이학박사 학위논문

Regulation of osteoclastogenesis and immune response to staphylococcal infection by cyclic dinucleotides

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Regulation of osteoclastogenesis and immune response to staphylococcal infection by cyclic dinucleotides

by

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Under the supervision of

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Department of Dental Science, School of Dentistry

The Graduate School, Seoul National University

Regulation of osteoclastogenesis and immune response to staphylococcal infection by cyclic dinucleotides

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이 논문을 이학박사 학위논문으로 제출함

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ABSTRACT

Regulation of osteoclastogenesis and immune response to staphylococcal infection by cyclic dinucleotides

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The Graduate School, Seoul National University

(Supervised by Professor Seung Hyun Han, Ph. D.)

Introduction

Microbe-associated molecular patterns (MAMPs), which are involved in host-microbe interaction, are recognized by its specific pattern recognition receptors, modulating host immune responses. Among MAMPs, cyclic dinucleotides (CDNs) are second messengers, which are widely expressed in bacteria, acting a critical role in bacterial growth, colonization, and biofilm formation. CDNs are recognized by stimulator of interferon (IFN) genes (STING) present in cytosol to promote host immune responses. However, a little is known about the effects of CDNs on

regulation of innate immunity by macrophages during osteoclast differentiation or bacterial infection. In the present study, the roles of CDNs on regulation of osteoclastogenesis and immune responses against staphylococcal infection in macrophages were investigated using *in vivo* and *in vitro* models.

Methods

Bone marrow-derived macrophages (BMMs) were stimulated with receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) in the presence or absence of CDN. Osteoclast differentiation was determined by tartrate-resistant acid phosphatase (TRAP) staining or RT-PCR. BMMs were transfected with STING targeting siRNA or non-targeting siRNA and then transfected BMMs were treated with CDN in the presence of RANKL. The cells were stained for TRAP. BMMs, prepared from wild-type or IFN-α/β receptor $(IFNAR1)^{-/-}$ mice, were treated with CDN in the presence of RANKL. Osteoclastogenesis or activation of IFNAR1 signaling pathway was determined by TRAP staining or Western blotting, respectively. A collagen sheet soaked with CDN in the absence or presence of RANKL was implanted in mouse calvaria. Resorptive area on the calvaria were analyzed by micro-CT scanning. In a separate experiment, mice were administrated with *Staphylococcus aureus* USA300 with or without CDN via intraperitoneal injection. Survival was monitored daily for 96 hours. In the same condition, serum was collected and cytokine production in serum was determined by Mouse Proteome Profiler Array kit and ELISA. BMMs were stimulated with CDN in the presence of heat-killed *S. aureus* (HKSa) wild-type or lipoprotein-deficient strain (Δ*lgt*), and IL-6 secretion in the cell culture supernatant was determined by ESLIA. In addition, IL-6 production by BMMs in response to

lipoprotein isolated from *S. aureus* or Pam2CSK4, a synthetic mimicking bacterial lipoprotein, with CDN was determined by ELISA. BMMs, pretreated with STING targeting siRNA or STING inhibitor, were incubated in the combination of CDN and Pam2CSK4 and then IL-6 production was determined by ELISA. Phosphorylation of transcriptional factors was determined by Western blotting or confocal laser microscope analysis. Pam2CSK4 and/or CDN with IL-6 neutralizing antibody were intraperitoneally administered to mice and survival rate was monitored for 96 hours.

Results

CDN inhibited RANKL-induced osteoclastogenesis from BMMs in a dose-dependent manner. In addition, CDN attenuated c-Fos and NFATc1, which are major transcriptional factors, induced by RANKL. CDN also inhibited osteoclast differentiation in osteoclast/osteoblast co-culture systems. Western blotting determined that CDN triggered STING signaling pathway during RANKL-induced osteoclast differentiation from BMMs. Indeed, the inhibitory effect of CDN on osteoclastogenesis was not observed in BMMs transfected with STING targeting siRNA, indicating that STING is essential for the inhibition of osteoclastogenesis by CDN. Moreover, CDNs induced the mRNA expression of IFN-β, which is a negative regulator of osteoclastogenesis. The inhibition of osteoclast differentiation was not observed in the presence of IFNAR1 neutralizing antibody or in macrophages derived from IFNAR1$^{-/-}$ mice. Furthermore, CDN ameliorated RANKL-induced calvarial bone resorption through osteoclastogenesis inhibition. In a separate experiment, when CDN was administered to C57BL/6 mice in combination with *S. aureus* USA300 by intraperitoneal injection, CDN plus *S. aureus* USA300

synergistically increased serum pro-inflammatory cytokine levels and mortality compared to *S. aureus* USA300 alone. Notably, BMMs treated with CDN and HKSa wild-type, but not Δ*lgt*, synergistically increased the IL-6 production *in vitro*. In addition, CDN together with lipoproteins isolated from *S. aureus* or Pam2CSK4 promoted the synergistic induction of IL-6. Moreover, CDNs potently activated NF-κB signaling pathway, which is critical for the IL-6 expression, in combination with Pam2CSK4. CDN failed to increase the Pam2CSK4-induced IL-6 production in STING targeting siRNA-transfected or STING inhibitor-pretreated BMMs. On the other hand, TBK1 and IRF3, mediators of canonical STING signaling pathway, were dispensable for the synergistic induction of IL-6 together with Pam2CSK4 since CDN synergistically induced the IL-6 even in the presence of TBK1 or IRF3 inhibitor. Administration of CDN with Pam2CSK4 potently increased the serum IL-6 levels and mortality, while survival rate was restored by IL-6 neutralizing antibody.

Conclusion

The present study demonstrates that CDNs regulate immune activity of macrophages to inhibit osteoclast differentiation and bone destruction induced by RANKL, while exacerbate morbidity and mortality due to excessive IL-6 production in bacterial infection.

Keywords: Cyclic dinucleotide; STING; Macrophage; Osteoclast; Infection.

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Abbreviations

BMM	Bone-marrow derived macrophage
c-di-AMP	Cyclic diguanylate monophosphate
c-di-GMP	Cyclic diguanylate monophosphate
CDN	Cyclic dinucleotide
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
ELISA	Enzyme-linked immunosorbent assay
IFN	Interferon
IL-6	Interleukin-6
IRF3	Interferon regulatory factor 3
M-CSF	Macrophage-colony stimulating factor
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-κB
RANKL	Receptor activator of NF-κB ligand
STING	Stimulator of interferon genes
TAK1	Transforming growth factor beta-activated kinase 1
TBK1	TANK-binding kinase 1
TLR	Toll-like receptor
TRAF6	TNF receptor associated factor 6
TRAP	Tartrate-resistant acid phosphatase

Literature review

1. Cyclic dinucleotides (CDNs)

1.1. General characteristics of CDNs

CDNs are nucleotide-based second messengers produced by bacteria. CDNs consist of two nucleotides that form cyclic compounds through cyclization by phosphodiester linkages.[1] CDNs can be classified by their constituent nucleotides as cyclic diadenylate monophosphate (c-di-AMP), cyclic diguanylate monophosphate (c-di-GMP), and cyclic guanosine monophosphate-adenosine monophosphate (Fig. 1*A*-*C*).[2] c-di-AMP is synthesized by diadenylate cyclase (DAC) containing DAC domain from two ATP. In addition, c-di-AMP is degraded by desert hedgehog protein (DHH) domain of phosphodiesterase into pApA (Fig. 2*A*).[3] Likewise, c-di-GMP is converted from two GTP via diguanylate cyclase containing GGDEF domain derived from its conserved sequence motif, Gly-Gly-Asp-Glu-Phe. Indeed, phosphodiesterase which contains Glu-Ala-Leu (EAL) or HD superfamily hydrolase (HD-GYP) domain degrades c-di-GMP into pGpG or two GMP, respectively (Fig. 2*B*).[4] Bacteria modulate the expression of CDNs in response to various environmental condition to maintain their life cycle.[2] CDNs were first discovered in 1987 as regulators of cellulose synthesis in *Acetobacter xylinum*.[5] Since then, accumulating reports have determined that CDNs are widely involved in regulation of bacterial physiology, such as proliferation, biofilm formation, DNA repair, gene expression, and antibiotic resistance.[6-10]

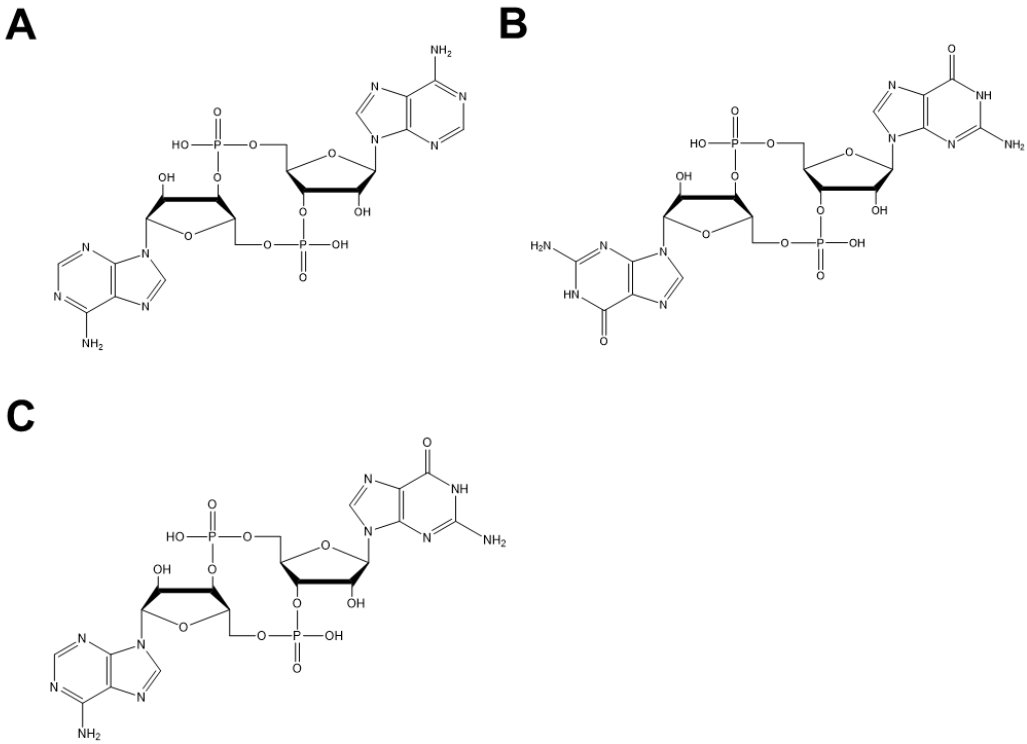

Figure 1. Structure of bacterial CDNs. CDNs consist of two nucleotides that form cyclic compounds through cyclization by 3'-5' linked phosphodiester bonds. CDNs can be distinguished by their constituent nucleotides as (*A*) cyclic diadenylate monophosphate, (*B*) cyclic diguanylate monophosphate, or (*C*) cyclic guanosine monophosphate-adenosine monophosphate.

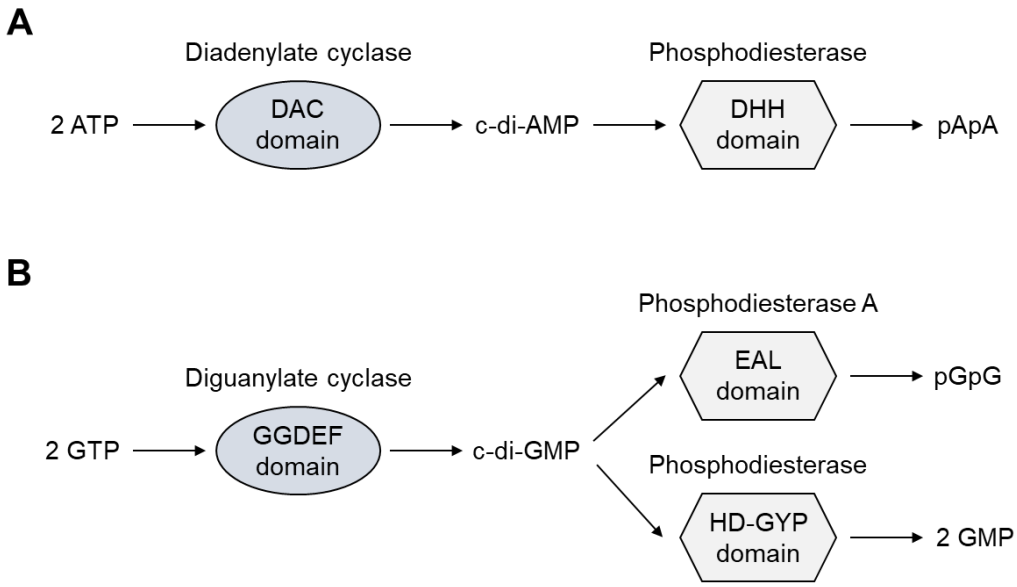

Figure 2. Schematic of CDNs synthesis and degradation. (*A*) c-di-AMP is synthesized by diadenylate cyclase containing DAC domain from two ATP. In addition, c-di-AMP is degraded by desert hedgehog protein (DHH) domain of phosphodiesterase into pApA. (*B*) c-di-GMP is converted from two GTP via diguanylate cyclase containing Gly-Gly-Asp-Glu-Phe (GGDEF) domain. Indeed, phosphodiesterase which contains Glu-Ala-Leu motif (EAL) or HD superfamily hydrolase (HD-GYP) domain degrades c-di-GMP into pGpG or two GMP, respectively.

1.2. Recognition of CDNs by STING

CDNs can regulate not only the bacterial behaviors but also host immune responses. Stimulator of interferon (IFN) genes (STING), also known as TMEM173, is a transmembrane protein which located at the endoplasmic reticulum of host cells.[11] STING includes an N-terminal four transmembrane domain, a CDN binding domain (CBD), and a C-terminal tail (CTT).[11] In fact, emerging evidence indicates that STING directly recognizes the bacterial CDNs to induce host immune responses.[12] Upon engaging CDNs on CBD of STING, STING recruits TANK-binding kinase 1 (TBK1) to the CTT.[13] Recruited TBK1 facilitates phosphorylation of IFN regulatory factor 3 (IRF3), inducing homo-dimerization of IRF3. Phosphorylated IRF3 homodimer translocate to the host nucleus to induce mRNA expression of IFN-β.[13] Collectively, STING has important role in host defense in response to bacteria by recognition of CDNs.

1.3. Immunostimulatory effects of CDNs during infection

Accumulating reports have determined that CDNs released from pathogenic bacteria influence the host immune responses during bacterial infection. For example, intracellular pathogen *Listeria monocytogenes* secrets c-di-AMP to host cytosol, inducing expression of IFN-β through STING signaling pathway in mouse cells.[14] In addition, obligate intracellular pathogen *Chlamydia trachomatis* increases cytosolic c-di-AMP levels and activates STING signaling pathway in mouse lung fibroblasts.[15] Moreover, facultative intracellular bacteria *Brucella abortus* triggers unfolded protein response by using c-di-GMP through STING signaling pathway.[16]

Interestingly, extracellular CDNs also activate the STING signaling pathway as much as intracellular CDNs. For instance, c-di-AMP is spontaneously released from *Staphylococcus aureus* biofilm, which is formed during bacterial infection, and recognized by STING in macrophages to induce type I IFN response.[17] Group B *Streptococcus* expresses ectonucleotidase to degrade own c-di-AMP and mutation in ectonucleotidase increases the secretion of c-di-AMP, resulting in activation of STING and upregulation of host immune responses.[18] In conclusion, CDNs secreted by pathogenic bacteria have immunostimulatory effects on the activation of host immune responses during infection.

1.4. Role of CDNs in regulation of host homeostasis

Numerous commensal microbiota live in human sites, such as skin, oral cavity, lung, gut, etc., and can interact with host through their molecules, such as cell wall components and secreted components.[19] Notably, CDNs, which are consistently released by bacteria, are abundantly existed in the gut.[20,21] Several studies indicate that CDNs released from commensal bacteria might have important roles in regulation of host immune system and tissue homeostasis. For example, microbiota-derived c-di-AMP and c-di-GMP can stimulate STING in murine intestinal cells, balancing the production levels of both pro-inflammatory cytokines and anti-inflammatory cytokines in the gut to maintain gut homeostasis.[22] Moreover, CDNs induce polarization of colonic epithelial cells.[23] Canesso *et al*. indicated that CDNs facilitate to preserve the protective mechanisms of gut, such as proliferation of goblet cells, mucus production, and IgA secretion.[20] In addition, CDNs regulate the

number of immune cells, including intraepithelial lymphocytes, group 1 innate lymphoid cells, and regulatory T cells, in the murine colon, resulting in controlling the gut homeostasis and inflammation.[20] Recently, it was reported that bacterial CDNs may also have important role in modulation of oral inflammation and immune defense.[24] c-di-AMP and c-di-GMP induce the immune response through STING signaling pathway in human dental pulp cells.[25] Further studies are needed to understand the role of CDNs on regulation of oral homeostasis.

1.5. Therapeutic use of CDNs

CDNs are considered as therapeutic agents and vaccine adjuvants for improving host immune defense against pathogenic microbe due to their immunostimulatory effects.[26] Administration of c-di-GMP via intranasal injection protects mice against *Klebsiella pneumoniae* by increasing bacterial clearance through stimulation of dendritic cells.[27] c-di-AMP significantly enhances resistance to tuberculosis caused by *Mycobacterium tuberculosis* infection through induction of autophagy and type I IFN response in macrophages.[28] In addition, a subunit vaccine ESAT-6 formulated with c-di-AMP as an adjuvant provides protective effect in *M. tuberculosis* challenge.[29] Moreover, c-di-GMP enhances potent antigen uptake through induction of pinocytosis *in vivo* and protects mice against *Streptococcus pneumoniae* infection.[30] Furthermore, CDNs show antiviral activity through STING activation in *Drosophila* enterocytes during Sindbis virus infection.[31] Notably, recent study determined that CDNs stimulate the IFN-mediated immune responses, suppressing SARS-CoV-2 replication in human respiratory cells.[32] Collectively, STING-

activating CDNs are promising therapeutic agents for the prevention and treatment against bacterial or viral infections.

2. Aims of the present study

CDNs are known as second messengers of almost all bacteria, playing important roles in regulation of bacterial physiology. In addition, CDNs activate the host immune responses through STING signaling pathway. Thus, CDNs could affect immune responses of macrophages, which is representative innate immune cells and osteoclast precursors. However, a little is known about the effects of CDNs on modulation of innate immune responses by macrophages during osteoclast differentiation or bacterial infection. Therefore, the aims of this study are to investigate (1) the effects of CDNs on osteoclast differentiation from macrophages and (2) the immunostimulatory effects of CDNs on macrophages during staphylococcal infection (Fig. 3).

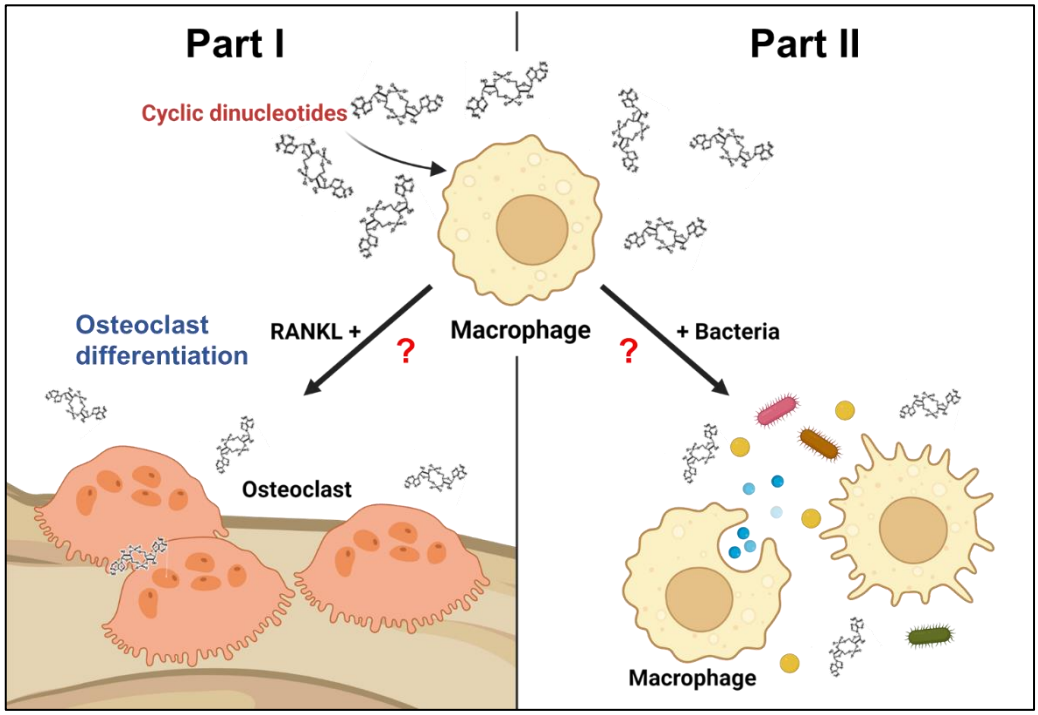

Figure 3. The aims of the present study. The aims of this study are to investigate (1) the effects of CDNs on osteoclast differentiation from macrophages and (2) the immunostimulatory effects of CDNs on macrophages during staphylococcal infection.

