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Human β -defensin 2 is involved in CCR2-mediated Nod2 signal transduction, leading to activation of the innate immune response in macrophages

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ARTICLE INFO

Keywords:

Adaptive immunity
Defensin
Innate immunity
Signaling
Virus

ABSTRACT

Beta-defensins contribute to host innate defense against various pathogens, including viruses, although the details of their roles in innate immune cells are unclear. We previously reported that human β -defensin 2 (HBD 2) activates primary innate immunity against viral infection and suggested that it plays a role in the induction of the adaptive immune response. We analyzed the mechanisms by which HBD 2 primes innate antiviral immunity and polarized activation of macrophage-like THP-1 cells using the receptor-binding domain (RBD) of Middle East respiratory syndrome coronavirus (MERS-CoV) spike protein (S RBD) as a model antigen. The expression of nucleotide-binding oligomerization domain containing 2 (Nod2), type I interferons, (IFNs), and proinflammatory mediators was enhanced in S RBD-HBD 2-treated THP-1 cells. S RBD-HBD 2 treatment also enhanced phosphorylation and activation of receptor-interacting serine/threonine-protein kinase 2 and IFN regulatory factor 3 compared to S RBD alone. Finally, HBD 2-conjugated S RBD interacted with C-C chemokine receptor 2 (CCR2), and Nod2 was involved in HBD 2-mediated CCR2 signaling, which was associated with the activation and M1 polarization of THP-1 cells. Therefore, HBD 2 promotes CCR2-mediated Nod2 signaling, which induces production of type I IFNs and an inflammatory response, and enhances primary innate immunity leading to an effective adaptive immune response to HBD 2-conjugated antigen.

1. Introduction

Antimicrobial peptides (AMPs) are a component of the primary host defense in the mucosa. AMPs are produced mainly by epithelial cells and the cells involved in innate immunity. The functions of AMPs in defense against various infections are better characterized than their role as innate immune modulators (Boyton and Openshaw, 2002). Defensins are small cationic AMPs and are found in various organisms including mammals, insects, and plants. Among them, β -defensins are primarily produced by epithelial cells and leukocytes in vertebrates (Stolzenberg et al., 1997). Immature dendritic cells (DCs) can be activated by β -defensins, and β -defensins also inhibit infection by *Haemophilus influenzae* and viruses (Zhao et al., 2016; Lafferty et al., 2017). Inactivation of human β -defensins (HBDs) leads to the recurrent airway infections experienced by patients with cystic fibrosis (Smith et al., 1996). Also, a lack of human β -defensin 2 (HBD 2) results in immune dysfunction, such as reduced numbers of B and regulatory T cells, resulting in decreased production of antigen (Ag)-specific

immunoglobulin A (Lugering et al., 2005; McDonald et al., 2007). Therefore, an understanding of the regulatory mechanism by which AMPs modulate the immune response during the early stage of viral infection is critically needed to deal with various diseases caused by virus infection.

Virus infection of host cells triggers innate antiviral responses, which are initiated via pattern recognition receptor (PRR) signaling pathways (Ishii et al., 2008). Among the PRR families, nucleotide-binding oligomerization domain containing 2 (Nod2) is an important mediator of the innate immune response to viral infection, and induces expression of type I interferons (IFNs) to promote the expression of proinflammatory cytokines and restrict viral replication (Wiese et al., 2017). Furthermore, Nod2-deficient mice exhibit decreased production of IFNs and increased susceptibility to viral infection (Sabbah et al., 2009). Presumably, the ability of a virus to counteract innate antiviral immunity during the early stage of infection influences pathogenicity and disease severity (Perlman and Dandekar, 2005). Type I IFNs play a major role in the antiviral innate immune response by upregulating the

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<https://doi.org/10.1016/j.imbio.2019.05.004>

Received 8 April 2019; Received in revised form 13 May 2019; Accepted 16 May 2019

Available online 18 May 2019

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production of antiviral proteins and the recruitment of immune cells (Haller et al., 2006). Production of type I IFN is initiated by ubiquitously expressed cytoplasmic viral sensors in response to detection of viral pathogen-associated molecular patterns such as double-stranded RNA (Kato et al., 2011; Li and Zhong, 2018). Stimulated viral sensors activate downstream signaling pathways, leading to expression of transcription factors including IFN regulatory factor 3 (IRF3) and nuclear factor- κ B (NF- κ B), which drive IFN- β expression (Yoneyama et al., 2004). However, some viruses, including Middle East respiratory syndrome coronavirus (MERS-CoV), inhibit these type I IFN induction pathways (Zielecki et al., 2013). For example, various proteins of MERS-CoV, including M protein, papain-like protease protein (PLpro), and accessory proteins 4a and 4b, are antagonists of IFNs (Shokri et al., 2019). Accordingly, the virulence of MERS-CoV is linked to its immune evasion mechanisms, such as suppression of IFN production during the early stage of infection, induction of macrophage apoptosis, and inactivation of T cells with downregulation of Ag presentation (Niemeyer et al., 2013).

