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Report

Antibiotic Vancomycin Promotes the Gene Expression of NOD-Like Receptor Families in Macrophages

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Vancomycin (VCM), a glycopeptide antibiotic, is commonly applied to infectious diseases caused by Gram-positive bacteria, in particular including methicillin-resistant *Staphylococcus aureus* (MRSA). However, VCM treatment sometimes causes adverse side effects, such as nephropathy and deafness. Although both side effects have been implicated in inflammatory processes, the underlying mechanisms remain unknown. Here, we investigate the cellular responses induced by VCM, especially focusing on the inflammatory responses, and found that VCM promotes the gene expression of NOD-like receptor (NLR) and absent in melanoma 2 (AIM2)-like receptor (ALR) families that mediate release of pro-inflammatory cytokines, interleukin-1β (IL-1β) and IL-18, by forming the multiprotein complexes, called inflammasomes. Thus, our findings suggest that VCM promotes IL-1β- and IL-18-mediated inflammation through the upregulation of the components of inflammasomes, which provides insight into the VCM-induced inflammation-related side effects.

Key words vancomycin, NOD-like receptor, interleukin-1β, inflammation

INTRODUCTION

Vancomycin (VCM) is one of the glycopeptide antibiotics, originally isolated from *Streptomyces orientalis*.[1] VCM inhibits cell wall synthesis of gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA).[2-4] On the other hand, VCM has been reported to cause adverse side effects, including nephropathy, deafness, hypotension, pancytopenia, skin rash, and red man syndrome.[5] In particular, nephropathy occurs in 5-25% of the patients treated with VCM, and the auditory toxicity is dose-dependent and can be exacerbated by the combination treatment with aminoglycosides.[6-8] However, the underlying mechanisms of these adverse side effects remain to be elucidated.

The nuclear factor-κB (NF-κB) and the mitogen-activated protein kinase (MAPK) signaling pathways play pivotal roles in mediating inflammatory responses.[9,10] A wide variety of pro-inflammatory mediators, including tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β), and toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS), strongly activate these pathways, and then induce the inflammatory responses.[11-13] In addition, interferon (IFN) signaling pathways also mediate the inflammatory responses by promoting expression of IFN-responsive genes.[14,15]

The inflammasomes are cytosolic multiprotein complexes consisting of the sensor proteins, such as NOD-like receptor (NLR) or absent in melanoma 2 (AIM2)-like receptor (ALR) family proteins, the adaptor protein ASC, and the downstream effector caspase-1.[16] The inflammasome formation can be triggered by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), leading to the auto-cleavage and subsequent activation of caspase-1. Finally, activated caspase-1 induces the processing and secretion of pro-inflammatory cytokines, IL-1β and IL-18.[17] On the other hand, excessive activation of the inflammasomes has been associated with a wide variety of diseases, including chronic inflammation, autoimmune diseases, metabolic diseases, and neurodegenerative diseases.[18-20] Thus, the inflammasomes are potential drug targets for these diseases.

In this study, we found that VCM upregulates the gene expression of NLRs and AIM2 in macrophages. Interestingly, the VCM-induced upregulation of these genes is likely mediated by alternative pathways rather than major inflammatory signaling pathways, such as NF-κB, MAPK, and IFN signaling pathways.

MATERIALS AND METHODS

Cell Culture and Reagents HEK293 TLR4/MD-2/CD14-stable cells purchased from InvivoGen were cultured in DMEM containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin solution. RAW264.7 and THP-1 cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin solution. All cells were cultured at 37 °C in 5% CO_2 atmosphere. For experiments, THP-1 cells differentiated for 24 h with 100 nM PMA on the day before stimulation. VCM was purchased from Wako. LPS was purchased from InvivoGen.

Immunoblot Cells were lysed with the 1% Triton X-100 buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Tri-

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ton-X100, 10% Glycerol, and 1% protease inhibitor cocktails (Nacalai)]. After centrifugation, the cell extracts were resolved by SDS-PAGE and analyzed as described previously.[21] The antibodies used were against phospho-p65, phospho-IκBα, phospho-p38, phospho-JNK, phospho-ERK (Cell Signaling Technology), and β-actin (Wako). The blots were developed with ECL (Merck Millipore).