PART I

Cyclic dinucleotides inhibit osteoclast differentiation through STING-mediated interferon-β signaling

The contents of part I consist of an article, published in Journal of Bone and Mineral Research [34(7):1366-1375] with a minor modification as a partial fulfillment of Yeongkag Kwon's Ph.D. program

Abstract

Cyclic dinucleotides (CDNs) such as cyclic diadenylate monophosphate and cyclic diguanylate monophosphate are commensal bacteria-derived second messengers in the gut that modulate bacterial survival, colonization, and biofilm formation. Recently, CDNs have been discovered to have an immunomodulatory activity by inducing the expression of type I interferon (IFN) through STING signaling pathway in macrophages. Since CDNs are possibly absorbed and delivered into the bone marrow, where bone-resorbing osteoclasts are derived from monocyte/macrophage lineages, CDNs could affect bone metabolism by regulating osteoclast differentiation. The present study investigated the effect of CDNs on the differentiation and function of osteoclasts and osteoblasts. When bone marrow-derived macrophages (BMMs) were differentiated into osteoclasts with M-CSF and RANKL in the presence of CDNs, the differentiation was inhibited by CDNs in a dose-dependent manner. In contrast, CDNs did not influence the differentiation of committed osteoclasts or osteoblast precursors. STING signaling pathway appeared to be critical for CDNs-mediated inhibition of osteoclast differentiation since CDNs induced the phosphorylation of TBK1 and IRF3, a representative feature of STING activation, and osteoclast differentiation was restored in STING knockdown BMMs with siRNA. Moreover, CDNs increased the mRNA expression of STING-meditated IFN-β, which is a negative regulator of osteoclastogenesis. In addition, CDNs also induced the phosphorylation of STAT1, which mediates IFN-α/β receptor (IFNAR) signal transduction. The inhibitory effects of CDNs on osteoclast differentiation were not observed in the presence of antibody blocking IFNAR or in macrophages derived

from IFNAR1$^{-/-}$ mice. Experiments using a mouse calvarial implantation model showed that RANKL-induced bone resorption was inhibited by CDNs. Taken together, these results suggest that CDNs inhibit osteoclast differentiation and bone resorption through induction of IFN-β via the STING signaling pathway.

KEY WORDS: CYCLIC DINUCLEOTIDE; OSTEOCLAST; BONE RESORPTION

Introduction

Bone homeostasis is maintained through the continuous balance between bone-forming osteoblasts and bone-resorbing osteoclasts.[33] Osteoclasts are multinucleated giant cells derived from the monocyte/macrophage lineage of hematopoietic stem cells.[34] Macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are essentially required for osteoclast differentiation. Indeed, M-CSF provides a signal for survival and proliferation of osteoclast precursors,[35] and RANKL supports osteoclast differentiation and activation.[36] RANKL induces the expression of c-Fos and NFATc1, which are the major transcription factors for the induction of osteoclast-specific genes, such as tartrate-resistant acid phosphatase (TRAP), dendritic cell-specific transmembrane protein (DC-STAMP), and cathepsin K.[37,38] Osteoblasts originate from mesenchymal stem cell lineages[39] and modulate the osteoclast differentiation by regulating the ratio of RANKL and its decoy receptor osteoprotegerin (OPG).[40] Dysregulation of bone-forming osteoblasts and bone-resorbing osteoclasts can lead to various bone diseases such as osteoporosis, periodontitis, and rheumatoid arthritis.[41]

Cyclic dinucleotides (CDNs), including cyclic diadenylate monophosphate (c-di-AMP) and cyclic diguanylate monophosphate (c-di-GMP), are second messengers.[1] Recently, it has been reported that CDNs, secreted by commensal gut microbiota, may play an important role in the maintenance of gut microbial homeostasis and the protection against colitis.[20,22] CDNs regulate the survival, colonization, and biofilm

formation of bacteria.[6,42] Increased expression of CDNs promotes extracellular matrix production and biofilm formation.[43-45] CDNs released from bacterial biofilm can also activate host immune responses.[17] Previous studies showed that CDNs could be used as novel vaccine adjuvants or immunostimulatory molecules for host defense.[46-49] Intranasal administration with c-di-GMP enhances bacteria clearance and protects mice from *Klebsiella pneumoniae* infection through stimulation of dendritic cell-meditated responses.[27] In addition, c-di-AMP increases autophagy in macrophages and attenuates bacterial virulence through the cytoplasmic surveillance pathway during *Mycobacterium tuberculosis* infection.[28]

Stimulator of interferon genes (STING), which is located at the endoplasmic reticulum of the host cells, directly recognizes CDNs or cytosolic DNA, which is critical for production of type I interferons (IFNs).[15,50] Upon activation, STING stimulates TANK-binding kinase 1 (TBK1) to phosphorylate IFN regulatory factor 3 (IRF3), which induces the gene expression of IFN-β.[13] IFN-β binds to the IFN-α/β receptor (IFNAR) to induce various cytokines through the Jak-STAT signaling pathway.[51] IFN-β, produced by the activated STING signaling pathway, provides anti-leukemic effects by inducing dendritic cell maturation and leukemia-specific T cell activation.[52]

It has been reported that microbiota-derived DNAs and CDNs are present at high levels in the gut,[20,21] delivered into the blood of host through extracellular vesicles,[53] and stable in the serum or tissues.[46] Thus, it could be hypothesized that CDNs may also be delivered into the bone marrow and influence bone metabolism.

However, the role of CDNs in bone metabolism has been poorly studied. In this study, the effect of CDNs on the differentiation and function of osteoclasts and osteoblasts was investigated using *in vitro* and *in vivo* experimental models.

Material and methods

Materials

c-di-AMP, c-di-GMP, and Pam2CSK4 were purchased from InvivoGen (San Diego, CA, USA). Recombinant murine RANKL and M-CSF were obtained from Peprotech (Rocky Hill, NJ, USA) and CreaGene (Seongnam, Republic of Korea), respectively. Methylthiazolyldiphenyl-tetrazolium bromide (MTT), β-glycerophosphate, ascorbic acid, 1α,25-dihydroxyvitamin D_3, an alkaline phosphatase (ALP) staining kit, and a TRAP staining kit were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Antibodies specific to phospho-IRF3, phospho-TBK1, phospho-STAT1, STAT1, and STING were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific to c-Fos, NFATc1, IRF3, TBK1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) and goat anti-mouse IgG-HRP were purchased from SouthernBiotech (Birmingham, AL, USA). Anti-mouse IFNAR1 antibody and its isotype control antibody (mouse IgG1, κ) were obtained from BioLegend (San Diego, CA, USA).

Preparation of osteoclasts

Animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-160524-3), Korea. Six-week-old C57BL/6 male mice were obtained from Orient Bio (Seongnam, Republic of Korea). $IFNAR1^{-/-}$ C57BL/6 mice[54] were obtained from the Scripps Research Institute (La Jolla, CA, USA). Bone marrow-derived macrophages (BMMs) and committed

osteoclast precursors were prepared as described previously.[55] Briefly, Bone marrow cells were obtained from mouse femur and tibia. After removal of red blood cells (RBC) using RBC lysis buffer (Sigma-Aldrich Inc.), the cells were incubated in α-MEM medium (Welgene, Daegu, Republic of Korea) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin Solution (HyClone, Logan, UT, USA) in the presence of 5 ng/ml M-CSF for 1 day. Non-adherent cells (stroma-free bone marrow cells) were incubated with 20 ng/ml M-CSF for 4 days to differentiate into BMMs, as osteoclast precursors. The BMMs were then seeded onto a 96-well culture plate at 2.5×10^4 cells/0.3 ml/well and differentiated into mature osteoclasts with 20 ng/ml RANKL and 20 ng/ml M-CSF in the presence or absence of c-di-AMP or c-di-GMP for 3 days. In a parallel experiment, BMMs were seeded onto an OsteoAssay™ Human Bone Plate (Lonza, Allendale, NJ, USA) coated with a thin layer of human bone and differentiated into osteoclasts with c-di-AMP or c-di-GMP in the presence of 20 ng/ml M-CSF and 20 ng/ml RANKL for 5 days. To generate committed osteoclast precursors, the BMMs were stimulated with 20 ng/ml M-CSF and 20 ng/ml RANKL for 2 days. The committed osteoclast precursors were washed with fresh media and differentiated into mature osteoclasts with 20 ng/ml RANKL and 20 ng/ml M-CSF in the presence or absence of c-di-AMP or c-di-GMP for 1 day.

Preparation of osteoblasts

Mouse osteoblast precursors were isolated from 1-day-old C57BL/6 mice calvaria as described previously.[56] The osteoblast precursors were seeded onto a 48-well culture plate at 2×10^4 cells/0.4 ml/well and incubated with 10 mM β-

glycerophosphate and 50 μg/ml ascorbic acid in the presence or absence of c-di-AMP or c-di-GMP for 7 days. For osteoclast/osteoblast co-cultures, the osteoblasts were seeded onto a 48-well culture plate at 2×10^4 cells/0.4 ml/well with BMMs (1×10^5 cells/well) and stimulated with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 100 ng/ml 1α,25-dihydroxyvitamin D_3 in the presence or absence of c-di-AMP or c-di-GMP for 8 days.

TRAP staining

The cells were fixed and stained with a TRAP staining kit. TRAP-positive multinucleated cells (MNCs) with three or more nuclei were counted as mature osteoclasts through microscopic analysis.[57]

***In vitro* bone resorption assay**

Stroma-free bone marrow cells were incubated with 20 ng/ml M-CSF for 4 days to differentiate into BMMs. The BMMs were then seeded onto a 96-well plate coated with calcium phosphate (Corning, Tewksbury, MA, USA) at 2.5×10^4 cells/0.3 ml/well and stimulated with 20 ng/ml RANKL and 20 ng/ml M-CSF in the presence or absence of c-di-AMP or c-di-GMP for 6 days. The cells were lysed with 5% sodium hypochlorite for 10 minutes and then washed with distilled water. After drying, the resorption pits were photographed and the areas were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). In a separate experiment, BMMs were seeded onto a dentin slice with 20 ng/ml M-CSF and 20 ng/ml RANKL to differentiate into mature osteoclasts for 3 days. The cells were

washed with fresh media and stimulated with 20 ng/ml RANKL and 20 ng/ml M-CSF in the presence or absence of c-di-AMP or c-di-GMP for 3 days. The cells were lysed with 5% sodium hypochlorite for 10 minutes and then washed with distilled water. After drying, the resorption pits were photographed by a confocal laser scanning microscope (LSM; LSM 5 Pascal, Carl Zeiss, Oberkochen, Germany) and analyzed with LSM Browser software.

In vivo bone resorption assay

A collagen sheet soaked with 200 nmol of c-di-AMP or c-di-GMP[46] in the presence of 10 μg RANKL was implanted on mouse calvaria for 7 days. The calvarial bone was fixed in 4% paraformaldehyde and then scanned by micro-computed tomography (micro-CT; Skyscan1172 scanner, Skyscan, Kontich, Belgium). Three-dimensional images of the calvarial bones were obtained using CT-volume software (Skyscan) and the resorbed area of the calvarial bone was analyzed using ImageJ software.

Western blotting

BMMs were differentiated into osteoclasts by 20 ng/ml RANKL and 20 ng/ml M-CSF in the presence or absence of c-di-AMP or c-di-GMP. Western blotting was performed as described previously.[58] Briefly, the lysates were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), the membrane was incubated with primary

antibodies specific to various signaling molecules or β-actin at 4°C overnight. The membrane was washed with TBST and incubated with HRP-conjugated secondary antibodies. Immunoreactive bands were detected using the GeneGnome XRQ System (Syngene, Cambridge, UK).

Reverse transcription-polymerase chain reaction (RT-PCR)

BMMs or committed osteoclasts were incubated with 20 ng/ml M-CSF and 20 ng/ml RANKL for 6 or 12 hours in the presence or absence of c-di-AMP or c-di-GMP. Total RNA was extracted and subjected to RT-PCR as described previously.[59] The sequences of each primer are as follows: IFN-β: forward 5'-TCCAAGAAAGGACGAACATTCG-3' and reverse 5'-TGAGGACATCTCCCACGTCAA-3'; and β-actin: forward 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse 5'-CTCCTTAATGTCACGCACGATTTC-3'. In a separate experiment, the mRNA expression of c-Fos, NFATc1, TRAP, DC-STAMP, cathepsin K, and GAPDH in osteoclast was determined by using real-time RT-PCR as described previously.[60] The sequences of each primer are as follows: c-Fos: forward 5'-CCAGTCAAGAGCATCAGCAA-3' and reverse 5'-AAGTAGTGCAGCCCGGAGTA-3'; NFATc1: forward 5'-CCGTCACATTCTGGTCCATAC-3' and reverse 5'-CCAATGAACAGCTGTAGCGTG-3'; TRAP: forward 5'-TGTGAGGGAGGAGGCGTCTGC-3' and reverse 5'-CGTTCCCAAGAAAGCTCTACC-3'; DC-STAMP: forward 5'-TATCTGCTGTATCGGCTCAT-3' and reverse 5'-

AGAATAATACTGAGAGGAACCCA-3'; cathepsin K: forward 5'-GTGTCCATCGATGCAAGCTTGGCA-3' and reverse 5'-GCTCTCTCCCCAGCTGTTTTTAAT-3'; and GAPDH: forward 5'-ACCCAGAAGACTGTGGATGG-3' and reverse 5'-CACATTGGGGGTAGGAACAC-3'.

Transfection with small interfering RNA (siRNA)

BMMs were seeded onto a 60 mm dish at 2.5×10^5 cells/ml in 3.5 ml media. The cells were transfected with 25 nM of ON-TARGETplus siRNA targeting STING or ON-TARGETplus non-targeting control siRNA (Dharmacon, Lafayette, CO, USA) using the DharmaFECT 1 transfection reagent (Thermo Fisher Scientific, Lafayette, CO, USA) according to the manufacturer's instructions. After 24 hours, the cells were transferred to a 96-well culture plate at 2.5×10^4 cells/0.3 ml/well and then differentiated into osteoclasts with 20 ng/ml RANKL and 20 ng/ml M-CSF in the presence or absence of c-di-AMP or c-di-GMP for 3 days. The target sequences of STING siRNA are as follows: 5'-UCAAUCAGCUACAUAACAA-3', 5'-GCAUCAAGAAUCGGGUUUA-3', 5'-AACAUUCGAUUCCGAGAUA-3', and 5'-CCAACAGCGUCUACGAGAU-3'.

Enzyme-linked immunosorbent assay (ELISA)

Mouse osteoblast precursors were seeded onto a 48-well culture plate at 2×10^4 cells/0.4 ml/well with BMMs (1×10^5 cells/well) and stimulated with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 100 ng/ml 1α,25-dihydroxyvitamin

D_3 in the presence or absence of c-di-AMP or c-di-GMP for 2 days. Cell culture supernatants were collected and OPG and RANKL in the cell culture supernatants were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

The results are the mean value ± standard deviation of triplicate samples. Statistical significance was examined using the t test. Asterisks indicate statistical significance at $p < 0.05$ compared with the control group.

Results

CDNs inhibit osteoclast differentiation

Osteoclast differentiation can be separated into three stages of pre-osteoclast, committed osteoclast, and mature osteoclast.[61] First, to investigate the effect of CDNs on osteoclast differentiation, BMMs, as pre-osteoclasts, were differentiated into osteoclasts by M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP for 3 days. After TRAP staining, the osteoclasts were enumerated by counting the number of TRAP-positive MNCs. Both c-di-AMP and c-di-GMP significantly inhibited RANKL-induced osteoclast differentiation in a dose-dependent manner (Fig. 4*A*). In addition, CDNs suppressed the RANKL-induced mRNA expression of c-Fos, NFATc1, TRAP, DC-STAMP, and cathepsin K at the early stage of osteoclast differentiation (Fig. 4*B* and *C*). Notably, CDNs did not affect the viability of BMMs (Fig. 4*D*), indicating that the inhibition of osteoclast differentiation by CDNs was due to down-regulation of osteoclast-specific genes without affecting cell cytotoxicity. To further determine bone-resorptive capacity, BMMs were seeded on calcium phosphate-coated plates and differentiated into osteoclasts with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP. As shown in Fig. 4*E*, resorption pits were formed in the presence of RANKL. In contrast, under the same conditions, resorption pit areas were significantly decreased in the presence of c-di-AMP or c-di-GMP. Moreover, when BMMs were cultured on human bone layer-coated plates with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP, both c-di-AMP and c-di-GMP potently inhibited the osteoclast formation (Fig. 4*F*). However, interestingly, neither c-di-AMP nor c-di-GMP influenced the differentiation of

committed osteoclasts into mature osteoclasts (Fig. 4*G*) and the mRNA expression of osteoclast-specific genes (Fig. 4*H*). In addition, applying c-di-AMP or c-di-GMP to mature osteoclasts on dentin slice did not affect osteoclast activity or bone resorption (Fig. 4*I*), indicating that the inhibitory effect of CDNs on resorptive activity is due to suppression of osteoclast differentiation, but not due to the suppression of osteoclast activity. Therefore, CDNs appeared to inhibit the early stage of osteoclast differentiation from macrophages, but not the late stage of osteoclast differentiation from committed osteoclasts. These results suggest that CDNs effectively inhibit bone-resorptive capacity through down-regulation of osteoclast differentiation at the early phase.

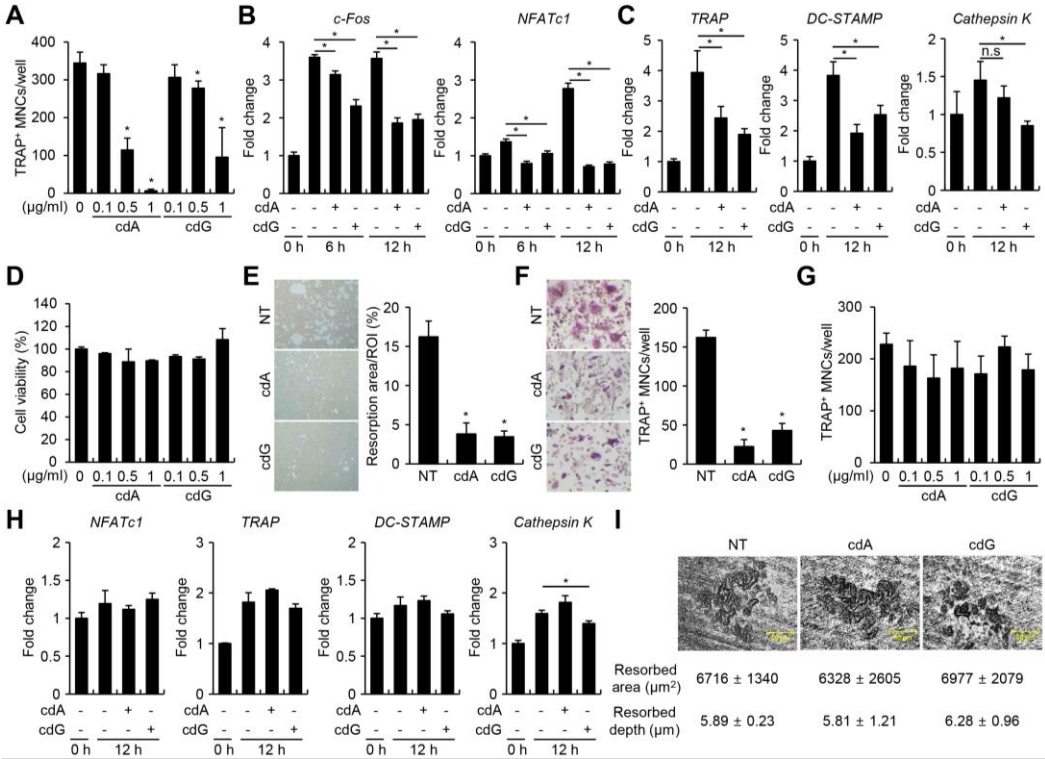

Figure 4. CDNs inhibit RANKL-induced osteoclast differentiation. (*A*) BMMs were differentiated into osteoclasts with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP for 3 days. The cells were fixed and subjected to TRAP staining. The cells were observed under an inverted phase-contrast microscope and TRAP-positive MNCs with three or more nuclei were enumerated through microscopic analysis. (*B*) BMMs were incubated with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP for 6 or 12 hours. The mRNA expression levels of c-Fos, NFATc1, and GAPDH were determined by real-time RT-PCR. (*C*) BMMs were incubated with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP for 12 hours. The mRNA expression levels of TRAP, DC-STAMP, cathepsin K, and GAPDH were determined by real-time RT-PCR. (*D*) BMMs were incubated with c-di-AMP or c-di-GMP for 2 days. Cell viability was determined using MTT assay. (*E*) BMMs seeded on a calcium phosphate-coated plate were differentiated into osteoclasts with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP for 6 days. The resorbed areas were photographed and the total resorption areas were

analyzed using ImageJ software. ROI = region of interest. (*F*) BMMs seeded on a human bone layer-coated plate were incubated with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP for 5 days. The cells were stained for TRAP and TRAP-positive MNCs with three or more nuclei were enumerated under an inverted phase-contrast microscope. (*G*) Committed osteoclasts were treated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for 1 day. The cells were stained for TRAP and TRAP-positive MNCs with three or more nuclei were enumerated under an inverted phase-contrast microscope. (*H*) Committed osteoclasts were incubated with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP for 12 hours. The mRNA expression levels of NFATc1, TRAP, DC-STAMP, cathepsin K, and GAPDH were determined by real-time RT-PCR. (*I*) BMMs on dentin slice were differentiated into osteoclasts with RANKL and M-CSF for 3 days. After observation of osteoclast formation, the cells were treated with c-di-AMP or c-di-GMP in the presence of RANKL and M-CSF for 3 days. The resorbed pits were photographed by a confocal LSM and analyzed with LSM Browser software. $*p < 0.05$. n.s = not significant. cdA = c-di-AMP. cdG = c-di-GMP. One of three similar results is shown.

