TNFα concentrations were determined by ELISA from supernatants harvested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

matory responses, we investigated whether **1** could inhibit the induction of IFNγ production elicited by LPS. Total splenocytes were treated with LPS and **1**, and 16 h later, the amount of IFNγ in the culture supernatant was measured by ELISA. Treatment with **1** suppressed LPS-induced IFNγ production in a dose-dependent manner in total splenocytes (Figure 3(a)). Because NK and NKT cells are the cellular source of IFNγ following LPS stimulation, we investigated whether **1** stimulation could restrain LPS-induced IFNγ production in NK and NKT cells. Treatment with **1** repressed IFNγ production by NK and NKT cells in a dose-dependent manner (Figure 3(b) and 3(c)). Thus, our results show that **1** inhibits IFNγ 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 IFNγ 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 IFNγ upon stimulation. Thus, we decided to examine whether **1** 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 **1** 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 **1** (Figure 4(a)), **1** dramatically reduced IFNγ production from $NK1.1^+$ DCs following LPS stimulation (Figure 4(b)). Our results clearly demonstrated that **1** 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 IFNγ signaling by **1** in LPS-stimulated NKDCs might contribute to the decreased activation of NKT cells, ultimately resulting in the suppression of cytokine

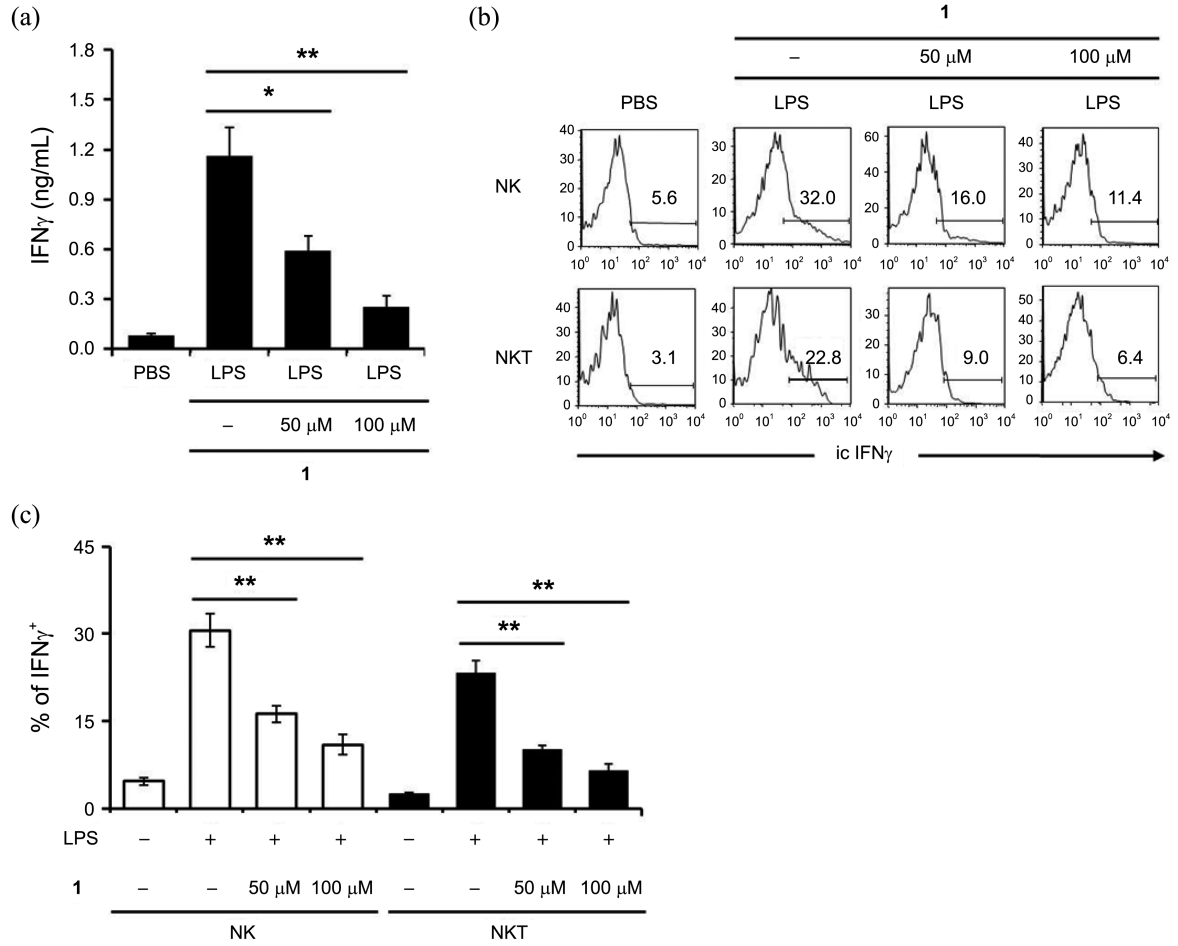

Figure 3. Suppression of IFNγ production in LPS-activated NK and NKT cells by **1**. Splenocytes were isolated from B6 mice and cultured in the absence or in the presence of LPS (1 μg/mL)/**1** (0, 50, or 100 μM) for 16 h. (a) IFNγ concentrations were determined by ELISA from supernatants harvested at 16 h after stimulation. The mean value ± SD is shown (n = 3, *P < 0.05, **P < 0.01). (b) Intracellular IFNγ production was analyzed in NK cells ($NK1.1^+CD3^-$) and NKT cells ($NK1.1^+CD3^+$). Representative results from three independent experiments are shown. (c) The graph represents mean percentage ± SD for the proportion of $IFN\gamma^+$ in NK and NKT cells (n = 3, **P < 0.01).