Luciferase Assay The reporter assays were performed essentially as described.[22] Cells were transfected with NF-κB-luc, IFN regulatory factor 3 (IRF3)-luc or IFNβ-luc using Polyethylenimine "Max" (Cosmo Bio) according to the manufacturer's instructions. After 24 h, cells were treated with VCM for 12 h or LPS for 6 h and then assayed their luciferase activities using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer´s instructions.

Quantitative Real-Time PCR Total RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque) and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Template cDNA was amplified by quantitative real-time PCR using KAPA SYBR FAST qPCR Kits (KAPA Biosystems) according to the manufacturer's instructions. Primers used for qRT-PCR were listed on Table 1. Each gene expression levels were normalized to that of *β-actin* or *GAPDH*.

RESULTS AND DISCUSSION

To investigate mechanisms by which VCM promotes inflammation, we examined VCM-induced cellular responses, in particular focusing on major pro-inflammatory signaling pathways, such as NF-κB, MAPK, and IFN signaling pathways. As shown in Fig. 1A, VCM failed to enhance phosphorylation levels of p65 and IκBα that are typical indicators of the activation of NF-κB signaling pathways, whereas LPS, a representative PAMP derived from Gram-negative bacteria, clearly enhanced these pathways. In addition, VCM also failed to activate the MAPKs, such as p38 MAPK, c-Jun N-terminal protein kinase (JNK), and extracellular signal-regulated kinase (ERK) (Fig. 1B). Moreover, luciferase assays revealed that VCM does not upregulate the gene promoter activity of not only NF-κB but also IRF3 and IFNβ (Fig. 1C). Consistent with these observations, VCM did not affect gene expression of IL-1β and TNF-α, whose induction is highly dependent on the NF-κB and MAPK signaling pathways, whereas LPS clearly upregulated the expression of these genes (Fig. 2). Collectively, these observations suggest that VCM does not affect the activation of these signaling pathways.

A previous report has demonstrated that VCM promotes IL-1β release in macrophages.[23] We thus speculated that VCM promotes IL-1β release by stimulating other mechanisms rather than the major pro-inflammatory signaling pathways, and then examined whether VCM affects the expression levels of NLR and ALR family genes that are critical components for IL-1β release. The listed genes in Fig. 3, except AIM2, belong to the NLR family. Interestingly, VCM significantly increased the mRNA levels of *NLRP1*, *NLRC4*, and *AIM2* in human monocytic THP-1 cells, suggesting that VCM has an ability to promote IL-1β release through the upregulation of the NLR and ALR family genes (Fig. 3). In particular, it has been reported that NLRP1, NLRC4, and AIM2 mediate IL-1β release induced by a component of bacterial peptidoglycan muramyl dipeptide (MDP), bacterial flagellin, and virus DNA, respectively, raising the possibility that VCM potentiates pathogen-induced IL-1β release.[24-27] Moreover, we found that VCM increased the mRNA levels of *NLRP7* and *NLRP12* (Fig. 3). Although the functions of NLRP7 and NLRP12 are poorly characterized, previous studies have demonstrated that both are involved in the inflammatory responses.[28-30] In particular, both NLRs promote IL-1β release by recognizing specific pathogens, suggesting that VCM-mediated upregulation of NLRP7 and NLRP12 might also potentiate pathogen-induced IL-1β release.[31-33] Taken together, our results provide evidence that VCM has an ability to upregulate various inflammasome components, which proba-