CDNs inhibit osteoclast differentiation in the osteoclast/osteoblast co-culture system without affecting osteoblasts.

Next, to examine whether CDNs also have an effect on osteoblast differentiation, osteoblast precursors were differentiated with β-glycerophosphate and ascorbic acid in the presence of c-di-AMP or c-di-GMP. After 6 days, osteoblast differentiation was determined by ALP staining. Unlike osteoclast differentiation, osteoblast differentiation was not affected by c-di-AMP or c-di-GMP (Fig. 5*A*). Then, the effect of CDNs on osteoclast differentiation was examined in the osteoclast/osteoblast co-culture system. Both c-di-AMP and c-di-GMP inhibited osteoclast differentiation in the co-culture system (Fig. 5*B*). Under the same conditions, no change in RANKL or OPG was observed in the culture supernatants (Fig. 5*C*). These results indicate that CDNs directly inhibit osteoclast differentiation without affecting osteoblasts.

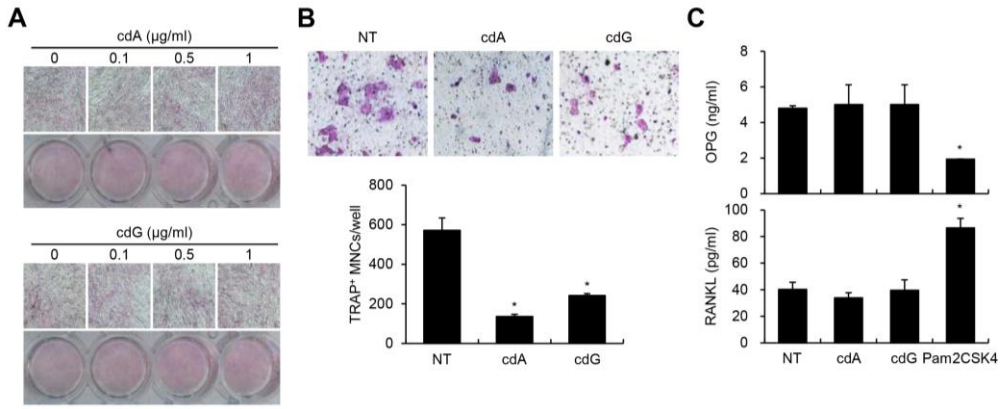

Figure 5. CDNs inhibit osteoclast differentiation in the osteoclast/osteoblast co-culture system. (*A*) Osteoblast precursors from mouse calvaria were differentiated into osteoblasts with ascorbic acid and β-glycerophosphate in the presence of c-di-AMP or c-di-GMP for 7 days. The cells were fixed and subjected to ALP staining. (*B, C*) BMMs were co-cultured with calvarial osteoblasts with c-di-AMP or c-di-GMP in the presence of ascorbic acid, β-glycerophosphate, and 1α,25-dihydroxyvitamin D_3 for 8 days. (*B*) The cells were stained for TRAP and TRAP-positive MNCs with three or more nuclei were enumerated under an inverted phase-contrast microscope. (*C*) The culture supernatants were collected to measure the expression of OPG and RANKL by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. cdG = c-di-GMP. One of three similar results is shown.

STING is required for inhibition of osteoclast differentiation by CDNs.

It is well known that CDNs are directly recognized by the cytosolic sensor STING and activate the TBK1-IRF3 signaling cascade.[62] Therefore, STING expression in BMMs and osteoblasts was determined. As shown in Fig. 6*A*, Western blot analysis demonstrated expression of STING in BMMs, but not (or little if it is) in osteoblasts. Next, to investigate whether CDNs trigger osteoclast differentiation through the STING signaling pathway, the activation of STING was examined using specific antibodies to the phosphorylated form of TBK1 or IRF3. Both c-di-AMP and c-di-GMP induced the phosphorylation of TBK1 and IRF3 in a time-dependent manner, indicating activation of the STING signaling pathway (Fig. 6*B*). Furthermore, to validate whether STING is specifically involved in the inhibition of osteoclast differentiation by CDNs, BMMs were transfected with STING-targeting siRNA and then differentiated into osteoclasts with M-CSF and RANKL in the presence of c-di-AMP or c-di-GMP. The expression of STING was knocked down by STING-targeting siRNA (Fig. 6*C*, upper), where the inhibitory effect of c-di-AMP or c-di-GMP on osteoclast differentiation was diminished (Fig. 6*C*, lower). These results suggest that CDNs inhibit osteoclast differentiation through the STING-dependent signaling pathway.

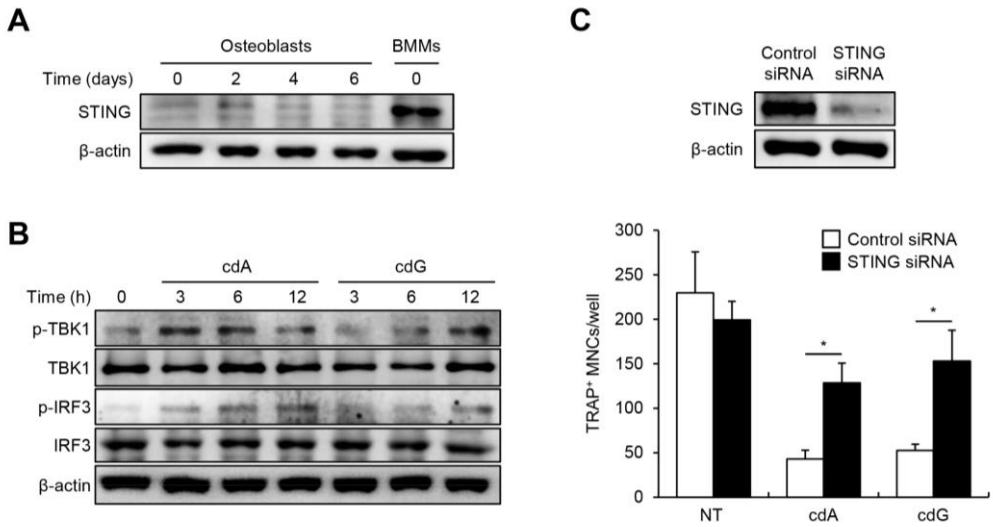

Figure 6. STING is required for inhibition of osteoclast differentiation by CDNs. (*A*) Osteoblast precursors from mouse calvaria were differentiated into osteoblasts with ascorbic acid and β-glycerophosphate for 2, 4, or 6 days. The whole-cell lysates were subjected to Western blotting using specific antibody to STING or β-actin. (*B*) BMMs were treated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for 3, 6, or 12 hours. The whole-cell lysates were subjected to Western blotting using specific antibodies to the phosphorylated or non-phosphorylated form of TBK1, IRF3, or β-actin. (*C*) BMMs were transfected with STING targeting siRNA or non-targeting siRNA for 24 hours. Transfected BMMs were treated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for 3 days. The knockdown of STING expression in whole-cell lysates was confirmed by Western blotting (upper). The cells were stained for TRAP and TRAP-positive MNCs with three or more nuclei were enumerated (lower). $*p < 0.05$. NT = non-treatment. cdA = c-di-AMP. cdG = c-di-GMP. One of three similar results is shown.

IFN-β is essential for inhibition of osteoclast differentiation by CDNs.

CDNs are potent inducers of IFN-β through activation of the STING signaling pathway.[62] Moreover, it has been demonstrated that IFN-β is a negative regulator of osteoclastogenesis.[63] Therefore, it could be hypothesized that IFN-β mediates the inhibition of osteoclast differentiation by CDNs. First, it has been examined whether CDNs modulate the expression of IFN-β during osteoclast differentiation. When BMMs were stimulated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL, both c-di-AMP and c-di-GMP potently induced the mRNA expression of IFN-β at the early stage of osteoclast differentiation (Fig. 7*A*). In addition, both c-di-AMP and c-di-GMP maintained the induction of IFN-β mRNA expression at the late stage of osteoclast differentiation (Fig. 7*B*). When BMMs were differentiated into osteoclasts in the presence of anti-IFNAR1 antibody, the inhibition of osteoclastogenesis by CDNs was restored. However, its isotype control antibody did not affect the inhibition of osteoclastogenesis by CDNs (Fig. 7*C*). To provide further validation, BMMs derived from wild-type or IFNAR1$^{-/-}$ mice were treated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL. As shown in Fig. 7*D*, both c-di-AMP and c-di-GMP failed to inhibit the differentiation of IFNAR1$^{-/-}$ BMMs into osteoclasts. STAT1 is known to mediate signal transduction upon stimulation of IFNAR with type I IFNs.[64] Stimulation with c-di-AMP or c-di-GMP induced the phosphorylation of STAT1, but such effects were not observed in IFNAR1$^{-/-}$ BMMs under the same condition (Fig. 7*E*). In addition, the expression of c-Fos and NFATc1 was inhibited by c-di-AMP or c-di-GMP in RANKL-stimulated wild-type BMMs. In contrast, the expression of c-Fos and NFATc1 was not

suppressed by CDNs in RANKL-stimulated IFNAR1$^{-/-}$ BMMs (Fig. 7*F*). These results suggest that CDN-induced IFN-β is responsible for the down-regulation of osteoclast differentiation through the IFNAR signaling pathway.

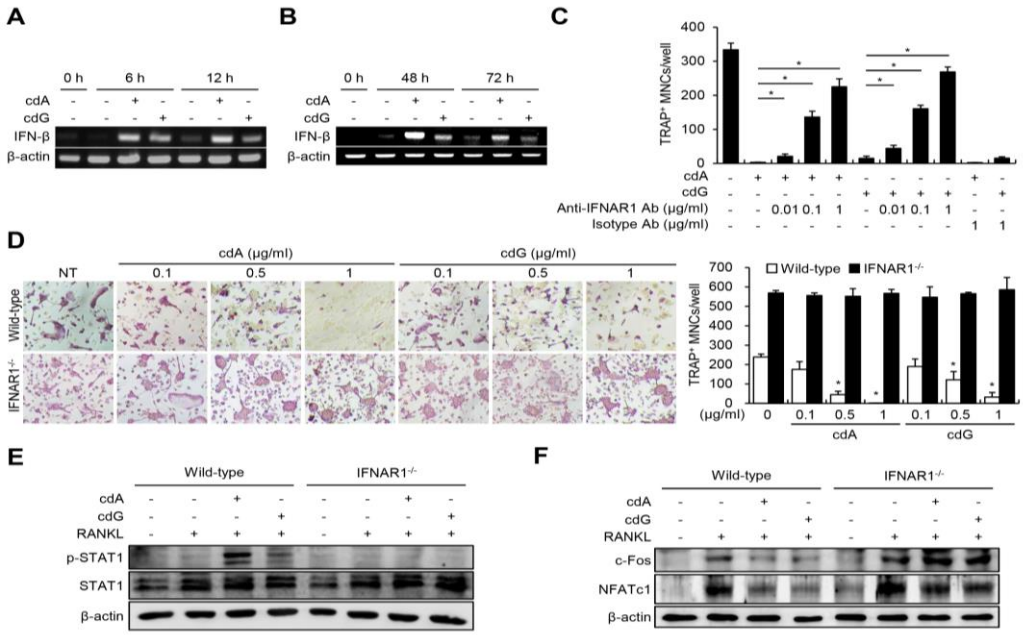

Figure 7. Type I IFN signaling is essential for inhibition of osteoclast differentiation by CDNs. (*A, B*) BMMs were incubated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for (*A*) 6 or 12 hours or (*B*) 48 or 72 hours. The mRNA expression level of IFN-β and β-actin was determined by RT-PCR. (*C*) BMMs were pretreated with anti-IFNAR1 antibody or isotype antibody for 1 hour and then treated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for 3 days. The cells were stained for TRAP and TRAP-positive MNCs with three or more nuclei were enumerated. (*D-F*) BMMs were prepared from wild-type or IFNAR1$^{-/-}$ mice. (*D*) BMMs were treated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for 3 days. The cells were stained for TRAP and TRAP-positive MNCs with three or more nuclei were enumerated. (*E*) BMMs were treated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for 6 hours. Western blot analysis was performed with antibodies specific to the phosphorylated or non-phosphorylated form of STAT1 or β-actin. (*F*) BMMs were incubated with c-di-AMP or c-di-GMP in the presence of M-CSF and RANKL for 2 days. The expression levels of c-Fos, NFATc1 and β-actin were determined by Western blotting. cdA = c-di-AMP. cdG = c-di-GMP. One of three similar results is shown.

CDNs alleviate RANKL-induced calvarial bone resorption *in vivo*.

To demonstrate the anti-resorptive activity of CDNs *in vivo*, a collagen sponge soaked with c-di-AMP or c-di-GMP plus RANKL was implanted it onto mouse calvaria. CDNs alone did not cause any resorption of mouse calvarial bone (Fig. 8*A*). Remarkably, both c-di-AMP and c-di-GMP decreased RANKL-induced bone resorption pits (Fig. 8*B*). Under the same condition, the TRAP-stained area was significantly decreased by c-di-AMP or c-di-GMP (Fig. 8*C*). These results indicate that CDNs inhibit RANKL-induced bone resorption and osteoclast differentiation *in vivo*.

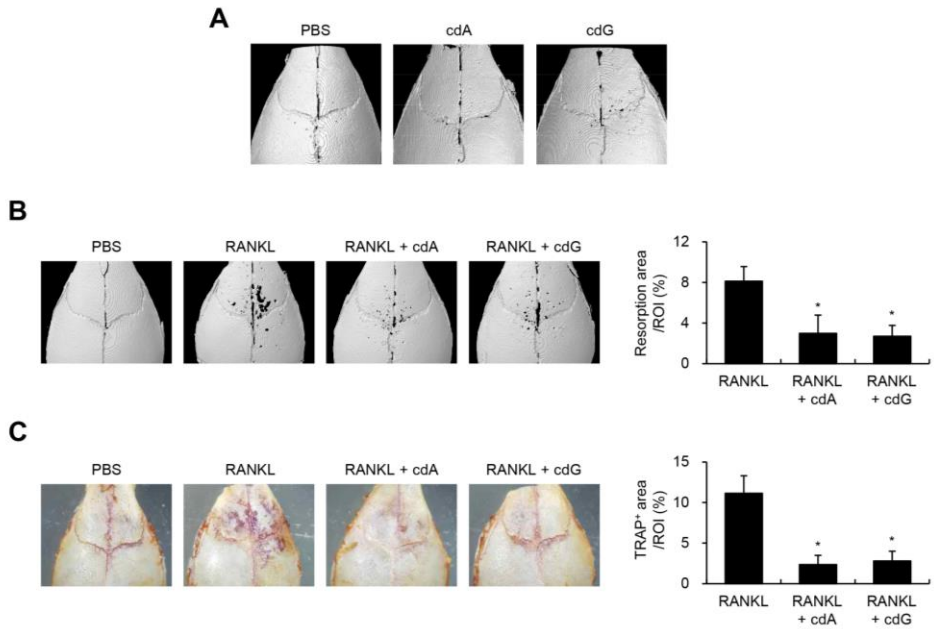

Figure 8. CDNs inhibit RANKL-induced calvarial osteoclast differentiation and bone resorption *in vivo*. A collagen sheet soaked with -di-AMP or c-di-GMP in the absence or presence of RANKL was implanted in mouse calvaria for 7 days. The calvaria were scanned by micro-CT (*A, B*) or subjected to TRAP staining and photographed (*C*). The resorption area or TRAP-stained area was analyzed by ImageJ software. *$p < 0.05$. ROI = region of interest. NT = non-treatment. cdA = c-di-AMP. cdG = c-di-GMP. One of five similar results is shown.

Discussion

In this study, it was demonstrated that CDNs effectively inhibit osteoclast differentiation and bone resorption in both *in vitro* and *in vivo* experiments. As summarized in Fig. 9, the results demonstrated that CDNs stimulate the cytosolic DNA sensor STING, leading to the induction of IFN-β followed by down-regulation of c-Fos and NFATc1, which are required for osteoclast differentiation. Therefore, the results suggest that CDNs inhibit osteoclast differentiation through induction of IFN-β via the STING signaling pathway.

The results showed that STING is required for inhibition of osteoclast differentiation by CDNs. In general, CDNs are recognized by STING and subsequently trigger the STING-TBK1-IRF3 signaling cascade.[62,65] In the present study, the results showed that CDNs induced the phosphorylation of TBK1 and IRF3, implying that CDNs activate the STING signaling pathway during osteoclast differentiation. In addition, CDN-induced inhibition of osteoclast differentiation was decreased by STING knockdown with its siRNA. Notably, unlike osteoclast differentiation, CDNs had no effect on osteoblast differentiation, which might be due to the low STING activity in osteoblasts. Thus, CDNs affect the differentiation of osteoclasts only, which express STING. Therefore, these reults suggest that the STING signaling pathway is critical for inhibition of osteoclastogenesis by CDNs. Concordant with the present study, the association of STING activation in bone metabolism has been reported. Overexpression of STING in osteoclast precursors attenuates osteoclast differentiation by inhibiting osteoclast-specific gene

expression.[66] In contrast, STING deficiency inhibits bone accrual due to attenuation of pro-osteogenic gene expression.[67] Collectively, these results propose that targeting the STING signaling pathway in the early stage of osteoclast differentiation by CDNs is a novel strategy for regulation of osteoclastogenesis.

The study demonstrated that inhibition of osteoclast differentiation by CDNs is fully dependent on the IFNAR signaling pathway. In the present study, CDNs efficiently induced the expression of IFN-β during osteoclast differentiation. In addition, the inhibition of osteoclast differentiation by CDNs was restored after incubation of macrophages with anti-IFNAR1 antibody. The inhibitory effect of CDNs on osteoclastogenesis was not observed in IFNAR1$^{-/-}$ macrophages. A previous study showed that bone volume is reduced by excessive osteoclastogenesis in IFNAR1$^{-/-}$ mice.[63] IFN-β, as a negative regulator of osteoclastogenesis, activates Jak-STAT signaling, which is known to inhibit c-Fos expression through the induction of protein kinase R.[63,68] Concordant with the present study, lipopolysaccharide (LPS) and flagellin also interfere with osteoclastogenesis through IFN-β.[69,70] Therefore, these results suggest that CDN-induced IFN-β is essential for inhibition of osteoclast differentiation from BMMs.

The study found that CDNs only affect the differentiation of BMMs into osteoclasts, but not of committed osteoclasts. Osteoclast formation undergoes the three stages: pre-osteoclast, committed osteoclast, and mature osteoclast,[61] and each stage exhibits different stage-specific effects when treated with stimuli. For example, LPS inhibits the differentiation of BMMs into osteoclasts through the induction of IFN-

β, while it promotes the differentiation of committed osteoclasts into mature osteoclasts.[69,71] Unlike LPS, previous study demonstrated that serum amyloid A only inhibits the differentiation of BMMs into osteoclasts, but has a poor effect on committed osteoclasts.[59] In the present study, CDNs inhibited BMM differentiation into osteoclasts through the induction of IFN-β. However, although CDNs potently induced the mRNA expression of IFN-β in committed osteoclasts (data not shown), they did not affect osteoclast differentiation from committed osteoclasts. One possible mechanism is the ubiquitin-mediated degradation of Jak1, which is a major mediator of the IFNAR signaling pathway, in committed osteoclasts.[72] Because of the degradation of Jak1 in committed osteoclasts, CDN-induced IFN-β might not be able to initiate the Jak-STAT signaling pathway that is necessary for inhibition of osteoclast differentiation. On the other hand, Kobayashi *et al*. reported that intraperitoneal administration of c-di-GMP modulates the bone marrow environment by inducing mobilization of hematopoietic stem cells and progenitor cells, which can be osteoclast precursors, through STING pathway.[73] Thus, further study is needed to determine the exact molecular and cellular mechanism by which CDNs modulate the bone marrow environment affecting osteoclast precursors.