Macrophages are professional phagocytes capable of internalizing and degrading pathogens and apoptotic cells. Macrophages are present in the respiratory mucosa, such as in the lung and various fluid compartments, where the detection of and the response to infection occur. Due to their location, macrophages detect viral Ags first and promote an antiviral innate immune response as well as an Ag-specific adaptive immune response by presenting Ags to T-cells (Manicassamy et al., 2010). We previously reported that HBD 2 promotes an antiviral innate immune response in macrophage-like THP-1 cells and elicits an enhanced Ag-specific and virus-neutralizing antibody (Ab) response *in vivo* using the receptor binding domain (RBD) of MERS-CoV spike protein (S RBD) as a model Ag (Kim et al., 2018). Moreover, the type I IFN response and the production of primary antiviral molecules such as Nod2 were enhanced by S RBD-HBD 2 treatment of THP-1 cells (Kim et al., 2018). Consequently, we assumed that modulation of Nod2 signaling by HBD 2 would prevent infection by viruses that suppress innate antiviral immunity. In this study, we investigated the mechanism by which HBD 2 enhances the type I IFN immune response in THP-1 cells by modulating Nod2 signaling pathways using HBD 2-conjugated S RBD of MERS-CoV.

2. Materials and methods

2.1. Experimental animals and materials

Macrophage-like THP-1 (ATCC[®] TIB-202[™]) and Vero E6 (ATCC[®] CRL-1586[™]) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MERS-CoV (1-001-MER-IS-2015001) was provided by the Korean Center for Disease Control and Prevention (KCDC). All experiments using MERS-CoV were performed in accordance with the World Health Organization recommendations in a biosafety level 3 facility in the Korea Zoonosis Research Institute at Chonbuk National University (Iksan, Korea). The chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and SPL Life Sciences (Pocheon, Korea), respectively, unless otherwise specified.

2.2. Recombinant protein production

Production of recombinant MERS-CoV S RBD with or without HBD 2 at the C-terminus (residues 291–725) of the S1 domain were performed as described previously with minor modifications (Ma et al., 2014). Briefly, the gene encoding S RBD was synthesized with codon optimization based on the MERS-CoV S protein sequence (GenBank AKL59401.1; GenScript, Piscataway, NJ, USA). The S RBD gene with the HBD 2 gene at its 3' terminus was amplified by polymerase chain reaction (PCR) using forward and reverse primers reported previously (Kim et al., 2018). The amplified genes were cloned into the pColdII

Escherichia coli expression vector (TaKaRa Bio, Shiga, Japan). Recombinant proteins with an N-terminal His tag were purified using Ni-NTA Superflow (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

2.3. Cell culture

THP-1 cells were cultured in RPMI medium (Welgene, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37 °C in a CO₂ incubator. The cells were treated with phorbol-12-myristate-13-acetate (1 µg/mL for 1 × 10⁶ cells) for 2–3 days to induce differentiation into macrophages (Daigneault et al., 2010). The cultures were replenished with fresh medium, maintained for 3 days, and incubated with recombinant S RBD or S BRD-HBD 2 (1 µg/mL per 1 × 10⁶ cells) with or without inhibitors (RS 102,895 for CCR2 and GSK 717 for Nod2). The cells were harvested after 6 or 24 h and subjected to quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) or western blotting.

2.4. RNA extraction and quantitative real-time RT-PCR

RNA was extracted using TRIzol[®] reagent (Thermo-Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA was used to synthesize cDNA with an MMLV Reverse Transcription Kit (Promega, Fitchburg, WI, USA). Gene expression levels were measured by quantitative real-time RT-PCR with the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) and an ABI 7500 system (Applied Biosystems, Foster City, CA, USA) using 50 ng of first-strand cDNA under the following conditions: 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. The expression levels were normalized to that of β -actin (hACTB) using 7500 FAST software version 2.0.6 (Applied Biosystems). The primer sets used to amplify target genes are listed in Table 1.

2.5. Protein preparation and western blotting

Cells were washed twice with cold phosphate-buffered saline (PBS), and lysed in a lysis buffer containing 1% Triton X-100 supplemented

Table 1
Sequences of the primers used for qRT-PCR. Primers used to measure the expression levels of genes associated with antiviral innate immune responses and macrophage differentiation. The β -actin gene (hACTB) was used as an endogenous control.