Table 1. Primers for Quantitative Real-Time PCR

gene	Forward	Reverse
IL-1β	5'-GTCCTGCGTGTTGAAAGATGATAAG-3'	5'-TTCTGCTTGAGAGGTGCTGATG-3'
TNF-α	5'-CGAGTGACAAGCCTGTAGCC-3'	5' -TTGAAGAGGACCTGGGAGTAGATG-3'
NLRP1	5'-AAGACCAGCTGTTCTCGGAGTT-3'	5'-AGGCATGAGATCTCCTGGTTTC-3'
NLRP2	5'-TGAGGAAACCACTGTGCAACTT-3'	5'-AACTGAACGGAGGGATGGAA-3'
NLRP3	5'-GAAGAAAGATTACCGTAAGAAGTACAGAAA-3'	5'-CGTTTGTTGAGGCTCACACTCT-3'
NLRP4	5'-AACTACCCAGCAGGCAACGT-3'	5'-AATCAATGGGTGAGAGGTGACAA-3'
NLRP5	5'-CTGGACACGGCTGGCTGTGG-3'	5'-TGCCGGTTGCAGGAAAGGGC-3'
NLRP6	5'-GACCCTCAGTCTGGCCTCTGT-3'	5'-TCCGGCTTTGCTCTCTTCAC-3'
NLRP7	5'-CTTCTGTGCGGATTCTTTGTGA-3'	5'-TTTTTAATCTCCACTTTCTGCAGATG-3'
NLRP8	5'-AGGCACCCTCAGTGCAAACT-3'	5'-CCCGTCAAAACACCGATTAAG-3'
NLRP9	5'-CGCATGTGTGTGGAGAATATCTTT-3'	5'-CCCGCCAGTAGACGAGCTT-3'
NLRP10	5'-CAAGGGCTTGAAGGTCATGAA-3'	5'-CGCACATGCTCTCGGTATACTT-3'
NLRP11	5'-CGCACACTCAAGTTGTCCTATGTC-3'	5'-ACGAGCCAAAGCCTTGAGTAAG-3'
NLRP12	5'-CCAGAAACTGTGGCTGGATAGC-3'	5'-GCGTTGTTGGTCAGGTAAAGG-3'
NLRP13	5'-CTCTGAAACCACATCGTGCATT-3'	5'-GCAAGCAGTTGTCAGATTGCAT-3'
NLRP14	5'-TCAGAGGCTCGGGTTGGA-3'	5'-TGCAGATAAGAGCAGAGGAGAGATC-3'
NLRC4	5'-TAGCCGAGCCCTTATTCAAA-3'	5'-ACCTTCTCGCAGCAAATGAT-3'
AIM2	5'-ATGTGAAGCCGTCCAGA-3'	5'-CATCATTTCTGATGGCTGCA-3'
β-actin	5'-GCCAACACAGTGCTGTCTG-3'	5'-CCTGCTTGCTGATCCACATC-3'
GAPDH	5'-AACAGCCTCAAGATCATCAGC-3'	5'-GGATGATGTTCTGGAGAGCC-3'

The mRNA expression of *NLRP4, NLRP5, NLRP8, NLRP9, NLRP10, NLRP13* and *NLRP14* was not detected by these primers in PMA-differentiated THP-1 cells.