The study found that CDNs negatively regulate the expression of osteoclast-specific genes during RANKL-induced osteoclast differentiation from BMMs. Activation of transcription factors c-Fos and NFATc1 is essential for osteoclast differentiation,[37,38] whereas down-regulation of c-Fos and NFATc1 leads to inhibition of osteoclastogenesis[57] or elicitation of an osteopetrotic phenotype in mice.[37,74] Therefore, the inhibitory effect of CDNs on osteoclastogenesis might be due to the

down-regulation of c-Fos and NFATc1. Moreover, the results showed that CDNs suppressed RANKL-induced bone resorptive activity through inhibition of osteoclastogenesis in both *in vitro* and *in vivo* experimental models. Blocking osteoclastogenesis or osteoclast activity is a common therapeutic strategy for the treatment of osteoporosis.[75] Therefore, the results suggest that CDNs have a potential as therapeutic agents against bone diseases including osteoporosis and periodontitis.

In conclusion, the study demonstrated that CDNs inhibit the differentiation of BMMs into osteoclasts and STING-mediated IFN-β has a critical role in the inhibitory effect. Gut microbiota-derived CDNs might be delivered into bone marrow and activate the STING signaling pathway in myeloid cells and bone cells.[53] In addition, oral microbiota also secret CDNs, activating STING signaling pathway in cells which are present in oral cavity.[24] Thus, microbiota-derived CDNs could be important for the maintenance of bone homeostasis by regulating osteoclastogenesis. Furthermore, CDNs, as a potential suppressor of osteoclastogenesis, may be used as a biocompatible therapy for the treatment of bone diseases caused by excessive osteoclast activity.

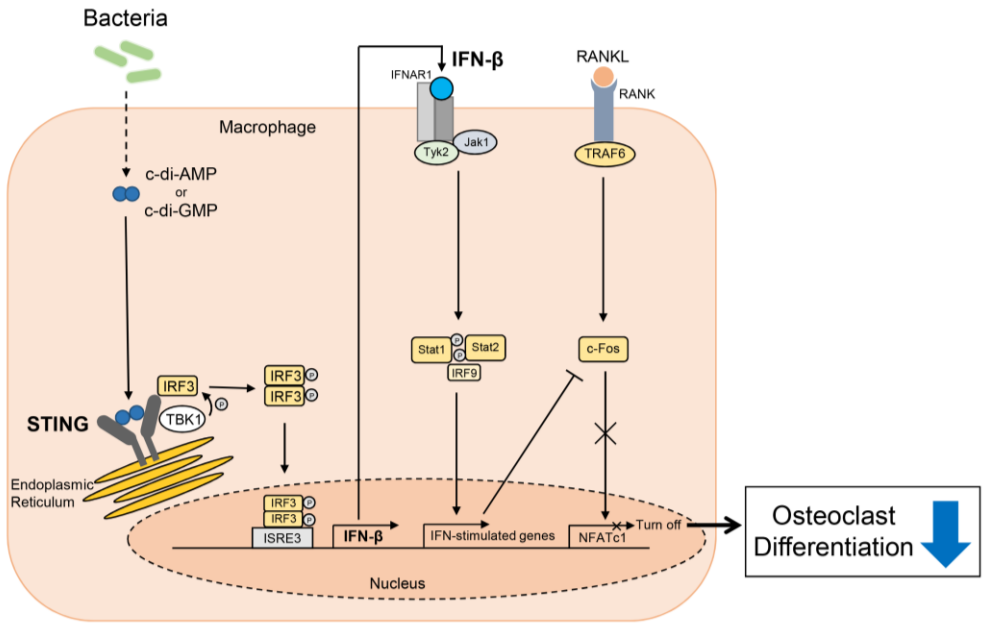

Figure 9. Schematic illustration of the proposed action mechanism. CDNs trigger the STING-TBK1-IRF3 signaling cascade to express IFN-β in macrophages during osteoclast differentiation. CDN-induced IFN-β inhibits osteoclast differentiation by down-regulation of c-Fos and NFATc1 through the Jak-STAT signaling pathway.

PART II

Cyclic dinucleotides potentiate bacterial lipoprotein-induced pro-inflammatory cytokine production through STING signaling

The contents of part II will be published elsewhere as a partial fulfillment of Yeongkag Kwon's Ph.D. program

Abstract

Microbe-associated molecular patterns (MAMPs) initiate the immune responses by activating each corresponding receptor during infection, causing inflammatory disorders. Among MAMPs, cyclic diadenylate monophosphate (c-di-AMP) is widely expressed in bacteria and plays important roles in bacterial physiology. In addition, c-di-AMP activates host immune responses through stimulator of interferon genes (STING) activation. Even though c-di-AMP can simultaneously activate the host immune response with other MAMPs during infection, the combined effects of c-di-AMP and other MAMPs on host immune responses are poorly understood. In this study, it has been investigated the role of c-di-AMP in combination with bacteria and their MAMPs on the host immune responses. When mice were intraperitoneally administrated with *Staphylococcus aureus* USA300 in the presence or absence of c-di-AMP, serum pro-inflammatory cytokines and mortality were synergistically increased by c-di-AMP plus *S. aureus* USA300. In addition, c-di-AMP exhibited the synergistic production of IL-6 combined with wild-type *S. aureus*, but not lipoprotein-deficient strain, in bone marrow-derived macrophages (BMMs). Moreover, lipoproteins isolated from *S. aureus* or Pam2CSK4, a mimicking bacterial lipoprotein, plus c-di-AMP also synergistically induced the IL-6 production. The c-di-AMP together with Pam2CSK4 potently activated NF-κB signaling pathway, which is critical for the IL-6 expression, and induced translocation of NF-κB into nucleus. The synergistic effect by c-di-AMP and Pam2CSK4 was not observed in STING knockdown BMMs with siRNA. Notably, TBK1 and IRF3, mediators of canonical STING signaling pathway, were redundant in the synergistic induction of

IL-6 since c-di-AMP synergistically induced the IL-6 in the presence of TBK1 or IRF3 inhibitor. For the last, administration of c-di-AMP with Pam2CSK4 in mice potently increased the serum IL-6 levels and mortality, while survival rate was restored by anti-IL-6 neutralizing antibody. Taken together, these results suggest that c-di-AMP exacerbates Gram-positive bacteria-induced sepsis through synergistic induction of IL-6 mediated by STING signaling pathway.

KEY WORDS: CYCLIC DINUCLEOTIDE; SEPSIS; IL-6; STING

Introduction

Sepsis is a life-threatening disorder with dysregulated host immune responses.[76] More than 1.7 million people develop sepsis annually, resulting 270,000 people death in United States.[77] It is mainly caused by bacterial infection and *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Escherichia coli* are commonly isolated from sepsis patients.[78] During bacterial infection, various microbe-associated molecular patterns (MAMPs), including cell wall components and secretory molecules, activate host immune responses through pattern recognition receptors.[79] Activated host immune responses have important role on host protection against bacteria by causing inflammation through production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6.[80] Even though these cytokines exhibit the protective effect on bacterial infection, excessive pro-inflammatory cytokines can elicit tissue and organ damage, which are critical cause of morbidity and mortality in sepsis.[81,82]

Bacterial lipoproteins, which are anchored on cytoplasmic membrane of bacteria, are composed of protein with lipid moieties.[79] Lipoproteins play important role in bacterial physiology, such as adherence, nutrient acquisition, bacterial growth, and pathogenesis.[83] Moreover, lipoproteins can be recognized by toll-like receptor (TLR) 2 that triggers MyD88-mediated signaling transduction and subsequently activates mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) to induce the pro-inflammatory cytokines.[84] The previous reports suggest that lipoproteins are major virulence factors that potently stimulate host immune

responses. For instance, staphylococcal lipoprotein induces inflammation by upregulation of inflammatory mediators through TLR2 signaling pathway.[85,86]

Cyclic dinucleotides (CDNs) are second messengers that modulate bacterial life cycle.[1] CDNs can be distinguished by their constituent component: cyclic diadenylate monophosphate (c-di-AMP); cyclic diguanylate monophosphate (c-di-GMP); and cyclic guanosine monophosphate-adenosine monophosphate (cGAMP).[2] Like other MAMPs, CDNs are also considered as immunostimulatory molecules to promote host immune responses.[46-48] Previous reports suggest that CDNs released from both intracellular and extracellular bacteria can be recognized by stimulator of interferon (IFN) genes (STING) located in the cytosol of host cells during infection.[17,87] Upon recognition, CDNs activate STING-mediated TANK-binding kinase 1 (TBK1)-IFN regulatory factor 3 (IRF3) signaling cascade to induce the expression of type I IFN, which controls pro- and anti-inflammatory processes of the host.[79,88]

Since almost all bacteria consistently expresses the lipoproteins and CDNs due to their critical roles on bacterial physiology,[2,83] it is possible that lipoproteins and CDNs can simultaneously activate the host immune responses in infection situation. However, previous reports have mainly focused on the single effect of lipoproteins or CDNs on the host immune responses. In the present study, the combined effect of bacterial lipoproteins and CDNs on the activation of inflammatory responses was investigated using *in vivo* and *in vitro* experimental models.

Material and methods

Materials

c-di-AMP, c-di-GMP, 2'3' cGAMP, 3'3' cGAMP, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), Pam2CSK4, and Pam3CSK4 were purchased from InvivoGen (San Diego, CA, USA). Recombinant murine M-CSF were obtained from JW CreaGene (Seongnam, Republic of Korea). Lipopolysaccharide (LPS) from *E. coli* was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Antibodies specific to phospho-IRF3, phospho-TBK1, phospho-P38, P38, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-NF-κB p65, NF-κB p65, phospho-IκB, IκB, and STING were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific to IRF3, TBK1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) and goat anti-mouse IgG-HRP were purchased from SouthernBiotech (Birmingham, AL, USA). Anti-mouse IFN-α/β receptor (IFNAR1) antibody, anti-mouse IL-6 antibody, and its isotype control antibody (mouse IgG1,κ or rat IgG1,κ, respectively) were obtained from BioLegend (San Diego, CA, USA).

Cell culture

The murine macrophage cell line, RAW 264.7, and murine alveolar macrophage cell line, MH-S cells, were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were incubated in DMEM medium (Welgene, Daegu, Republic of Korea) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin Solution

(HyClone, Logan, UT, USA) at 37℃ in a humidified incubator with 5% CO_2.

Preparation of primary cells

Animal experiments were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-180516-2), Republic of Korea. Six-week-old C57BL/6 male mice were from DooYeol biotech (Seoul, Republic of Korea). IFNAR1$^{-/-}$ C57BL/6 mice[54] were obtained from the Scripps Research Institute (La Jolla, CA, USA). Bone marrow-derived macrophages (BMMs) were prepared as described previously.[89] Bone marrow cells were isolated from femur and tibia. After removal of red blood cells (RBC) using RBC lysis buffer (Sigma-Aldrich Inc.), the cells were incubated in α-MEM medium (Welgene) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin Solution (HyClone) in the presence of 5 ng/ml M-CSF for 1 day at 37℃ in a humidified incubator with 5% CO_2. Non-adherent cells were differentiated into BMMs with 20 ng/ml M-CSF for 4 days at 37℃ in a humidified incubator with 5% CO_2.

Preparation of killed bacteria

To prepare heat-killed bacteria, bacterial cells were cultured at 37℃ to mid-log phase and then washed with phosphate-buffered saline (PBS). The cells were incubated at 65ºC for 2 hours. To prepare ethanol- or formaldehyde-killed bacteria, bacterial cells were cultured at 37℃ to mid-log phase and then washed with PBS. The cells were incubated with ice-cold 70% ethanol for 3 hours or treated with 0.2% formaldehyde

in PBS for 4 hours, respectively. Complete killing of bacteria was verified by plating the killed bacteria on agar plates at 37℃. No colonies were observed at 3 days after plating (data not shown).

Isolation of bacterial lipoprotein

Isolation of lipoproteins from *S. aureus* RN4220 or USA300 was performed as described previously.[90] *S. aureus* RN4220 or USA300 was incubated in Luria Bertani broth (LPS solution, Daejeon, Republic of Korea) or trypticase soy broth (TSB; BD Biosciences, Franklin Lakes, NJ, USA) for 12 hours at 37℃. The bacterial cells were collected and suspended in Tris-buffered saline (TBS) containing protease inhibitors, then sonicated for 15 minutes. Bacterial lysates were resuspended in 2% Triton X-114 and incubated at 4℃ for 2 hours. After centrifugation, the Triton X-114 phase was further incubated with an equal volume of TBS at 37℃ for 15 minutes. The lipoprotein containing phase was collected after centrifugation and mixed with methanol at −20℃ overnight. Triton X-114 extracts were precipitated by centrifugation and dissolved in octyl β-d-glucopyranoside.

Intraperitoneal infection

S. aureus USA300 was cultured to mid-log phase at 37℃ in TSB. The bacterial pellet was collected and washed three times with PBS. Mice were administrated with 1×10^8 CFU of *S. aureus* USA300 with or without 200 nmol c-di-AMP in 300 μl PBS via intraperitoneal injection. Serum was collected on 6 hours after administration. The expression of IL-6 or TNF-α in the serum was measured by enzyme-linked

immunosorbent assay (ELISA) kit (BioLegend) according to the manufacturer's instructions. Survival was monitored daily for 96 hours. In a separate experiment, Mice were intraperitoneal administered with 100 μg Pam2CSK4 and/or 200 nmol c-di-AMP in the presence of 50 μg anti-IL-6 antibody or its IgG isotype antibody. Survival was monitored daily for 96 hours.

ELISA

BMMs were plated onto a 96-well plate (Corning, Tewksbury, MA, USA) at 2.5 × 10^4 cells/0.3 ml/well and stimulated with 20 ng/ml M-CSF and 2 μg/ml c-di-AMP in the presence or absence of indicated stimuli for 24 hours. Cell culture supernatants were collected and IL-6 in the cell culture supernatants were measured using an ELISA kit (BioLegend) according to the manufacturer's instruction. In a separate experiment, *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus mutans*, or *S. aureus* USA300 was cultured at 37℃ to mid-log phase. The culture supernatants were collected to measure the production of c-di-AMP or c-di-GMP by using ELISA kit (Cayman chemical, Ann Arbor, MI, USA) according to the manufacturer's instruction.

Proteome profiler mouse cytokine array

Mice were randomly divided into four groups and administrated with 1 × 10^8 CFU of *S. aureus* USA300 with or without 200 nmol c-di-AMP in 300 μl PBS via intraperitoneal injection. Serum was collected on 6 hours after administration. Expression of cytokines in serum was determined by a mouse cytokine array kit (Proteome Profiler™ Mouse XL Cytokine Array Kit; R&D systems, Minneapolis,

MN, USA) according to the manufacturer's instructions. In a separate experiment, BMMs were plated onto a 96-well plate at 2.5×10^4 cells/0.3 ml/well and stimulated with 20 ng/ml M-CSF and 2 μg/ml c-di-AMP in the presence or absence of heat-killed *S. aureus* (HKSa) USA300 for 24 hours. Cell culture supernatants were collected and cytokines in the cell culture supernatants were measured using a mouse cytokine array kit (Proteome Profiler™ Mouse XL Cytokine Array Kit) according to the manufacturer's instructions. Immunoreactive spots were detected using the Fusion FX6.0 (Vilber Lourmat, France). Mean pixel density of each dots was measured by ImageJ program (National Institutes of Health, Bethesda, MD, USA). The mean pixel density of protein dots was divided by the mean pixel density of reference dots for each membrane.

Western blotting

BMMs were seeded onto a 60 mm dish at 1×10^6 cells/4 ml and treated with 20 ng/ml M-CSF and 2 μg/ml c-di-AMP in the presence or absence of 50 ng/ml Pam2CSK4 for indicated time. Western blotting was performed as described previously.[91] Briefly, the lysates were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% skim milk or bovine serum albumin (BSA) in TBS containing 0.05% Tween-20 (TBST), the membrane was incubated with primary antibodies specific to TBK1, IRF3, or β-actin at 4°C overnight. The membrane was washed three times with TBST and incubated with HRP-conjugated secondary antibodies. Immunoreactive bands were detected using Fusion FX6.0.

Real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR)

BMMs were plated onto a 35 mm dish at 5×10^5 cells/2 ml and stimulated with 20 ng/ml M-CSF and 2 μg/ml c-di-AMP in the presence or absence of 50 ng/ml Pam2CSK4 for 6 hours. Total RNA was extracted and subjected to real-time RT-PCR as described previously.[92] The sequences of each primer are as follows: IL-6: forward 5'-CCGGAGAGGAGACTTCACAG-3' and reverse 5'-GGAAATTGGGGTAGGAAGGA-3'; IFN-β: forward 5'-AACTCCACCAGCAGACAGTG-3' and reverse 5'-CATCCAGGCGTAGCTGTTGT-3'; and GAPDH: forward 5'-ACCCAGAAGACTGTGGATGG-3' and reverse 5'-CACATTGGGGGTAGGAACAC-3'.

Transfection with small interfering RNA (siRNA)

BMMs were plated onto a 6-well culture plate at 3×10^5 cells/2 ml/well. The cells were transfected with 25 nM of ON-TARGETplus siRNA targeting STING or ON-TARGETplus non-targeting control siRNA (Dharmacon, Lafayette, CO, USA) using the Viromer Green transfection reagent (Lipocalyx GmbH, Halle, Germany) according to the manufacturer's instructions. After 24 hours, the cells were seeded on a 96-well culture plate at 2.5×10^4 cells/0.3 ml/well and then stimulated with 20 ng/ml M-CSF and 2 μg/ml c-di-AMP in the presence or absence of 50 ng/ml Pam2CSK4 for 24 hours. The target sequences of STING siRNA are as follows: 5'-UCAAUCAGCUACAUAACAA-3', 5'-GCAUCAAGAAUCGGGUUUA-3', 5'-AACAUUCGAUUCCGAGAUA-3', and 5'-CCAACAGCGUCUACGAGAU-3'.

Immunofluorescence staining

BMMs were seeded on coverslip in a 24-well culture plate at 1×10^5 cells/0.5 ml/well and incubated with 20 ng/ml M-CSF and 2 μg/ml c-di-AMP in the presence or absence of 50 ng/ml Pam2CSK4 for 1 hour. The cells were fixed with 4% formaldehyde for 15 minutes and permeabilized with TBS containing 0.3% Triton X-100 (TBST) for 5 minutes. After blocking with 5% BSA in TBST, the fixed cells were incubated with antibody specific to NF-κB p65 at 4°C overnight. The fixed cells were stained with Alexa fluor 568-conjugated secondary antibody for 1 hour and the nuclei were counterstained with HOECHST 33258. The cells were examined by LSM 800 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) and analyzed with LSM Browser software.

Statistical analysis

The results are the mean value ± standard deviation of triplicate samples. Statistical significance was examined using the t test. Asterisks indicate statistical significance at $p < 0.05$ compared with the control group.

Results

CDNs are produced by bacteria and exogenous c-di-AMP does not affect growth of *S. aureus* USA300.

To determine which CDNs are produced at bacterial growth, *S. gordonii*, *S. sanguinis*, *S. mutans*, or *S. aureus* USA300 was cultured at 37℃ to mid-log phase. The production of c-di-AMP or c-di-GMP was measured by ELISA. As shown in Fig. 10*A*, *S. gordonii*, *S. sanguinis*, *S. mutans*, and *S. aureus* produced c-di-AMP during bacterial growth. In addition, *S. gordonii*, *S. sanguinis*, and *S. mutans* also produced c-di-GMP. However, *S. aureus* produced c-di-GMP less than streptococci. Next, to examine the effect of exogenous c-di-AMP on growth of *S. aureus*, *S. aureus* was incubated with various doses of c-di-AMP at 37℃ for 24 hours. c-di-AMP did not influence the growth of *S. aureus* USA300 *in vitro* (Fig. 10*B*). These results indicate that *S. aureus* could promote host immune responses through production of c-di-AMP without affecting bacterial growth.

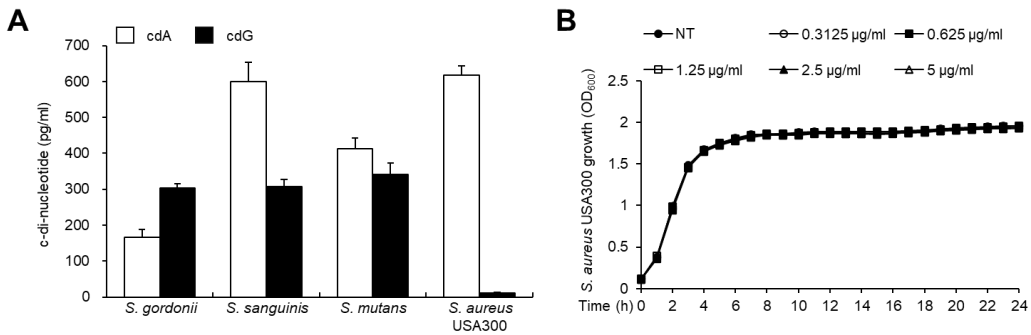

Figure 10. CDNs are produced by bacteria and exogenous c-di-AMP does not affect growth of *S. aureus* USA300. (*A*) *S. gordonii*, *S. sanguinis*, *S. mutans*, or *S. aureus* USA300 was cultured at 37℃ to mid-log phase. The culture supernatants were collected to measure the production of c-di-AMP or c-di-GMP by ELISA. (*B*) *S. aureus* USA300 was cultured in the presence or absence of different doses of c-di-AMP for 24 hours. Optical density at 600 nm was measured. One of three similar results is shown. cdA = c-di-AMP. cdG = c-di-GMP.

c-di-AMP exacerbates mortality with potent cytokine induction during *S. aureus* USA300 infection.