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 septic shock 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,[8,10,11] 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

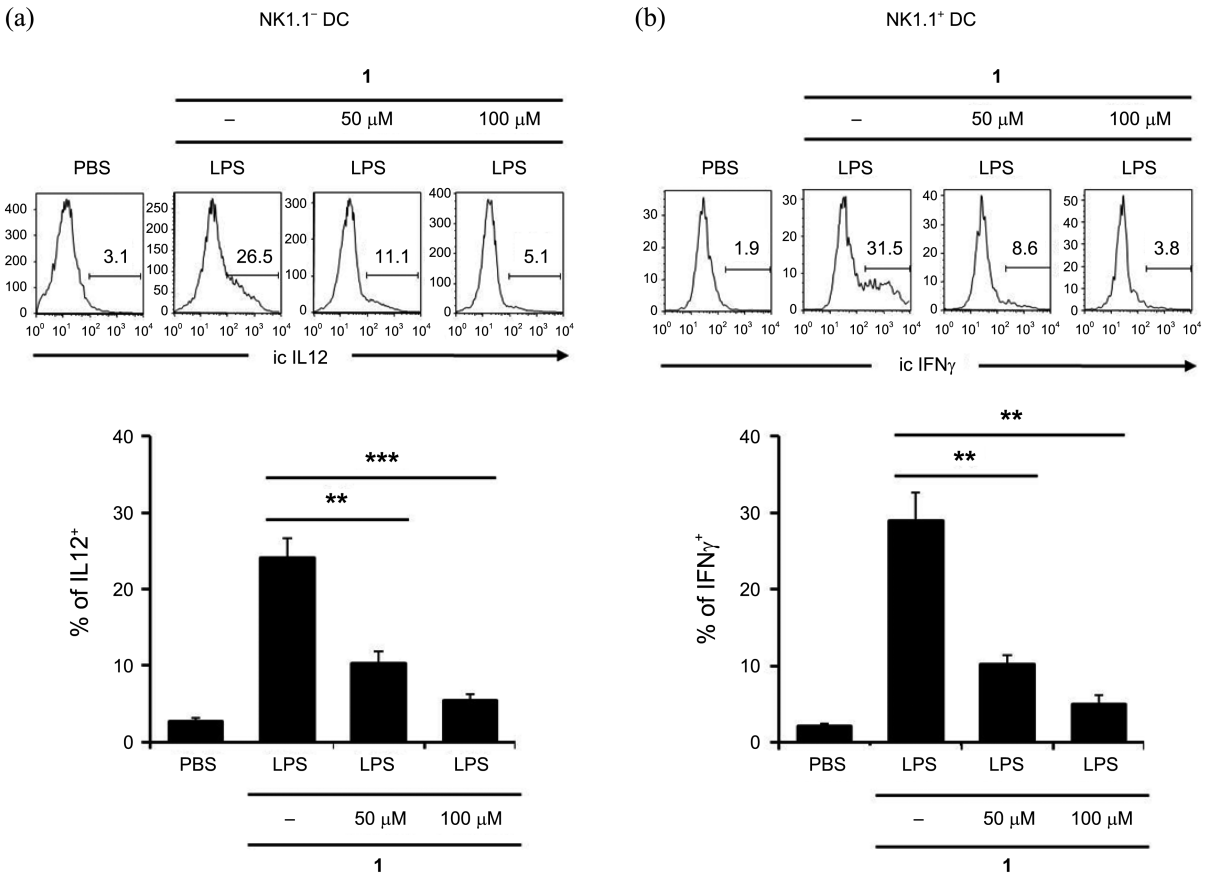

Figure 4. Suppression of the production of IL12 on NK1.1$^-$ DC and IFNγ on NK1.1$^+$ DC by LPS stimulation by **1**. CD11c$^+$ splenic DCs from B6 mice were enriched and cultured in the absence or in the presence of LPS (1 μg/mL)/**1** (0, 50, or 100 μM) for 16 h. (a) Intracellular IL12 production on NK1.1$^-$ DC (NK1.1$^-$CD11c$^+$) was evaluated by flow cytometry. The graphs in the lower panel represents mean percentage ± SD for the proportion of IL12$^+$ (n = 3, **P < 0.01 ***P < 0.001). (b) Intracellular IFNγ production on NK1.1$^+$ DC (NK1.1$^+$CD11c$^+$) was evaluated by flow cytometry. The graph in the lower panel represents mean percentage ± SD for the proportion of IFNγ (n = 3, **P < 0.01).

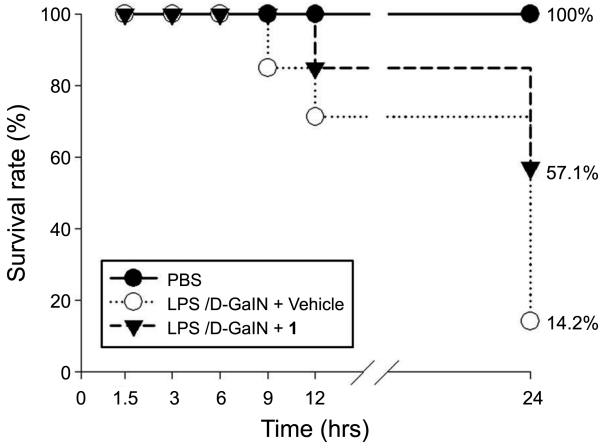

Figure 5. Improvement of survival after LPS/D-GalN-induced endotoxic shock by **1**. B6 mice were injected intraperitoneally without (n = 4) or with LPS (2 μg/mouse)/D-GalN (25 μg/mouse) plus vehicle (n = 7) or **1** (5 mg/kg) (n = 7). Survival of these mice was monitored until 24 h after LPS challenge.

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.

Acknowledgments. This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (#2010-0023683).

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