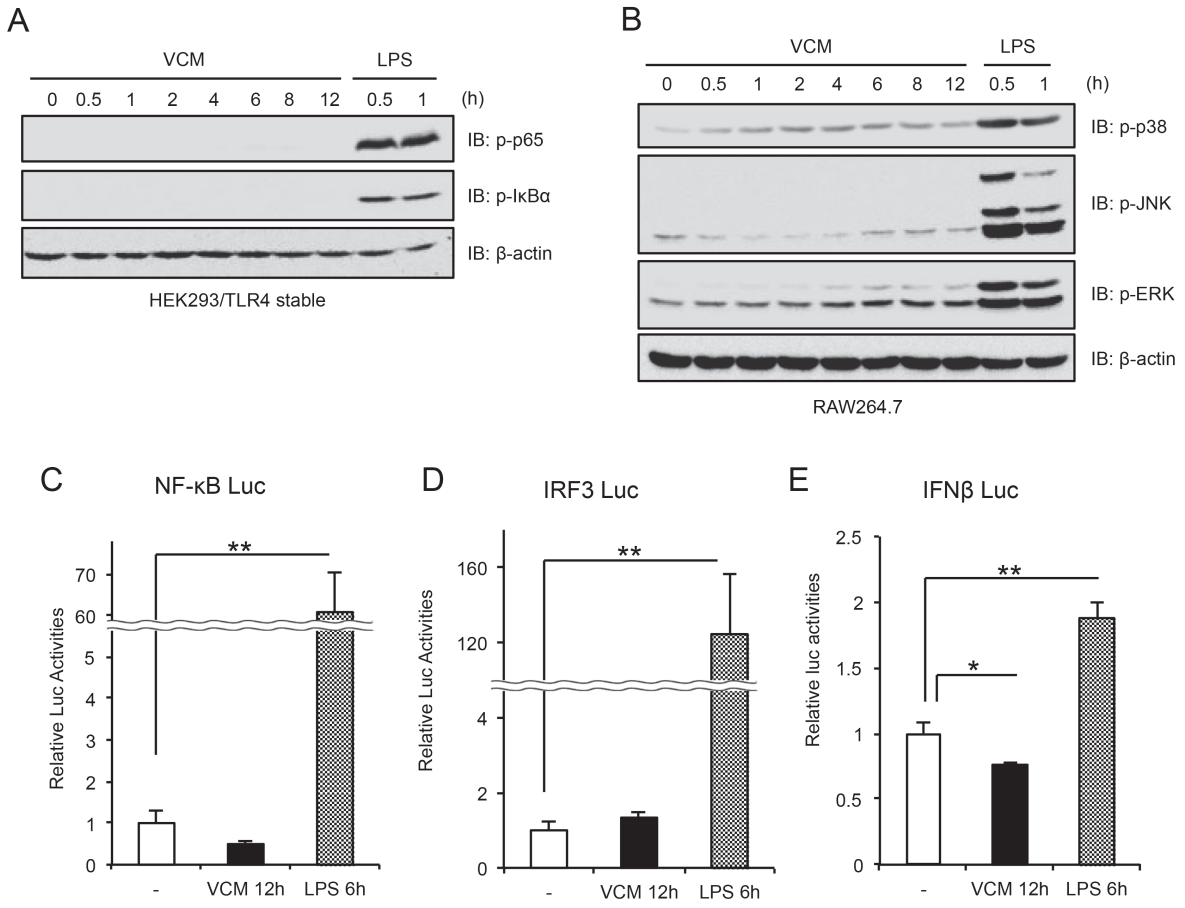

Fig. 1. Effects of VCM on the Inflammatory Signaling Pathways

(A)(B) TLR4-stable HEK293 cells (A) or RAW264.7 cells (B) were treated with VCM (1 mg/mL) or LPS (100 ng/mL) for the indicated periods. Total cell lysates were then analyzed by western blot. (C)(D)(E) TLR4-stable HEK293 cells were transfected with NF-κB-, IRF3- or IFNβ-luciferase reporter vector. After 24 h, cells were treated with VCM (1 mg/mL) for 12 h or LPS (100 ng/mL) for 6 h, and then their luciferase activities were analyzed. Graphs are shown as mean ± S.D. (n=3). Statistical significance was determined by student's t-test. **p < 0.01.

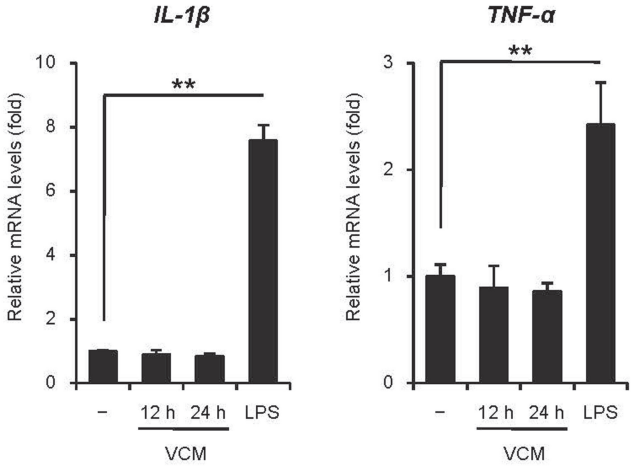

Fig. 2. Effects of VCM on the Gene Expression of IL-1β and TNF-α

RAW264.7 cells were treated with VCM (1 mg/mL) for the indicated periods or LPS (100 ng/mL) for 6 h. The mRNA levels of the indicated genes were then analyzed by quantitative real-time PCR (normalized with *β-actin* mRNA levels). Graphs are shown as mean ± S.D. (n=3). Statistical significance was determined by student's t-test. **$p < 0.01$.

bly enhances the pathogen-induced IL-1β release, and may help to explain why VCM causes the inflammatory side effects.

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Author Contributions Author contributions: Y.K., C.I., T.N., and A.M. conceived and designed research; Y.K., C.I., K.M., A.N., and T.N. performed research; Y.H., T.N., and A.M. analyzed data; T.N. and A.M. wrote the paper.

Conflict of Interest The authors declare no conflict of interest.