To examine an effect of c-di-AMP on bacterial infection, *S. aureus* USA300 was administered with/without 200 nmol of c-di-AMP via intraperitoneal injection in mouse. After 6 hours, blood samples were taken and sera were examined for the release of cytokines. In addition, survival rate was monitored as a percentage of each experimental group until 96 hours. As shown in figure 11*A*, survival rate was not changed when c-di-AMP alone or *S. aureus* USA300 alone was administrated. However, mice injected with c-di-AMP and *S. aureus* USA300 together showed the reduced survival rate to 50%. Moreover, co-administration of c-di-AMP and *S. aureus* USA300 further increased the cytokines in the serum, including IL-6, IL-16, CXCL1, CXCL2, CCL4, TIMP-1, and TNF-α, compared with those of c-di-AMP or *S. aureus* USA300 alone (Fig. 11*B* and *C*). In the same condition, quantitative ELISA data also showed that co-injection of c-di-AMP and *S. aureus* USA300 promoted excessive IL-6 production than *S. aureus* USA300 or c-di-AMP alone (Fig. 11*D*). These results indicate that c-di-AMP increases mortality and cytokine production during *S. aureus* infection.

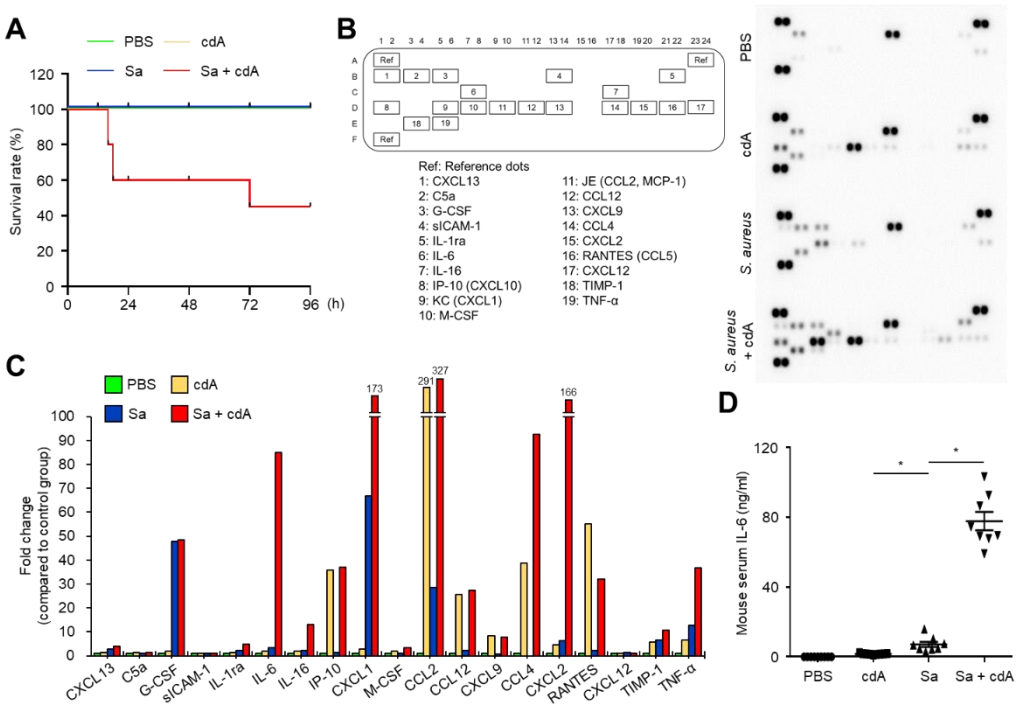

Figure 11. c-di-AMP increases mortality and cytokine production during *S. aureus* USA300 infection. Mice were administrated with *S. aureus* USA300 with or without c-di-AMP via intraperitoneal injection. Serum was collected on 6 hours after the administration. (*A*) Survival was monitored daily for 96 hours. Data shown represented the survival percent of 10 mice per group. (*B*, *C*) The expression of cytokines in serum was measured by Mouse Proteome Profiler Array kit. (*B*) Immunoreactive spots were detected using the Fusion FX6.0. (*C*) Mean pixel density of each dots was measured by ImageJ program. (*D*) The expression of IL-6 in the serum was measured by ELISA. *$p < 0.05$. One of seven similar results is shown. cdA = c-di-AMP. Sa = *S. aureus* USA 300.

c-di-AMP synergistically increases HKSa-induced cytokine production *in vitro*.

To confirm the combination effects of c-di-AMP and *S. aureus* on induction of excessive pro-inflammatory cytokines *in vitro*, BMMs were stimulated with c-di-AMP in the presence or absence of HKSa for 24 hours. Fig. 12*A* and *B* showed that c-di-AMP alone induced the expression of CCL1, IL-10, IL-16, IP-10, and IFN-γ. In addition, HKSa alone induced the expression of pro-inflammatory cytokines such as G-CSF, CCL1, IL-10, CXCL1, CXCL2, and TNF-α. Notably, c-di-AMP in combination with HKSa synergistically increased the expression of G-CSF, IL-6, CCL3, and RANTES. These results indicate that c-di-AMP synergistically increases the expression of cytokines induced by HKSa.

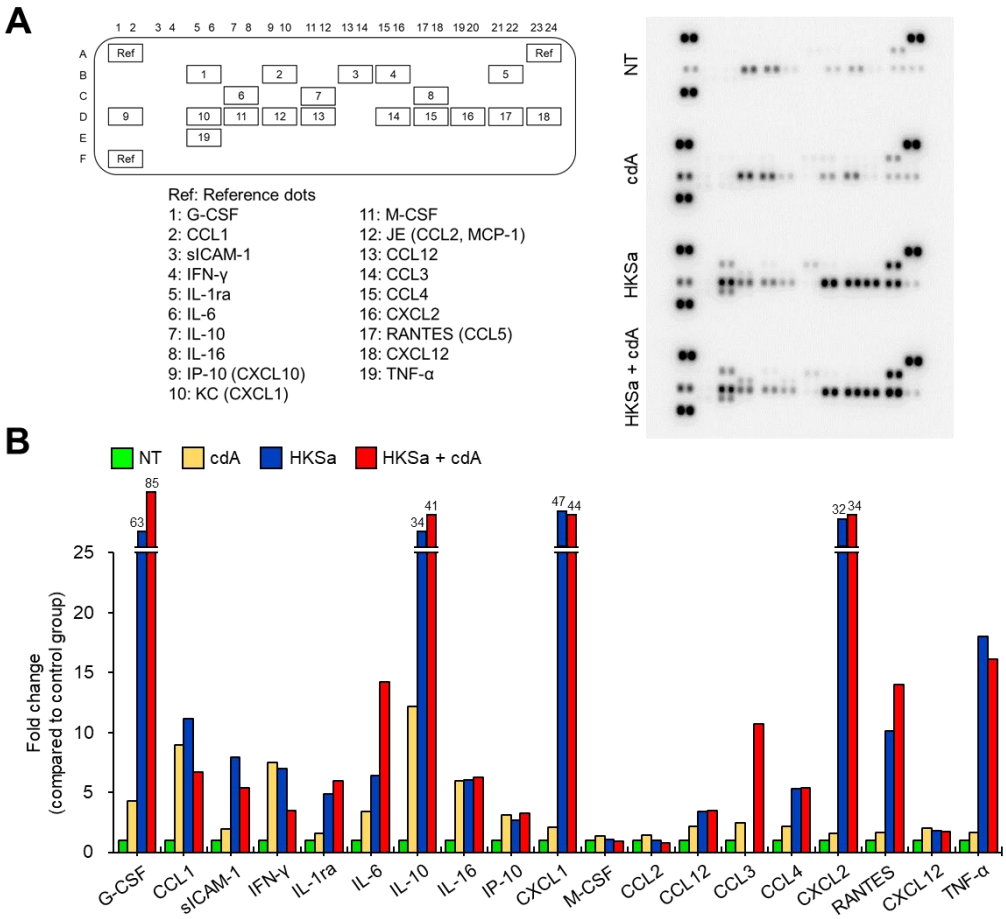

Figure 12. c-di-AMP potently enhances cytokine production induced by heat-killed *S. aureus in vitro*. BMMs were incubated with c-di-AMP in the presence or absence of HKSa for 24 hours. The culture supernatants were collected to measure the expression of cytokines by Mouse Proteome Profiler Array kit. (*A*) Immunoreactive spots were detected using the Fusion FX6.0. (*B*) Mean pixel density of each dots was measured by ImageJ program. NT = non-treatment. cdA = c-di-AMP. HKSa = heat-killed *S. aureus* USA 300.

c-di-AMP enhances IL-6 production in BMM treated with killed *S. aureus* regardless of killing methods.

To determine whether c-di-AMP synergistically increases the IL-6 production in combination with *S. aureus* USA300 killed by various methods, BMMs were stimulated with c-di-AMP in the presence of HKSa, ethanol-killed *S. aureus* (EKSa), or formaldehyde-killed *S. aureus* (FKSa) for 24 hours. c-di-AMP treatment alone did not induce the IL-6 production, while HKSa, EKSa, and FKSa induced IL-6 production. Notably, applying c-di-AMP with HKSa, EKSa, or FKSa in BMMs significantly enhanced the IL-6 production when compared with inactivated *S. aureus* alone (Fig. 13*A*-*C*). These results suggest that c-di-AMP synergistically increases the IL-6 production in BMM treated with inactivated *S. aureus*.

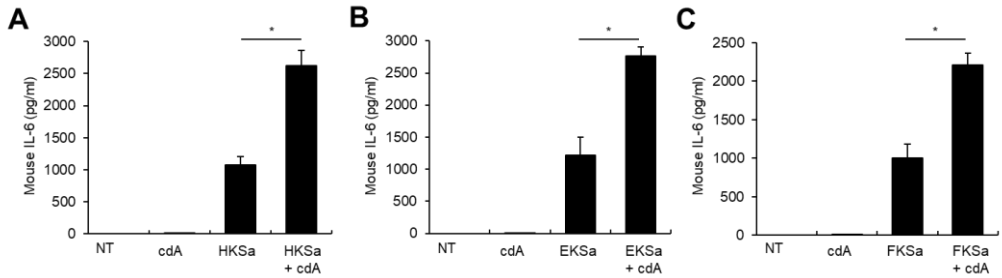

Figure 13. c-di-AMP potently enhances IL-6 production induced by killed *S. aureus* independent of inactivation method. BMMs were incubated with M-CSF and c-di-AMP in the presence of (*A*) HKSa, (*B*) EKSa, or (*C*) FKSa at 1×10^7 CFU/ml for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. HKSa = heat-killed *S. aureus* USA 300. EKSa = ethanol-killed *S. aureus* USA300. FKSa = formaldehyde-killed *S. aureus* USA300. One of three similar results is shown.

c-di-AMP increases the HKSa-induced IL-6 production in various immune cells.

To confirm the synergistic effect of c-di-AMP on HKSa-induced IL-6 production in various murine immune cells, c-di-AMP was treated on murine primary cells, including M2 macrophages, M1 macrophages, and DCs, or murine macrophage cell line RAW 264.7 cells in the presence or absence of HKSa for 24 hours. c-di-AMP augments the HKSa-induced IL-6 production in both M2 and M1 macrophages (Fig. 14*A* and *B*). Likewise, HKSa-induced IL-6 secretion was significantly upregulated by c-di-AMP in DCs (Fig. 14*C*). In addition, c-di-AMP also increased the HKSa-induced IL-6 production in RAW 264.7 cells (Fig. 14*D*). These data indicate that c-di-AMP potently upregulates the HKSa-induced IL-6 expression in various immune cells.

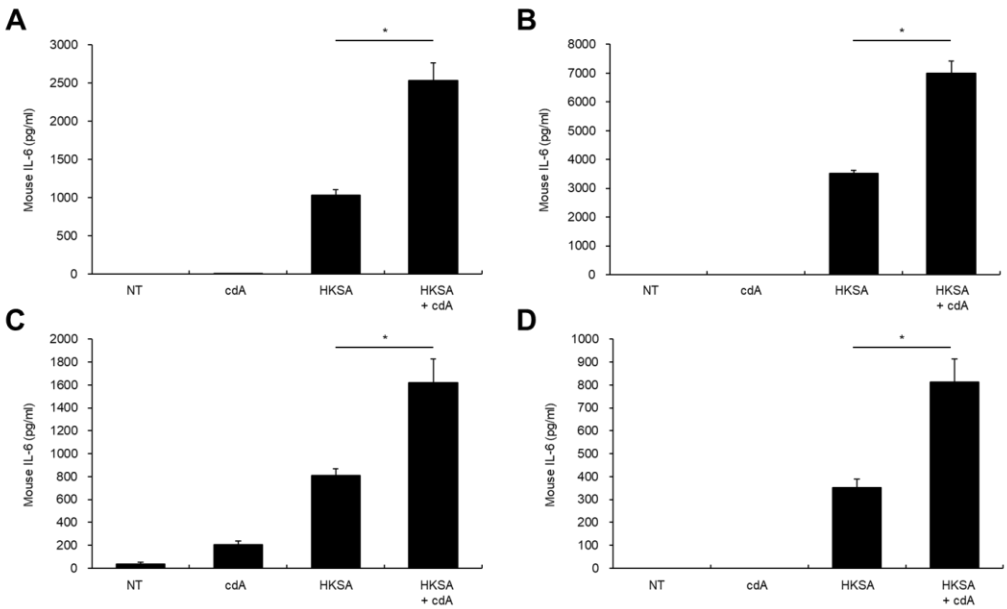

Figure 14. c-di-AMP enhances the secretion of IL-6 induced by heat-killed *S. aureus* in various immune cells. (*A*) M2 macrophages were incubated with M-CSF and c-di-AMP in the presence of HKSa for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*B*) M1 macrophages were cultured with GM-CSF and c-di-AMP in the presence of HKSa for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*C*) DCs were incubated with GM-CSF and c-di-AMP in the presence of HKSa for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*D*) RAW 264.7 cells were stimulated with c-di-AMP in the presence of HKSa for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. HKSa = heat-killed *S. aureus* USA 300. One of three similar results is shown.

Lipoproteins of *S. aureus* are critical for the synergistic induction of IL-6 by c-di-AMP.

Lipoproteins are known as responsible molecules for causing sepsis by activation of TLR2 signaling pathway, inducing pro-inflammatory cytokines.[93] To examine role of lipoproteins of *S. aureus* on synergistic induction of IL-6 in combination with c-di-AMP, BMMs were stimulated with HKSa wild-type or lipoprotein-deficient strain (Δ*lgt*) in the presence or absence of c-di-AMP. HKSa RN4220 wild-type increased the IL-6 secretion in a dose-dependent manner and it was augmented by c-di-AMP treatment. However, HKSa RN4220 Δ*lgt* was neither able to induce the IL-6 secretion nor c-di-AMP synergized with HKSa RN4220 Δ*lgt* (Fig. 15*A*). In addition, lipoproteins isolated from *S. aureus* RN4220 increased the production of IL-6 in a dose-dependent manner, and co-treatment of c-di-AMP further enhanced the IL-6 secretion (Fig. 15*B*). To further validate, BMMs derived from wild-type or $TLR2^{-/-}$ mice were stimulated with c-di-AMP in the presence of HKSa USA300. Fig. 15*C* showed that c-di-AMP with HKSa USA300 potently induced the IL-6 production in wild-type BMMs, while the IL-6 production was reduced in $TLR2^{-/-}$ BMMs. Furthermore, co-treatment of c-di-AMP and lipoproteins isolated from *S. aureus* USA300 also synergistically enhanced the IL-6 secretion in wild-type BMMs. In contrast, the IL-6 secretion and synergistic effect of c-di-AMP were not observed in $TLR2^{-/-}$ BMMs (Fig. 15*D*). These results suggest that lipoproteins of *S. aureus* are crucial for the synergistic effect on IL-6 production by c-di-AMP.

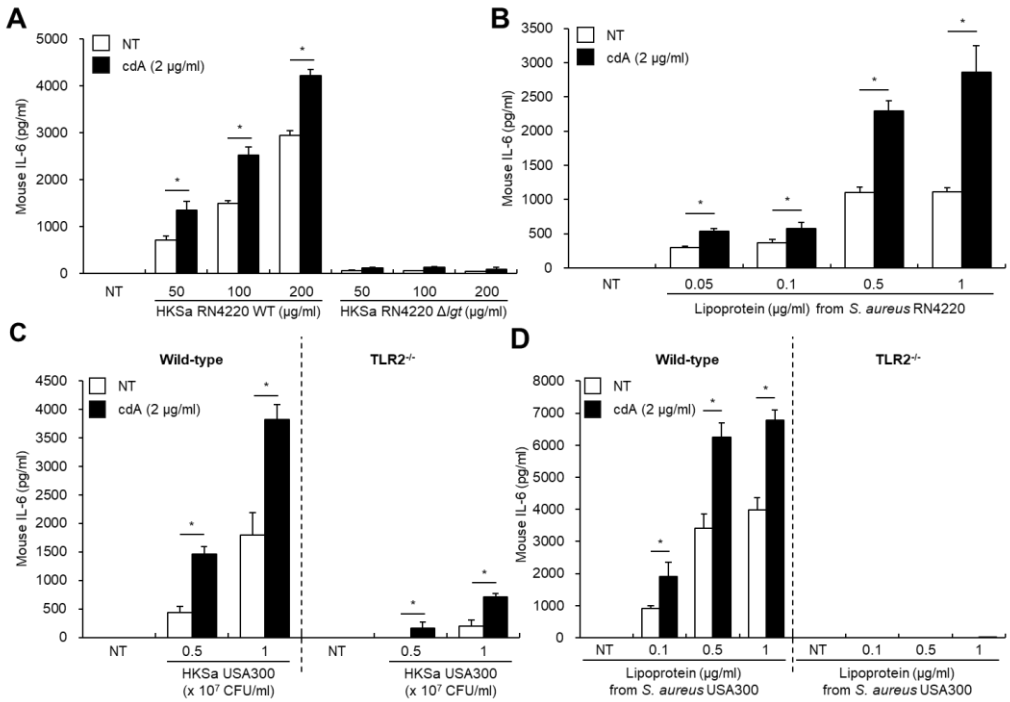

Figure 15. Lipoproteins of *S. aureus* are important for synergistic effect on Il-6 production by c-di-AMP. (*A*, *B*) BMMs were stimulated with c-di-AMP in the presence or absence of (*A*) HKSa RN4220 WT or Δ*lgt*, or (*B*) lipoproteins from *S. aureus* RN4220 for 24 hours. The culture supernatants were collected to measure the expression of IL-6. (*C*, *D*) BMMs prepared from wild-type or TLR2$^{-/-}$ mice were treated with c-di-AMP in the presence or absence of (*C*) HKSa USA300 or (*D*) lipoproteins from *S. aureus* USA300. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. HKSa = heat-killed *S. aureus*. One of three similar results is shown.

Co-treatment of c-di-AMP and synthetic lipopeptide synergistically induces IL-6 production.

Next, to investigate the synergistic expression of IL-6 in response to c-di-AMP and Pam2CSK4, which is a synthetic diacylated lipopeptide, BMMs were stimulated with or without c-di-AMP and/or Pam2CSK4 for 6 hours. As shown in Fig. 16*A*, Pam2CSK4-induced mRNA expression of IL-6 was enhanced by c-di-AMP compared with Pam2CSK4 alone. In addition, production of IL-6 in BMMs co-stimulated with c-di-AMP and Pam2CSK4 was increased in a time- (Fig. 16*B*) or dose-dependent manner (Fig. 16*C* and *D*). To further confirm the synergistic effects of c-di-AMP in combination with Pam2CSK4 on IL-6 production at different time points, BMMs were stimulated with c-di-AMP at 6, 3, or 1 hour pre-treatment or 0, 1, 3, or 6 hours post-treatment in the presence of Pam2CSK4. The synergistic effects of c-di-AMP were observed from 6 hours pre-treatment to 6 hours post-treatment with a maximal enhancement at 0 hour (Fig. 16*E*). In addition, c-di-AMP synergistically induced the IL-6 production in combination with Pam2CSK4 and Pam3CSK4, but not lipoteichoic acid isolated from *S. aureus* or LPS (Fig. 17). These results suggest that co-treatment of c-di-AMP and lipopeptide promotes the potent IL-6 production compared with lipopeptide treatment alone.