Gene	Primer sequences
hACTB	F: 5'-CCA ACC GCG AGA AGA TGA-3' R: 5'-TCC ATC ACG ATG CCA GTG-3'
IFN- α	F: 5'-GAA GCT TYC TCC TGY YTG AWG GAC AGA-3' R: 5'-GGG GAT CCT CTG ACA ACC TCC CAN GCA CA-3'
IFN- β	F: 5'-TTT CAG TGT CAG AAG CTC CT-3' R: 5'-TGG CCT TCA GGT AAT GCA GA-3'
TNF- α	F: 5'-GGA GAA GGG TGA CCG ACT CA-3' R: 5'-CTG CCC AGA CTC GGC AA-3'
Nod2	F: 5'-CGG CGT TCC TCA GGA AGT AC-3' R: 5'-ACC CCG GGC TCA TGA TG-3'
RIP2	F: 5'-CCA TTG AGA TTT CGC ATC CT-3' R: 5'-ATG CGC CAC TTT GAT AAA CC-3'
IRF3	F: 5'-TCT TCC AGC AGA CCA TCT CC-3' R: 5'-TGC CTC ACG TAG CTC ATC AC-3'
CD14	F: 5'-CGA GGA CCT AAA GAT AAC CGG C-3' R: 5'-GTT GCA GCT GAG ATC GAG CAC-3'
CD16	F: 5'-GAC AGT GTG ACT CTG ACT CC-3' R: 5'-GCA CCT GTA CTC TCC AC-3'
CD86	F: 5'-CTC TTT GTG ATG GCC TTC CTG-3' R: 5'-CTT AGG TTC TGG GTA ACC GTG-3'
upE	F: 5'-GCC TCT ACA CGG GAC CCA TA-3' R: 5'-GCA ACG CGC GAT TCA GTT-3'

F and R, sequences of the forward and reverse primers, respectively.

with a complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and 2 mM dithiothreitol. Total cell lysates were prepared by centrifugation at 10,000 rpm for 10 min. Equal amounts of lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon-P polyvinylidene difluoride membrane (Merck Millipore, Burlington, MA, USA), and immunoblotted using specific Abs. Primary Abs against human p-RIP2 and p-IRF3 were obtained from Abcam (Cambridge, MA, USA) and Abs against Nod2 and I κ B α were from Invitrogen (Grand Island, NY, USA) and Cell Signaling (Danvers, MA, USA), respectively. The primary Ab against human β -actin was purchased from Bioss (Woburn, MA, USA). Target proteins were detected by enhanced chemiluminescence (Thermo-Fisher Scientific).

2.6. Viral infection

MERS-CoV was propagated in Vero E6 cells, which were cultured in DMEM medium (Welgene) containing 10% FBS at 37 °C in a CO₂ incubator. To assess the viral loads and the expression levels of target genes in MERS-CoV-infected cells, MERS-CoV was passaged six times in Vero E6 cells and transferred to THP-1 cells (2×10^6 /well) in a tissue culture plate. After incubation for 24 h, we extracted total RNA and performed quantitative real-time RT-PCR using the primers in Table 1 to measure the expression levels of MERS-CoV upE and target genes (Kim et al., 2018).

2.7. Immunofluorescence assay

THP-1 cell monolayers in confocal dishes were fixed with 4% paraformaldehyde. The cells were permeabilized using buffer containing Triton X-100 (0.1%), blocked, and incubated with the recombinant protein and a specific primary Ab. Primary Abs against human C-C chemokine receptor 2 (CCR2) and Nod2 were purchased from Invitrogen. A Penta-His Ab conjugated to Alexa Fluor® 488 (Qiagen) and anti-rabbit and -mouse IgG conjugated to Alexa Fluor 555 or 568 (Invitrogen), respectively, were used as the secondary Abs. Finally, washed cells were stained with 4'6-diamidino-2-phenylindole (DAPI), covered with SlowFade Gold Antifade Reagent (Invitrogen), and observed using a confocal laser scanning microscope (CLSM, LSM 510, Carl Zeiss, Thornwood, NY, USA).

2.8. Statistical analysis

Statistical analyses were performed using Prism 7 (GraphPad, San Diego, CA, USA). Data are means \pm standard deviations (SDs). The significance of differences was assessed by two-way analysis of variance (ANOVA), and $p < 0.05$ was considered indicative of statistical significance.

3. Results

3.1. HBD 2-conjugated Ag stimulates the Nod2 signaling procedure, which leads to type I IFN production in macrophage-like THP-1 cells

MERS-CoV infection inhibits the production of IFN- α/β and the host antiviral immune response (Zielecki et al., 2013). We evaluated the influence of MERS-CoV infection of THP-1 cells on the innate immune response (Fig. 1). The expression level of the upE gene was assessed by quantitative real-time RT-PCR at 24 h post-infection of THP-1 cells with 10^3 , 10^4 , and 10^5 plaque-forming units (PFU) of MERS-CoV (Fig. 1A). Importantly, MERS-CoV infection dose-dependently decreased the expression of factors critical in antiviral innate immunity, such as Nod2, IFN- α , and IFN- β (Fig. 1B–D).