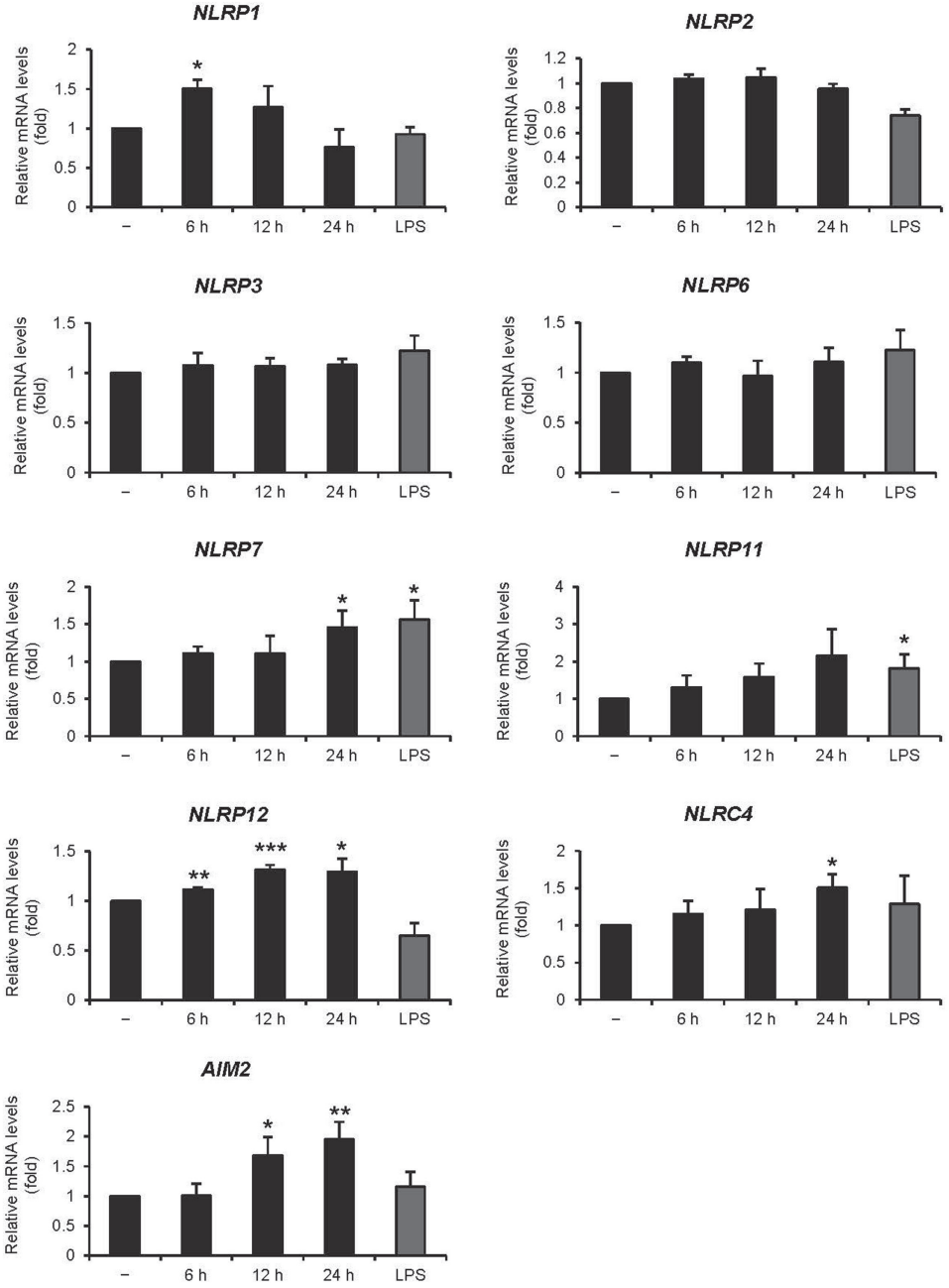

Fig. 3. Effects of VCM on the Gene Expression of NLRs and AIM2

THP-1 cells were treated with VCM (1 mg/mL) for the indicated periods or LPS (100 ng/mL) for 6 h. The mRNA levels of the indicated genes were then analyzed by quantitative real-time PCR (normalized with *GAPDH* mRNA levels). Graphs are shown as mean ± S.D. (n=3). Statistical significance was determined by student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (vs control).

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