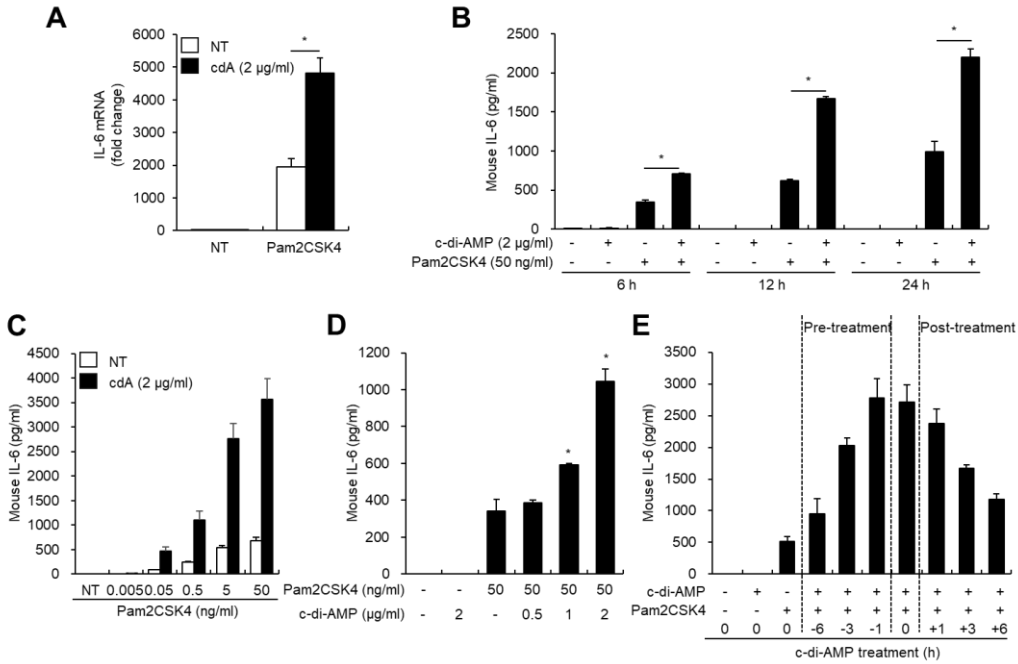

Figure 16. Co-treatment of c-di-AMP and synthetic diacylated lipopeptide synergistically induces IL-6 production. (*A*) BMMs were incubated with c-di-AMP in the presence or absence of Pam2CSK4 for 6 hours. The mRNA expression levels of IL-6 and GAPDH were determined by real-time RT-PCR. (*B*) BMMs were stimulated with c-di-AMP in the presence or absence of Pam2CSK4 for 6, 12, or 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*C*) BMMs were incubated with c-di-AMP in the presence of Pam2CSK4 at various doses for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*D*) BMMs were incubated with Pam2CSK4 in the presence of c-di-AMP at various doses for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*E*) BMMs were treated with c-di-AMP at -6, -3, or -1 hour pre-treatment or 0, +1, +3, or +6 hours post-treatment with Pam2CSK4. Total incubation time with Pam2CSK4 was 24 hours for all treatment groups. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. One of three similar results is shown.

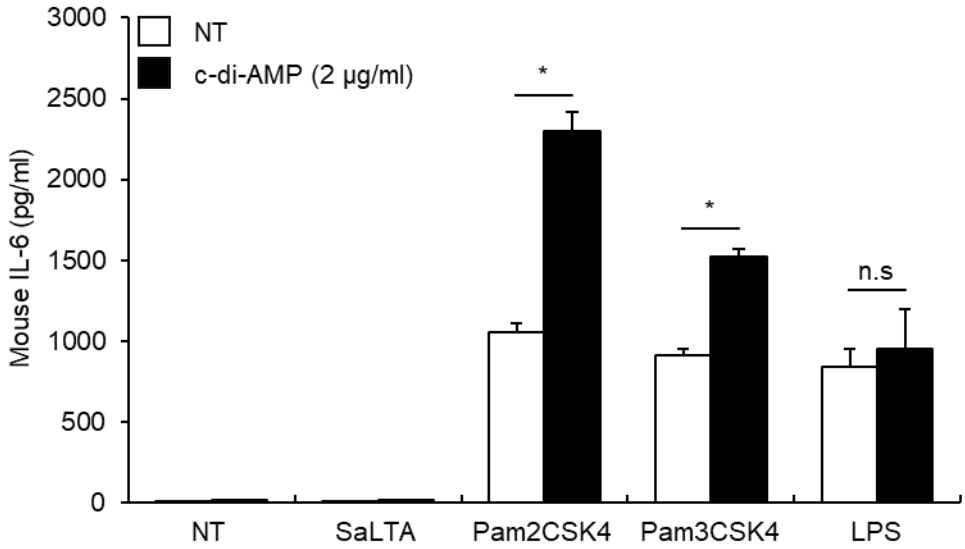

Figure 17. c-di-AMP in combination with lipopeptides elicits a synergistic induction of IL-6. BMMs were stimulated with SaLTA, Pam2CSK4, Pam3CSK4, or LPS, in the presence or absence of c-di-AMP for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. SaLTA = lipoteichoic acid isolated from *S. aureus*. LPS = lipopolysaccharide. n.s = not significant. One of three similar results is shown.

c-di-AMP enhances Pam2CSK4-induced IL-6 production in various immune cells.

To confirm the synergistic effect of c-di-AMP on Pam2CSK4-induced IL-6 production in various murine immune cells, c-di-AMP was treated on murine primary cells, such as M2 macrophages, M1 macrophages, and DCs, or murine macrophage cell line, including RAW 264.7 cells or MH-S cells, in the presence or absence of Pam2CSK4 for 24 hours. As shown in Fig. 18*A* and *B*, induction of IL-6 in M2 or M1 macrophages co-stimulated with c-di-AMP and Pam2CSK4 was more potent than with Pam2CSK4 alone. In addition, c-di-AMP increased the Pam2CSK4-induced IL-6 production in DCs (Fig. 18*C*). When the RAW 264.7 cells or MH-S cells, which are murine macrophage cell lines, were treated with c-di-AMP and Pam2CSK4, c-di-AMP synergistically upregulated the Pam2CSK4-induced IL-6 production (Fig. 18*D* and *E*). These data show that the c-di-AMP increases the Pam2CSK4-induced IL-6 production in various immune cells.

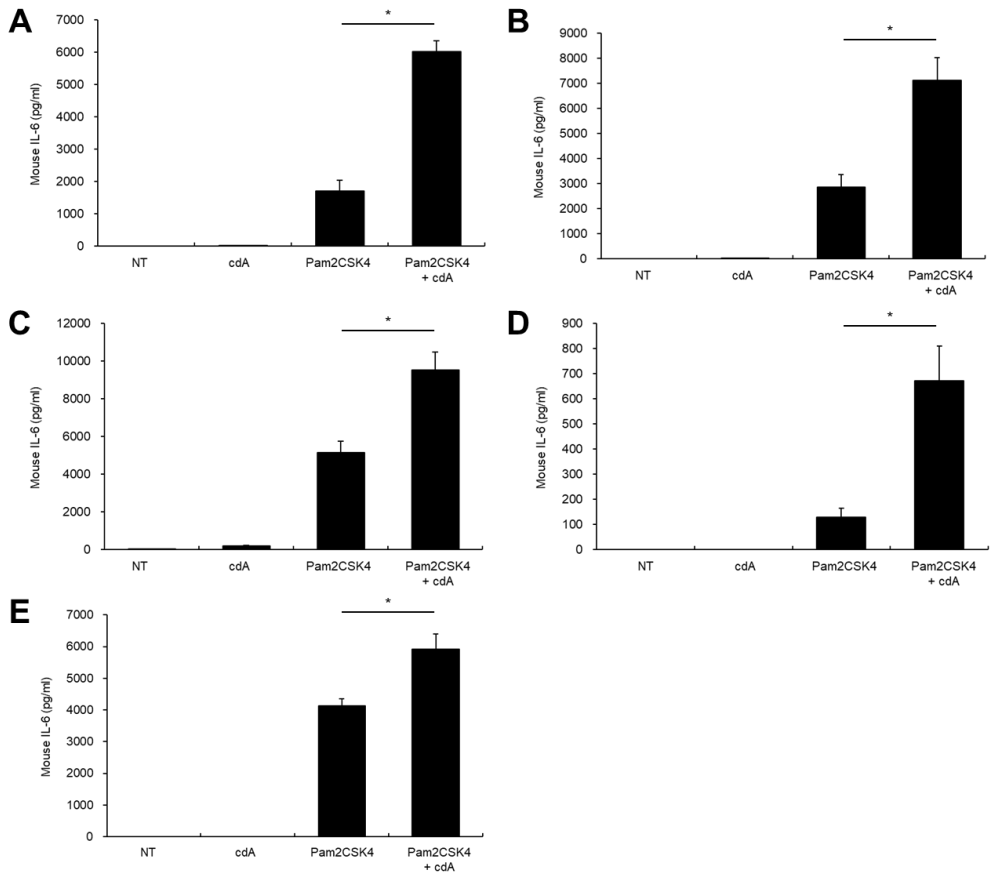

Figure 18. c-di-AMP increases Pam2CSK4-induced IL-6 production in various immune cells. (*A*) M2 macrophages, (*B*) M1 macrophages, (*C*) DCs, (*D*) RAW 264.7 cells, or (*E*) MH-S cells were stimulated with c-di-AMP in the presence or absence of Pam2CSK4 for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. One of three similar results is shown.

NF-κB, but not MAPK, signaling pathway is required for synergistic induction of IL-6 in BMMs treated with c-di-AMP and Pam2CSK4.

It has been demonstrated that the expression of IL-6 induced by lipoprotein is mediated by activation of MAPK and NF-κB signaling pathway.[94] Therefore, to investigate whether c-di-AMP affects MAPK or NF-κB signaling pathway activated by bacterial lipoproteins, BMMs were stimulated with c-di-AMP in the presence or absence of Pam2CSK4. As expected, Pam2CSK4 potently induced the phosphorylation of P38, ERK1/2, and JNK, while c-di-AMP did not affect the Pam2CSK4-induced phosphorylation of MAPK (Fig. 19*A*). In contrast, c-di-AMP synergistically upregulated the Pam2CSK4-induced phosphorylation of NF-κB in a time-dependent manner (Fig. 19*B*). In addition, stimulation of c-di-AMP in the combination with Pam2CSK4 enhanced a translocation of NF-κB to nuclei (Fig. 19*C*). These results indicate that c-di-AMP upregulates the Pam2CSK4-induced NF-κB activation, but not MAPK activation.

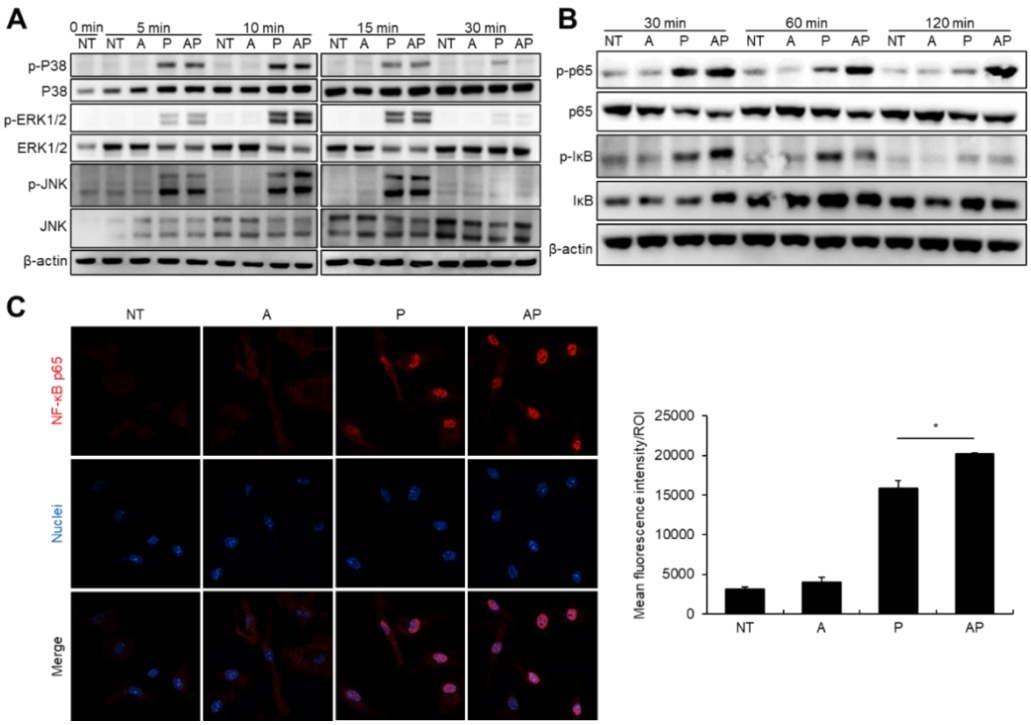

Figure 19. c-di-AMP only increases the Pam2CSK4-induced NF-κB signaling pathway activation. (*A*, *B*) BMMs were stimulated with c-d-AMP in the presence or absence of Pam2CSK4 for indicate times. (*A*) The whole-cell lysates were subjected to Western blotting using specific antibodies to the phosphorylated or non-phosphorylated form of P38, ERK1/2, JNK, or β-actin. (*B*) The whole-cell lysates were subjected to Western blotting using specific antibodies to the phosphorylated or non-phosphorylated form of NF-κB p65, IκB, or β-actin. (*C*) BMMs were plated onto a cover glass and then incubated with c-di-AMP and/or Pam2CSK4 for 1 hour. The cells were fixed and incubated with antibody specific to NF-κB p65. The fixed cells were stained with Alexa fluor 568-conjugated secondary antibody (red), and the nuclei were counterstained with HOECHST 33258 (blue). The stained cells were examined by confocal laser scanning microscope (left). Mean fluorescence intensity of Alexa fluor 568 in nuclei was analyzed with LSM Browser software (right). *$p < 0.05$. NT = non-treatment. A = c-di-AMP. P = Pam2CSK4. One of three similar results is shown.

Synergistic induction of IL-6 in BMMs treated with c-di-AMP and Pam2CSK4 is dependent on STING, but not canonical STING-TBK1-IRF3 axis.

It has been reported that c-di-AMP is directly recognized by STING, activating the STING-TBK1-IRF3 signaling cascade.[95] Thus, to investigate whether STING is essential for the synergistic effects of c-di-AMP on IL-6 production, BMMs were transfected with STING-targeting siRNA and then stimulated with c-di-AMP in the presence of Pam2CSK4. The expression of STING was knocked down by STING-targeting siRNA (Fig. 20*A*, left), and the synergistic effects of c-di-AMP on Pam2CSK4-induced IL-6 production were disappeared in STING-knock down BMMs (Fig. 20*A*, right). The activation of STING in BMMs co-stimulated with c-di-AMP and Pam2CSK4 was determined by phosphorylated form of TBK1 or IRF3. When the cells were co-treated with c-di-AMP and Pam2CSK4, phosphorylation of TBK1 or IRF3 was increased (Fig. 20*B*). Next, to validate if canonical STING-TBK1-IRF3 pathway is involved in the synergistic effects of c-di-AMP on IL-6 production, BMMs were stimulated with c-di-AMP in the presence of H-151, amlexanox, or GSK690693, which is an inhibitor of STING, TBK1, or IRF3, respectively. c-di-AMP-induced mRNA expression of IFN-β was diminished by the all three inhibitors (Fig. 20*C*, left). However, only H-151, but not amlexanox and GSK690693, suppressed the synergistic mRNA expression of IL-6 in response to c-di-AMP and Pam2CSK4 (Fig. 20*C*, right). In addition, synergistic production of IL-6 by c-di-AMP and Pam2CSK4 was inhibited by H-151, but not amlexanox and GSK690693 (Fig. 20*D*). These results indicate that c-di-AMP synergistically affects the Pam2CSK4-induced IL-6 production through the STING, but not canonical TBK1-IRF3 axis.

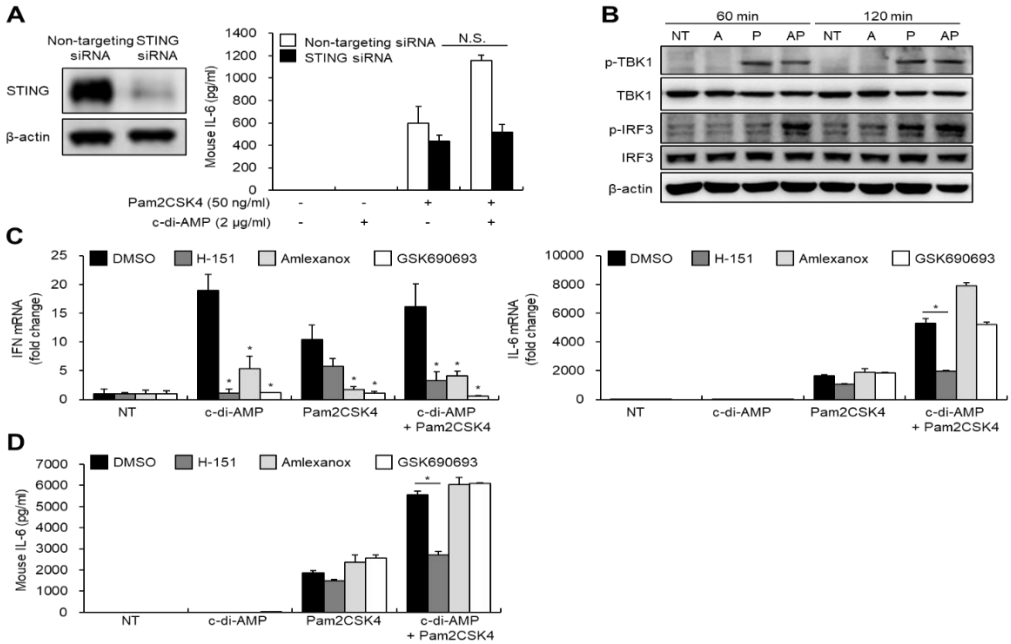

Figure 20. c-di-AMP synergistically induces the Pam2CSK4-induced IL-6 through the STING, but not canonical STING-TBK1-IRF3 signaling cascade. (*A*) BMMs were transfected with STING targeting siRNA or non-targeting siRNA for 24 hours and then treated with c-di-AMP in the presence or absence of Pam2CSK4 for additional 24 hours. The knockdown of STING expression in whole-cell lysates was confirmed by Western blotting (left). The culture supernatants were collected to measure the expression of IL-6 by ELISA (right). (*B*) BMMs were treated with c-di-AMP in the presence or absence of Pam2CSK4 for 60 or 120 minutes. The whole-cell lysates were subjected to Western blotting using specific antibodies to the phosphorylated or non-phosphorylated form of TBK1, IRF3, or β-actin. (*C*, *D*) BMMs were pretreated with H-151, amlexanox, or GSK690693 for 1 hour. (*C*) The cells were then stimulated with c-di-AMP in the presence or absence of Pam2CSK4 for 6 hours. The expression levels of IFN-β (left), IL-6 (right) and β-actin were determined by real-time RT-PCR. (*D*) The cells were incubated with c-di-AMP in the presence or absence of Pam2CSK4 for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. A = c-di-AMP. P = Pam2CSK4. One of three similar results is shown.

Type I IFN is not involved in synergistic production of IL-6 by c-di-AMP.

c-di-AMP is well known to induce type I IFN through the activation of STING signaling pathway.[62] Thus, to confirm whether c-di-AMP upregulates the expression of IFN-β, BMMs were treated with c-di-AMP in the presence or absence of Pam2CSK4 for 6 hours. As shown in Fig. 21*A*, mRNA expression of IFN-β was induced by co-treatment of c-di-AMP and Pam2CSK4, but the synergistic effects of c-di-AMP on the mRNA expression of IFN-β was not observed. Next, to investigate that c-di-AMP-mediated type I IFN is involved in the synergistic induction of IL-6, BMMs were pretreated with anti-IFNAR1 antibody or its isotype control antibody and then stimulated with c-di-AMP and Pam2CSK4. Interestingly, anti-IFNAR1 antibody failed to reduce the synergistic induction of IL-6 by c-di-AMP compared with its isotype control antibody (Fig. 21*B*). Moreover, when BMMs derived from wild-type or IFNAR1$^{-/-}$ mice were treated with c-di-AMP in the presence or absence of Pam2CSK4, c-di-AMP still synergized the Pam2CSK4-induced IL-6 production in IFNAR1$^{-/-}$ BMMs as much as in wild-type BMMs (Fig. 21*C*). These results suggest that type I IFN is dispensable for the synergistic induction of IL-6 by c-di-AMP.

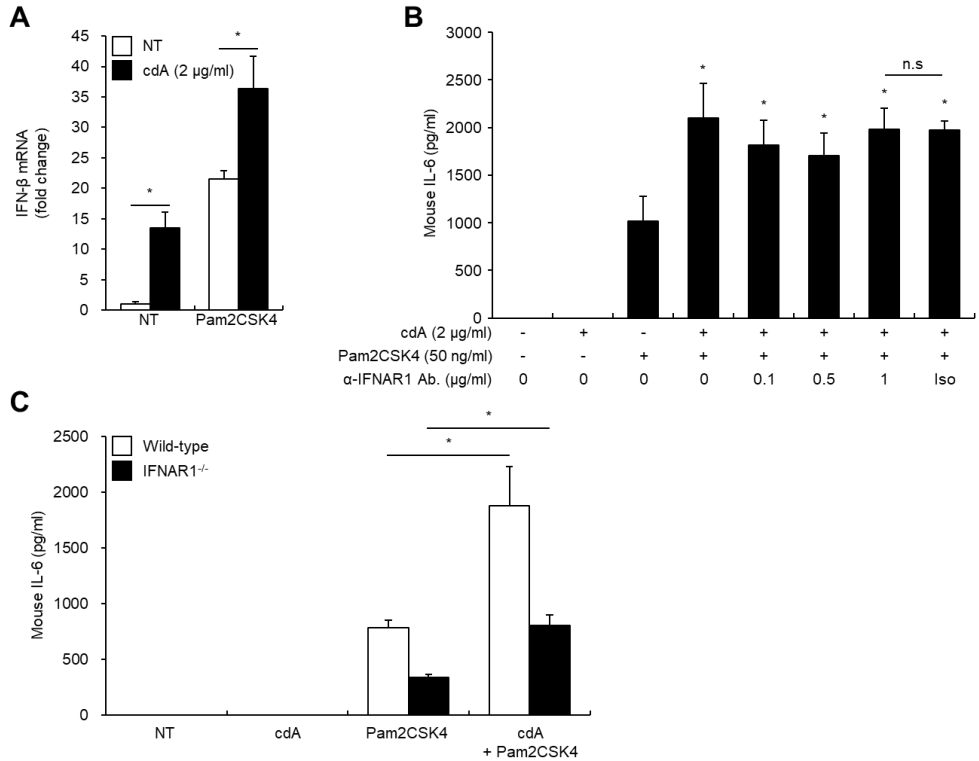

Figure 21. Type I IFN is not essential for the synergistic effects of c-di-AMP on Pam2CSK4-induced IL-6 production. (*A*) BMMs were incubated with c-di-AMP in the presence or absence of Pam2CSK4 for 6 hours. The expression level of IFN-β and β-actin was determined by real-time RT-PCR. (*B*) BMMs were pretreated with anti-IFNAR1 antibody or its isotype control antibody and then stimulated with c-di-AMP and Pam2CSK4 for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*C*) BMMs were prepared from wild-type or IFNAR1$^{-/-}$ mice. The cells were treated with c-di-AMP in the presence or absence of Pam2CSK4 for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. n.s = not significant. One of three similar results is shown.

Various STING ligands induce synergistic induction of IL-6.

To confirm if other STING ligands could induce the synergistic production of IL-6, BMMs were treated with various CDNs, such as c-di-AMP, c-di-GMP, 2'3' cGAMP, or 3'3' cGAMP, in the presence of Pam2CSK4. As shown in Fig. 22*A*, c-di-AMP, c-di-GMP, 2'3' cGAMP, or 3'3' cGAMP alone did not induced the IL-6 production. However, when BMMs were treated with CDNs and Pam2CSK4, Pam2CSK4-induced IL-6 production was significantly enhanced by c-di-AMP, c-di-GMP, 2'3' cGAMP, and 3'3' cGAMP (Fig. 22*A*). To provide further validation, BMMs were stimulated with DMXAA, which is a non-nucleotide STING ligand, in the presence of Pam2CSK4 or HKSa USA300 for 24 hours. Both Pam2CSK4- and HKSa USA300-induced IL-6 production was synergistically increased by DMXAA in a dose-dependent manner (Fig. 22*B* and *C*). These results suggest that not only nucleotide, but also non-nucleotide STING ligand increase the IL-6 production by Pam2CSK4 and HKSa.

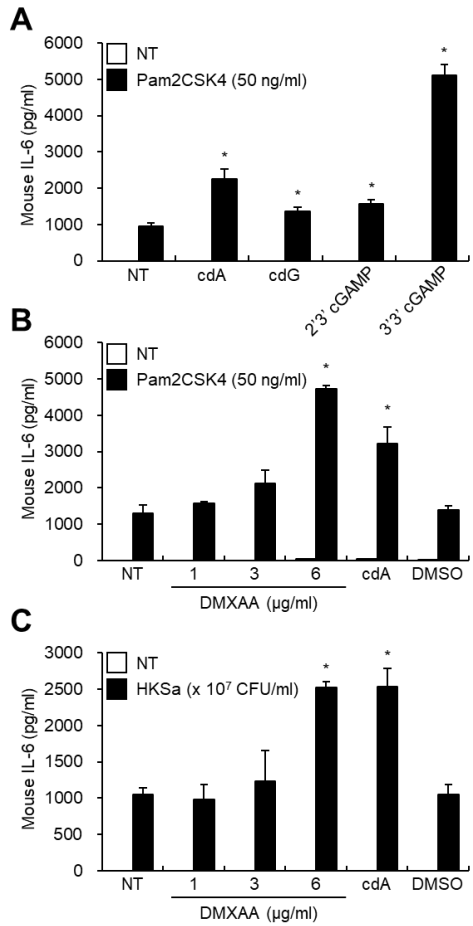

Figure 22. Various STING ligands induce the synergistic production of IL-6. (*A*) BMMs were stimulated with c-di-AMP, c-di-GMP, 2'3' cGAMP, or 3'3' cGAMP in the presence or absence of Pam2CSK4 for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. (*B, C*) BMMs were treated with c-di-AMP in the presence or absence of (*B*) Pam2CSK4 or (*C*) HKSa USA300 for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. cdG = c-di-GMP, HKSa = heat-killed *S. aureus* USA300. One of three similar results is shown.

Pam2CSK4 plus c-di-AMP exhibits no synergistic induction of IFN-β and IL-6 in the mouse calvarial osteoblasts.

To determine if c-di-AMP activates STING signaling pathway in osteoblasts, which have low STING activity, calvarial osteoblasts were stimulated with c-di-AMP in the presence or absence of Pam2CSK4. As shown in Fig. 23*A*, Pam2CSK4 significantly induced the mRNA expression of IFN-β in osteoblasts. However, c-di-AMP did not induce the mRNA expression of IFN-β in osteoblasts. In addition, Pam2CSK4 upregulated the mRNA expression of IL-6, but the synergistic expression of IL-6 by c-di-AMP in combination with Pam2CSK4 was not shown in osteoblasts (Fig. 23*B*). These results indicate that Pam2CSK4 together with c-di-AMP does not synergistically induce the expression of IFN-β and IL-6 probably due to low STING activity in osteoblasts.

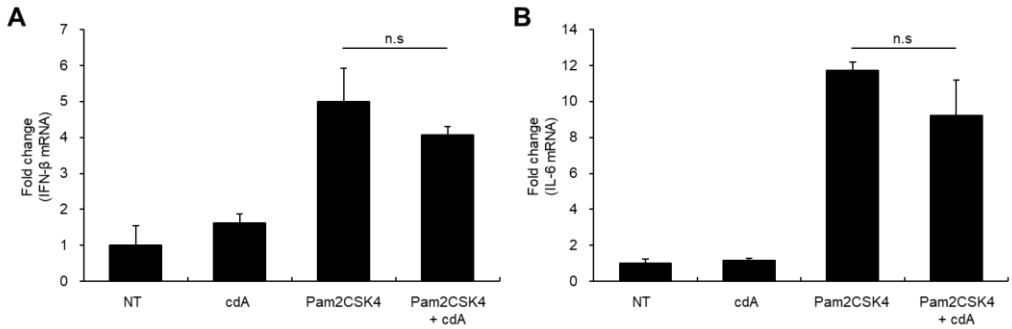

Figure 23. Pam2CSK4 together with c-d-AMP has no synergistic induction of IFN-β and IL-6 in the mouse calvarial osteoblasts. Primary calvarial osteoblasts were stimulated with cdA and/or Pam2CSK4 for 6 hours. (*A*) The mRNA expression levels of IFN-β and β-actin were determined by real-time RT-PCR. (*B*) The mRNA expression levels of IL-6 and β-actin were determined by real-time RT-PCR. NT = non-treatment. cdA = c-di-AMP. n.s = not significant. One of three similar results is shown.

c-di-AMP in combination with Pam2CSK4 synergistically increases the expression of various pro-inflammatory mediators.

To determine if c-di-AMP increases the expression of other pro-inflammatory mediators, BMMs were stimulated with c-di-AMP in the presence or absence of Pam2CSK4. c-di-AMP in combined with Pam2CSK4 synergistically induced the mRNA expression of inducible nitric oxide synthase at 12 hours (Fig. 24*A*). In addition, Pam2CSK4-induced nitric oxide production was significantly enhanced by c-di-AMP, c-di-GMP, and 3'3' cGAMP (Fig. 24*B*). Moreover, c-di-AMP synergistically enhanced the mRNA expression of IL-1β and TNF-α (Fig. 24*C* and *D*). These results suggest that STING agonists synergistically induce the expression of various pro-inflammatory mediators, such as nitric oxide, IL-1β, and TNF-α in combination with Pam2CSK4.

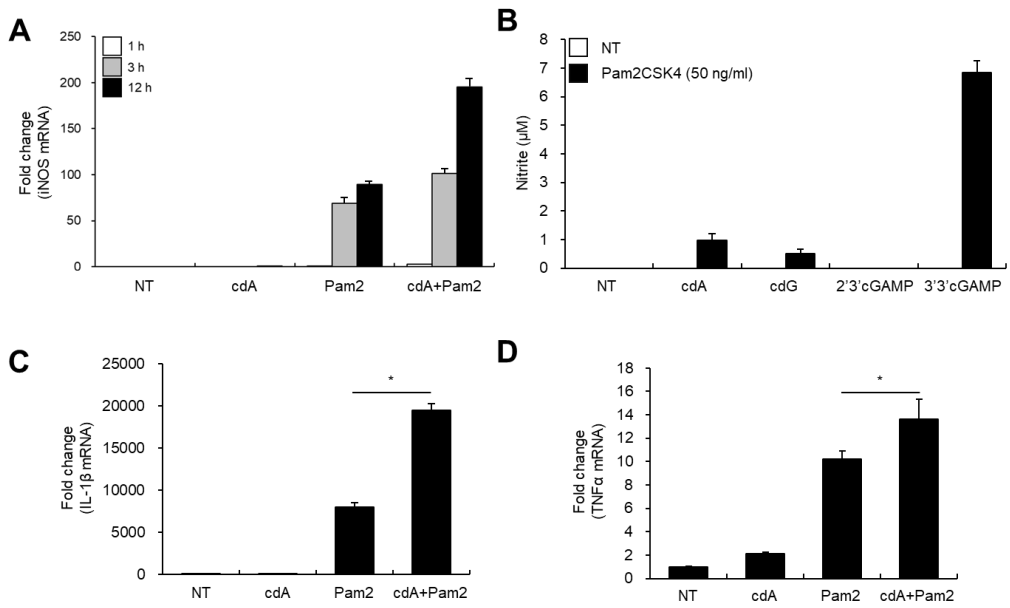

Figure 24. c-di-AMP together with Pam2CSK4 synergistically enhances the expression of various pro-inflammatory mediators. BMMs were stimulated with c-di-AMP in the presence or absence of Pam2CSK4 for 1, 3, or 12 hours. (*A*) The mRNA expression levels of inducible nitric oxide synthase and β-actin were determined by real-time RT-PCR. (*B*) BMMs were stimulated with c-di-AMP in the presence or absence of Pam2CSK4 for 24 hours. Nitric oxide in culture supernatant was measured using Griess reagent. (*C*, *D*) BMMs were stimulated with c-di-AMP in the presence or absence of Pam2CSK4 for 6 hours. (*C*) The mRNA expression levels of IL-1β and β-actin were determined by real-time RT-PCR. (*D*) The mRNA expression levels of TNF-α and β-actin was determined by real-time RT-PCR. **p* < 0.05. NT = non-treatment. cdA = c-di-AMP. Pam2 = Pam2CSK4. One of three similar results is shown.

c-di-AMP synergistically increases the IL-6 production in combination with various bacteria.

To determine if c-di-AMP increases the IL-6 production which induced by other bacteria, BMMs were stimulated with c-di-AMP in the presence or absence of various heat-killed bacteria. As shown in Fig. 25, c-di-AMP in combination with Gram-positive bacteria, including *Streptococcus mitis*, *S. sanguinis*, *S. gordonii*, *Bacillus cereus*, *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, synergistically increased the IL-6 production. In addition, c-di-AMP combined with Gram-negative bacteria, *Aggregatibacter actinomycetemcomitans*, also induced the synergistic production of IL-6. These results indicate that c-di-AMP synergizes with not only *S. aureus* but also other bacteria on the IL-6 production.

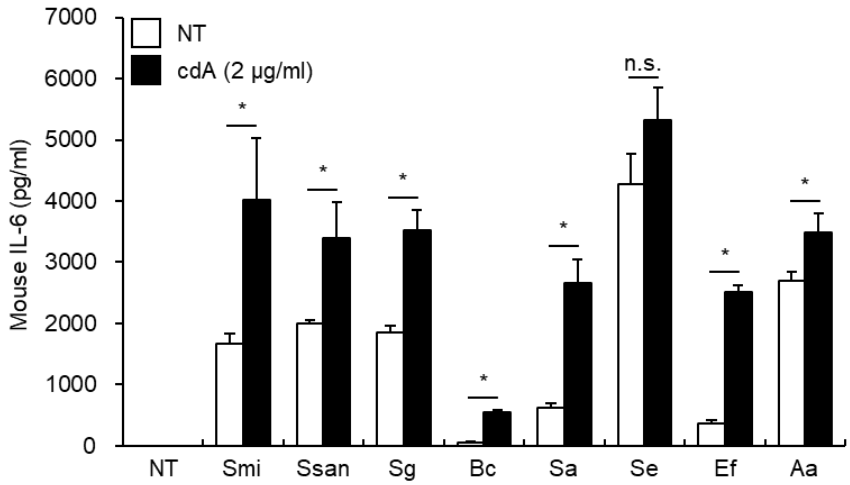

Figure 25. c-di-AMP synergizes with not only *S. aureus* but also other bacteria on the IL-6 production. BMMs were stimulated with c-di-AMP in the presence or absence of heat-killed bacteria for 24 hours. The culture supernatants were collected to measure the expression of IL-6 by ELISA. *$p < 0.05$. NT = non-treatment. cdA = c-di-AMP. Smi = *S. mitis*. Ssan = *S. sanguinis*. Sg = *S. gordonii*. Bc = *B. cereus*. Sa = *S. aureus*. Se = *S. epidermidis*. Ef = *E. faecalis*. Aa = *A. actinomycetemcomitans*. One of three similar results is shown.

c-di-AMP increases serum IL-6 levels and mortality in combination with Pam2CSK4.

It has been reported that excessive IL-6 production exhibits septic shock.[96] To investigate if c-di-AMP affects expression of serum IL-6 in combination with TLR2 agonist *in vivo*, mice were administrated with Pam2CSK4 in the presence or absence of c-di-AMP through intraperitoneal injection. Serum IL-6 levels were enhanced by c-di-AMP in combination with Pam2CSK4 on 6 hours (Fig. 26*A*). Next, to determine whether synergistic production of serum IL-6 levels by c-di-AMP was involved in symptom of sepsis, mice were intraperitoneally administrated with Pam2CSK4 and/or c-di-AMP in the presence or absence of anti-IL-6 antibody or its IgG isotype antibody for 96 hours. Pam2CSK4-administrated mice survived for 48 hours, and 75% of mice survived on 96 hours. In contrast, mice injected with c-di-AMP and Pam2CSK4 died within 72 hours and final survival rate was 37% at 96 hours, with severe morbidity (data not shown). Notably, when mice were administrated with Pam2CSK4 and c-di-AMP in the presence of anti-IL-6 antibody, survival rate was increased at 87% on 96 hours (Fig. 26*B*). These results suggest that c-di-AMP increases susceptibility to mortality through synergized induction of IL-6 in combination with Pam2CSK4.

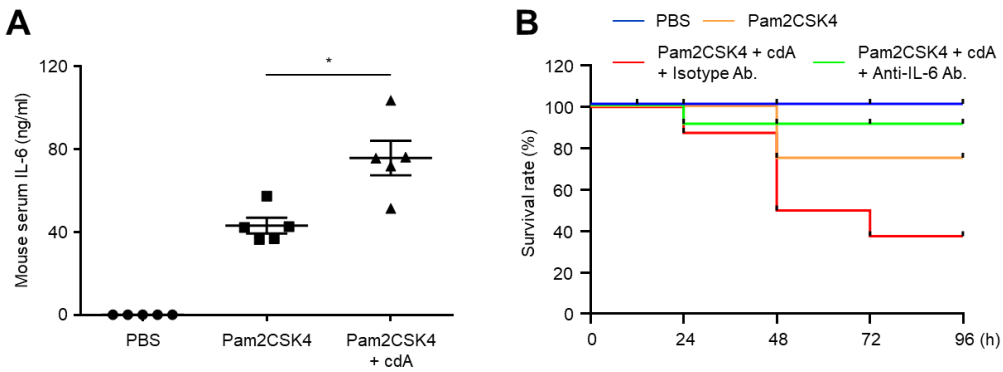

Figure 26. c-di-AMP increases mortality through enhancement of serum IL-6 expression induced by Pam2CSK4. (*A*) Mice were intraperitoneally administrated with Pam2CSK4 in the presence or absence of c-di-AMP. Serum was collected at 6 hours after the administration. Expression levels of IL-6 in the serum were determined by ELISA. One of five similar results is shown. (*B*) Mice were randomly divided into four groups and administrated with Pam2CSK4 and/or c-di-AMP in the presence of anti-IL-6 antibody or its IgG isotype antibody. Survival was monitored daily for 96 hours. Data shown represent the percent survival of 7 mice per group. $*p < 0.05$. cdA = c-di-AMP.

Discussion

This study demonstrated that c-di-AMP synergistically increased IL-6 production and mortality in combination with *S. aureus* or lipoproteins in both *in vivo* and *in vitro* experiments. Bacterial lipoproteins activate the TLR2-mediated MAPKs and NF-κB signaling pathway to induce IL-6 expression.[84] Upon TLR2 activation, unknown cell signaling mediator may be interacted with STING activated c-di-AMP, triggering the synergistic activation of NF-κB signaling pathway independent of TBK1-IRF3 cascade. (Fig. 27) Furthermore, blockade of serum IL-6 ameliorated morbidity and mortality. Therefore, the study suggests that c-di-AMP combined with lipoproteins synergistically enhances IL-6 production through up-regulation of NF-κB via STING signaling pathway, leading to increase mortality during bacterial infection.

The study showed that c-di-AMP exacerbated inflammatory conditions caused by *S. aureus* USA300 infection. Several studies suggest that cyclic dinucleotides have immunostimulatory roles in bacterial infection. Group B *Streptococcus* (GBS) degrades own c-di-AMP by ectonucleotidase and GBS ectonucleotidase mutant strain induces potent IFN-β expression, indicating that c-di-AMP from GBS is important for the host immune responses.[18] Similarly, *S. aureus* exposed to antibiotics elevates c-di-AMP production, leading to excessive airway inflammation.[97] In addition, extracellular c-di-AMP released from *S. aureus* biofilm, which is formed during bacterial infection, promotes host immune responses.[17,98] In the present study, c-di-AMP synergistically increased the serum

levels of pro-inflammatory cytokines. Indeed, c-di-AMP led to worsen mortality rate with symptoms of sepsis, such as reduced activity, lethargy, shivering, and face swelling (data not shown) during *S. aureus* USA300 infection. Thus, these results suggest that c-di-AMP is responsible molecule for causing sepsis by up-regulation of inflammatory conditions in bacterial infection.

Overwhelming production of pro-inflammatory cytokines and chemokines in serum is typical feature of septic shock.[99] Upon bacterial infection, chemokines recruit various immune cells to infected tissue or organ.[100] In addition, pro-inflammatory cytokines, including IL-1β, IL-6, IL-17, and TNF-α, stimulate various host cells, such as myeloid cell, lymphoid cell, hepatocyte, and keratinocyte, causing systematic inflammation.[101,102] Moreover, hyper-activated immune cells are implicated in causing multi-organ dysfunction and tissue damage.[103,104] In the present study, c-di-AMP elicited the synergistic production of chemokines, including CXCL1, CXCL2, and CCL4, during *S. aureus* USA300 infection. Indeed, c-di-AMP also synergistically enhanced the production of TNF-α and IL-6 induced by *S. aureus* USA300. Furthermore, blockade of serum IL-6, which is one of the key molecules in septic shock [105], ameliorated mortality caused by combination of Pam2CSK4 and c-di-AMP. Collectively, the results suggest that various inflammatory mediators enhanced by c-di-AMP is promising target for treatment of septic shock in bacterial infection.

Bacterial lipoprotein is critical for the synergistic effect by c-di-AMP. It has been reported that bacterial lipoproteins play an important role in promoting synergistic

immune responses with other MAMPs.[94,106-108] In the present study, c-di-AMP showed a synergistic effect on IL-6 production in combination with *S. aureus* wild-type, but not with lipoprotein-deficient *S. aureus*. In addition, c-di-AMP synergistically increased the IL-6 production in combination with lipoproteins isolated from *S. aureus* or Pam2CSK4 mimicking bacterial lipoprotein. Notably, intraperitoneal injection of Pam2CSK4 and c-di-AMP increased mortality and serum IL-6 levels. Concordant with the present study, it has been reported that intranasally administration of c-di-AMP with bacterial lipoprotein promotes neutrophil infiltration with potent pro-inflammatory production in murine airway.[97] Therefore, these results suggest that targeting bacterial lipoprotein and CDNs could be a novel strategy for treatment of severe inflammatory condition caused by bacterial infection.

STING, which is present on the host endoplasmic reticulum, directly recognizes viral nucleic acids [109], leading to the induction of host immune responses.[110] Moreover, not only viral nucleic acids but also bacterial CDNs can activate STING signaling pathway.[111] In the present study, the results showed that activation of STING is essential for the synergistic induction of IL-6 by bacterial lipoprotein. In addition, H-151, a STING inhibitor, potently reduced the synergistic induction of IL-6 by c-di-AMP and Pam2CSK4. c-di-AMP failed to increase the Pam2CSK4-induced IL-6 production in BMMs transfected with STING-targeting siRNA. In addition, other nucleotide STING ligands, including c-di-GMP, 2’3’ cGAMP, and 3’3’ cGAMP, also synergistically increased the Pam2CSK4-induced IL-6 production. Furthermore, it has been reported that STING activation also occurs by non-nucleotide STING ligand, such as DMXAA.[112] The results showed that DMXAA

synergized with *S. aureus* or Pam2CSK4 on the IL-6 production in BMMs. Taken together, activation of STING by its various ligands is critical for the synergistic induction of IL-6. Furthermore, it is likely that viral nucleic acid may be able to increase the pro-inflammatory cytokines through STING signaling pathway in combination with bacterial lipoproteins. Further study is needed to determine the role of STING in host immune response during superinfection.

Upon STING activation, canonical STING signaling pathway, a TBK1-IRF3 cascade, generally mediates to express the type I IFN.[13] However, in the present study, although TBK1 or IRF3 inhibitor successfully blocked the induction of IFN-β, c-di-AMP still showed synergistic effect on IL-6 production in combination with Pam2CSK4. Moreover, c-di-AMP synergistic enhanced the Pam2CSK4-induced IL-6 production in anti-IFNAR1 antibody treated BMMs or IFNAR1$^{-/-}$ BMMs, indicating that IFN-β is redundant for the synergistic effect by c-di-AMP. According to previous studies, STING rarely promotes the host immune responses independent of TBK1 or IRF3. Balka *et al*. demonstrated that TBK1 is unnecessary for STING-mediated pro-inflammatory cytokine production in myeloid cells.[113] In addition, non-canonical STING activation induces K63-linked ubiquitination on STING mediated by TRAF6, resulting in promoting NF-κB activation rather than IRF3 activation, after DNA damage.[114] Collectively, these results propose that canonical STING-TBK1-IRF3 axis and its end product, IFN-β, are not involved in the synergistic effect of c-di-AMP.

It is well known that TLR2 activated by bacterial lipoprotein activates MAPKs,

including p38, ERK1/2, and JNK, and NF-κB which are major transcriptional factors for the expression of IL-6.[94] The results showed that Pam2CSK4 increased the phosphorylation of MAPKs and NF-κB. Moreover, when BMMs were treated with c-di-AMP and Pam2CSK4, c-di-AMP upregulated the Pam2CSK4-induced phosphorylation of NF-κB, but not MAPKs. Possible mechanism for the upregulation of NF-κB activity by c-di-AMP in combination with Pam2CSK4 can be hypothesized. When TLR2 is activated by lipoprotein, TLR2 recruits TRAF6 and TAK1 to activate NF-κB signaling pathway.[115] It has been reported that TRAF6 is involved in STING activation to trigger NF-κB signaling pathway.[114,116] In addition, TAK1-IKK complex is critical for STING-mediated NF-κB activation.[113] Thus, it is likely that activation of STING by c-di-AMP could interact with TRAF6 or TAK1, which is recruited by Pam2CSK4-induced TLR2 activation, leading to synergistically amplify NF-κB signaling pathway. Further studies are needed to address the exact molecule which leads to synergize the IL-6 production by c-di-AMP and Pam2CSK4.

In conclusion, the results in the present study showed that CDNs combined with bacterial lipoproteins synergistically induce the IL-6 production, which is critical to exacerbate the mortality, through STING and TLR2 signaling pathway. Thus, targeting the STING and TLR2 signaling pathway could be a novel strategy for treatment of sepsis. Moreover, the co-stimulation of CDNs and lipoproteins which are abundantly expressed in most of bacteria may be important for the development of sepsis caused by bacterial infection. These findings place profound importance on acknowledging CDNs and lipoproteins as immunoregulatory therapeutics, thus

allowing efficient septic shock alleviation in bacterial infection.

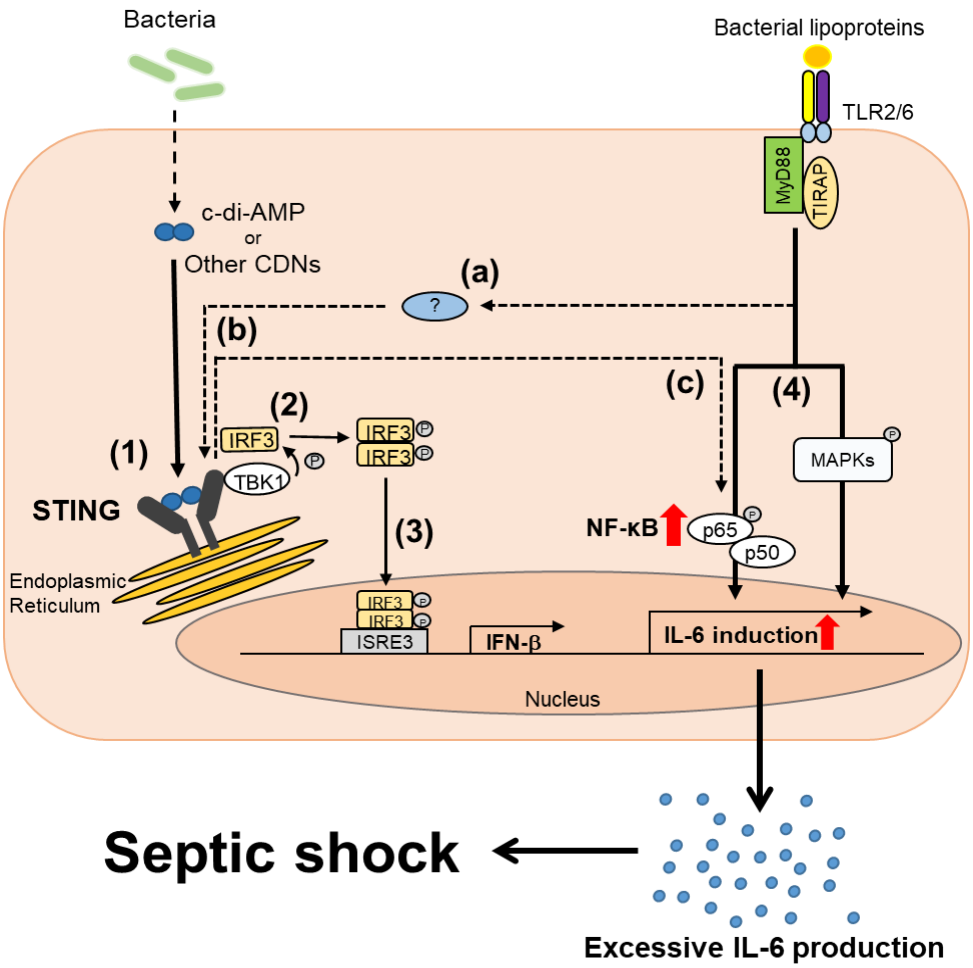

Figure 27. Schematic illustration of the proposed mechanisms for the synergistic effect of c-di-AMP and lipoprotein on IL-6 production. During bacterial infection, both cyclic dinucleotides and lipoprotein could activate the host immune responses. (1) Cytosolic cyclic dinucleotides are directly recognized by STING on host endoplasmic reticulum. (2) STING further recruits TBK1 and phosphorylates IRF3. (3) Phosphorylated IRF3 dimer entries to nucleus, inducing IFN-β. (4) Lipoprotein is recognized by TLR2, triggering the MAPKs and NF-κB signaling pathway to induce IL-6 expression. Upon TLR2 activation, (a) unknown cell signaling mediator may be activated and (b) interacted with STING, then (c) triggering the synergistic activation of NF-κB signaling pathway. The dotted lines indicate proposed mechanism of synergistic effect by c-di-AMP and lipoprotein on IL-6 production.

Conclusion

The present study demonstrates that CDN could modulate the innate immune response of macrophages to bacterial MAMPs, leading to the inhibition of osteoclast differentiation and bone destruction induced by RANKL (Fig. 28). Since CDNs are abundantly produced by commensal bacteria in the gut,[22] gut microbiota-derived CDNs might be delivered into the bone tissue.[53] Thus, gut microbiota-derived CDNs could be key molecules that are involved in gut-bone axis for maintaining bone homeostasis. On the other hand, CDNs in combination with bacterial lipopeptides exacerbate morbidity and mortality due to excessive IL-6 production in bacterial infection (Fig. 28). These results suggest that targeting CDNs could be important for the treatment of sepsis caused by bacterial infection. Furthermore, based on the mechanism study, CDNs modulate the osteoclast differentiation and immune responses through STING signaling pathway in macrophages. Therefore, controlling the STING signaling pathway by CDNs could be novel strategies for the treatment or prevention of bone or inflammatory diseases.

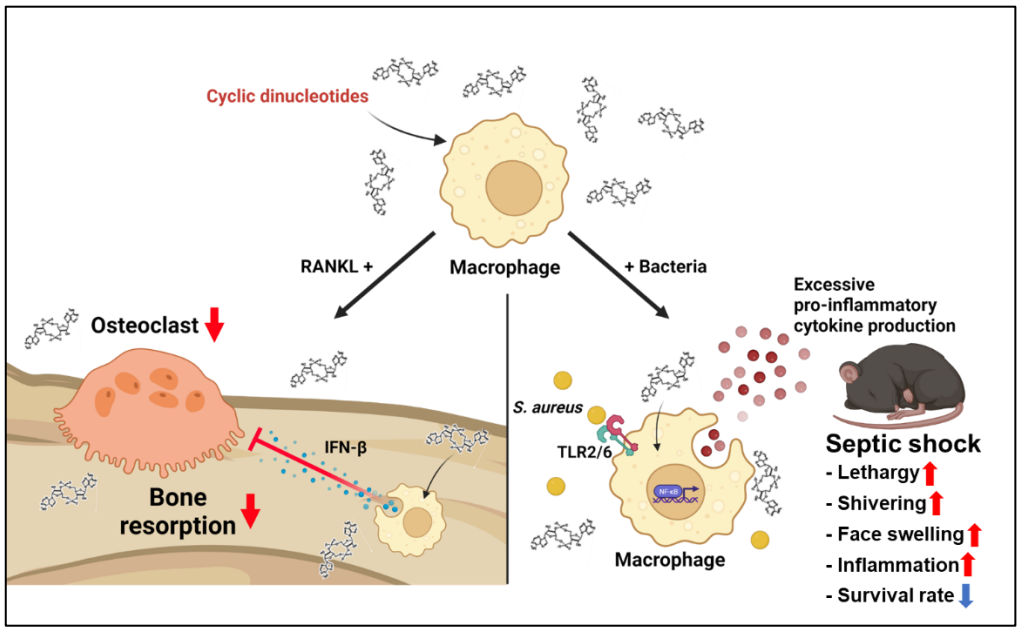

Figure 28. Schematic illustration of the proposed mechanisms. CDNs inhibits RANKL-induced osteoclastogenesis from macrophages through induction of type I IFN via STING signaling pathway. Moreover, CDNs attenuate the bone destruction by inhibiting osteoclast differentiation. On the other hand, CDNs combined with lipoproteins synergistically enhance IL-6 production through up-regulation of NF-κB via STING signaling pathway, leading to increase mortality during bacterial infection.

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국문초록

고리형 디뉴클레오타이드에 의한 파골세포 형성 및 포도상구균 감염 대응 면역반응 조절

권 영 각

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1. 목 적

숙주-세균 상호작용은 세균에 존재하는 미생물-연관 분자패턴이 숙주세포의 패턴인식수용체를 활성화시킴으로써 일어나게 된다. 다양한 미생물-연관 분자패턴 중 고리형 디뉴클레오타이드 (cyclic dinucleotdie, CDN)는 이차전달체로 대부분의 세균이 분비하고 있으며, 세균의 생장, 집락 형성, 바이오필름 형성 등에 필수적으로 관여하는 물질이다. 또한 분비된 CDN은 숙주 면역세포에 존재하는 stimulator of interferon genes (STING)에 의해 인지되고 면역반응을 활성화시켜 숙주-세균 상호작용에 중요한 역할을 한다고 알려져 있다. 하지만 기존 연구에서는 CDN 단일의 면역활성 효과만을 검증하였기 때문에 실제 숙주-세균 상호작용의 복합성을 대변하지 못하는 한계가 있다. 따라서 기존연구의 한계를 극복하기 위해 CDN과 다른 요인들의

복합적 효과를 확인하는 연구가 필요하다. 본 연구에서는 CDN이 파골세포분화유도인자 receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) 또는 세균 세포벽성분과 함께 대표적 선천면역세포인 대식세포의 파골세포 분화 및 면역반응 조절에 미치는 혼합효과를 분자적 수준에서 규명하고자 하였다.

2. 방 법

CDN이 RANKL에 의한 대식세포의 파골세포 분화를 조절하는지 평가하기 위해 골수유래 대식세포를 준비한 후 RANKL과 CDN을 동시처리하여 3일 동안 배양하였다. 파골세포의 분화양상은 TRAP염색법과 RT-PCR기법을 통해 분석하였다. CDN에 의한 영향이 특이적 인식수용체 STING에 의한 것인지 검증하기 위해 대식세포에 STING-siRNA를 처리한 후 CDN과 RANKL을 처리하여 파골세포로 분화를 유도하였으며 TRAP염색법을 통해 분화양상을 확인하였다. 파골세포 분화 중 CDN에 의한 STING 신호활성을 규명하기 위해 STING 하위 신호매개체인 TBK1과 IRF3의 인산화를 Western blotting으로 검증하였다. CDN에 의해 분비되는 인터페론이 파골세포에 영향을 미치는지 확인하기 위해 야생형 및 인터페론수용체결손 C57BL/6 쥐로부터 골수유래 대식세포를 얻고 CDN과 RANKL을 동시처리하여 파골세포로 분화를 유도하였다. 이후 TRAP염색법과 Western blotting을 통해 파골세포 분화양상 및 인터페론수용체 하위 신호매개체의 인산화 정도를 확인하였다. CDN에 의한 파골세포 분화조절 능력의 생체 내 효용성 검증을 위해 CDN과 RANKL을

C57BL/6 쥐의 두개골에 삽입한 후 마이크로 컴퓨터 단층촬영을 이용해 골파괴 정도를 비교분석 하였다. 다음으로 CDN이 감염상황에서 세균에 의한 면역반응에 영향을 미칠지 확인하기 위해 C57BL/6 쥐에 병독성 세균인 *Staphylococcus aureus*와 CDN을 복강주사 후 쥐의 생존을 시간대별로 확인하였다. 같은 조건에서 혈청을 얻고 CDN에 의한 혈청 내 IL-6 농도를 ELISA를 통해 확인하였다. CDN과 유의적인 혼합효과를 보이는 세균 유래물질을 규명하기 위해 CDN과 야생형 또는 지질단백질결손 *S. aureus* 사균을 골수유래 대식세포에 동시처리 후 ELISA를 통해 IL-6의 발현양상을 확인하였다. 또한 세균으로부터 지질단백질을 분리하여 CDN과 대식세포에 동시처리하고 ELISA를 통해 IL-6의 발현을 확인하였다. CDN에 의한 면역활성 변화에 핵심적으로 관여하는 인식수용체를 규명하기 위해 대식세포에 STING-siRNA 또는 억제제를 처리하고 CDN과 지질단백질로 자극시킨 후 ELISA로 IL-6의 발현을 비교분석하였다. CDN에 의해 변화하는 염증인자 발현양상에 대한 분자적 기전을 규명하기 위해 대식세포에 CDN과 지질단백질을 혼합처리하고 세포 내 신호전달 매개체 인산화 차이를 Western blotting과 공초점현미경을 통해 관찰하였다. 마지막으로 CDN과 지질단백질 및 IL-6 중성화항체를 쥐의 복강으로 투여하고 생존을 시간대별로 확인하였다.

3. 결 과

CDN은 RANKL에 의한 대식세포의 파골세포 분화를 농도의존적으로 억제하였다. 특히 CDN은 RANKL에 의해 유도되는 파골세포 분화

전사인자들을 유의적으로 억제하였다. CDN의 파골세포 분화억제 효능은 파골세포/조골세포 동시배양 환경에서도 확인되었다. 반면 CDN은 조골세포의 분화와 활성에는 아무런 영향을 주지 못하였다. 다음으로 CDN이 RANKL과 함께 대식세포에 처리되었을 때 CDN-특이적 수용체인 STING의 활성화가 나타남을 확인하였다. 또한 CDN의 파골세포 분화 억제효능은 STING-siRNA로 인해 STING의 발현이 감소한 대식세포에서는 관찰되지 않았다. 이로써 STING은 CDN에 의한 파골세포 억제효능에 필수적인 수용체임을 검증할 수 있었다. CDN이 RANKL과 함께 대식세포에 처리되었을 때 파골세포 분화 억제효과를 보이는 인터페론의 발현이 지속적으로 유도되었다. CDN에 의한 억제효과는 인터페론수용체 중화항체로 인하여 감소되었고, 이러한 현상은 인터페론수용체결손 대식세포에서도 확인되었다. CDN은 쥐 두개골에서 RANKL에 의한 파골세포의 분화를 억제하였으며 골 파괴 또한 유의적으로 감소시켰다. 다음으로 CDN을 *S. aureus*와 함께 복강으로 주사하였을 때, *S. aureus* 단독주사군에 비해 CDN 동시주사군에서 혈청 내 IL-6 발현의 상승효과가 관찰되었으며 최종 생존율은 감소하였다. 시험관 내 실험에서도 CDN과 *S. aureus* 사균의 동시처리는 대식세포에서 IL-6 발현에 대한 상승효과를 보였다. 반면 CDN은 지질단백질 결손 *S. aureus* 사균에 의한 대식세포의 IL-6 발현에 아무런 영향을 미치지 못하였다. CDN에 의한 IL-6의 상승효과는 대식세포에 정제된 지질단백질 또는 합성지질단백질이 동시처리 되었을 때도 관찰되었다. 따라서 CDN은 세균의 지질단백질과 혼합효과를 보이는 것으로 규명되었다. CDN에 의한 IL-6 발현의 상승효과는 STING-

siRNA 또는 STING억제제로 STING의 활성이 억제된 대식세포에서 보이지 않았다. 반면 STING의 하위 신호전달 매개체 억제제가 처리된 대식세포에서는 CDN과 지질단백질에 의한 IL-6 발현 상승효과가 유지되었다. 지질단백질은 신호전달 매개체 MAPK 또는 NF-κB의 인산화를 유도하였는데, CDN은 그 중 NF-κB의 인산화와 NF-κB의 핵 내 이동을 증가시켰다. 마지막으로 C57BL/6 쥐의 복강을 통해 지질단백질과 CDN을 주사하였을 때 혈청 내 IL-6가 지질단백질 단독주사군보다 증가하였다. 또한 CDN과 지질단백질의 동시주사로 인한 생존율은 지질단백질 단독주사군과 비교했을 때 감소하였으며, IL-6 중성화항체의 주사로 생존율이 회복됨을 관찰하였다.

4. 결 론

이상의 연구결과를 통해 CDN은 대식세포의 면역활성을 유도하여 파골세포 분화와 골파괴를 억제시키는 긍정적 효과가 있는 반면 세균감염 상황에서는 과도한 면역활성으로 생존율을 감소시키는 부정적 효과도 있음을 확인하였다. 이러한 복합적 결과는 추후 숙주-세균 상호작용을 연구하는데 있어 다중적 요인을 고려하는 것이 필수적이라는 통찰을 제시해 줄 것으로 기대된다.

주요어: 고리형 디뉴클레오타이드, 대식세포, 파골세포, 염증성 질환, STING

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