To analyze whether HBD 2 affects immune induction, we determined the expression levels of the IFN- β , Nod2, and tumor necrosis factor (TNF)- α genes in THP-1 cells treated with S RBD or S RBD-HBD 2

(Fig. 2). S RBD-HBD 2 significantly ($p < 0.01$) enhanced the expression of IFN- β and Nod2 by 2.18- and 7.35-fold, respectively, compared to S RBD alone-treated THP-1 cells (Fig. 2A and B). The expression of TNF- α , an NF- κ B-dependent proinflammatory cytokine, was increased in S RBD-HBD 2-treated cells compared with that in S RBD alone-treated cells, albeit not significantly so (Fig. 2C). We next assessed the levels of the Nod2-associated signal transducing mediators, receptor interacting protein-2 (RIP2) and IRF3, in S RBD- or S RBD-HBD 2-treated THP-1 cells (Fig. 3). As expected, treatment with S RBD-HBD 2 remarkably upregulated the phosphorylation of RIP2 and IRF3 and the Nod2 protein level. In addition, HBD 2-conjugated S RBD reduced I κ B α accumulation, degradation of which requires NF- κ B activation (Fig. 3C). Therefore, HBD 2-conjugated Ag treatment induces the production of the type I IFN, IFN- β , and the proinflammatory cytokine, TNF- α , through Nod2-associated RIP2 response by activating the transcription factors IRF3 and NF- κ B.

3.2. HBD 2-conjugated Ag-mediated induction of a type I IFN response was mediated by CCR2 signaling

β -Defensin-fused proteins retain their antibacterial and chemotactic activity for C-C chemokine receptor 6 (CCR6)-expressing cells, as do monocytes that do not express CCR6 (Röhrli et al., 2010a). In addition, the β -defensin-conjugated proteins induced CCR2-specific chemotaxis on CCR2-transfected HEK293 cells, human peripheral blood monocytes, and mouse peritoneal exudate cells in a dose-dependent manner (Röhrli et al., 2010b). Therefore, we determined whether HBD 2 binds to CCR2 rather than CCR6 (Fig. 4) by immunofluorescence assay in THP-1 cells (Fig. 4A). CCR2 and S RBD-HBD 2 co-localized, suggesting a direct interaction, but no such co-localization with CCR2 was observed for S RBD alone (data not shown). We next evaluated whether the interaction of HBD 2 with CCR2 mediates intracellular signaling. Interestingly, the significantly ($p < 0.01$) enhanced IFN- β expression by S RBD-HBD 2 was abrogated by the CCR2 antagonist, RS 102,895 (Fig. 4B). Moreover, the Nod2 expression and Nod2-associated RIP2 and IRF3 expression enhanced significantly ($p < 0.05$) by S RBD-HBD 2 was abrogated by the CCR2 antagonist (Fig. 4C–E), as was TNF- α , whose expression is dependent on NF- κ B activation (Fig. 4F). Thus, HBD 2 interacts directly with CCR2, which promotes signal transduction, activation of type I IFN, and an inflammatory response.

3.3. Nod2 is involved in HBD 2-mediated intracellular signaling

We next focused on endosomal and/or cytoplasmic signal transducers involved in HBD 2-mediated activation of primary antiviral responses. In our previous report, a proinflammatory cytokine response, which requires the activation of NF- κ B, was induced by HBD 2 (Kim et al., 2018). Additionally, early stimulation of an innate immune response is dependent on Toll-like receptor (TLR) 4 and/or Nod2-triggered NF- κ B signaling (Tsai et al., 2011). We thus determined whether the HBD 2-mediated enhancement is due to Nod2-associated signaling because Nod2 expression was enhanced by S RBD-HBD 2 (Fig. 5). S RBD-HBD 2 co-localized with Nod2, suggesting an interaction (Fig. 5A), but no such co-localization with Nod2 occurred for S RBD without HBD 2 conjugation (data not shown). Consequently, we speculated that Nod2 interacts with S RBD-HBD 2 and that Nod2-mediated signaling is involved in HBD 2-mediated activation of NF- κ B. We evaluated the influence of Nod2 signaling on HBD 2-mediated type I IFN and proinflammatory responses using the inhibitor of Nod2 signaling, GSK 717. GSK 717 abrogated the HBD 2-mediated ($p < 0.01$) enhanced expression of IFN- β , RIP2, and IRF3 (Fig. 5B–D). Additionally, a Nod2 inhibitor abrogated the enhanced expression of TNF- α in HBD 2-treated THP-1 cells (Fig. 5E). These observations suggest that Nod2 functions as an intracellular signal transducer in HBD 2-induced activation of type I IFN production and the inflammatory response.

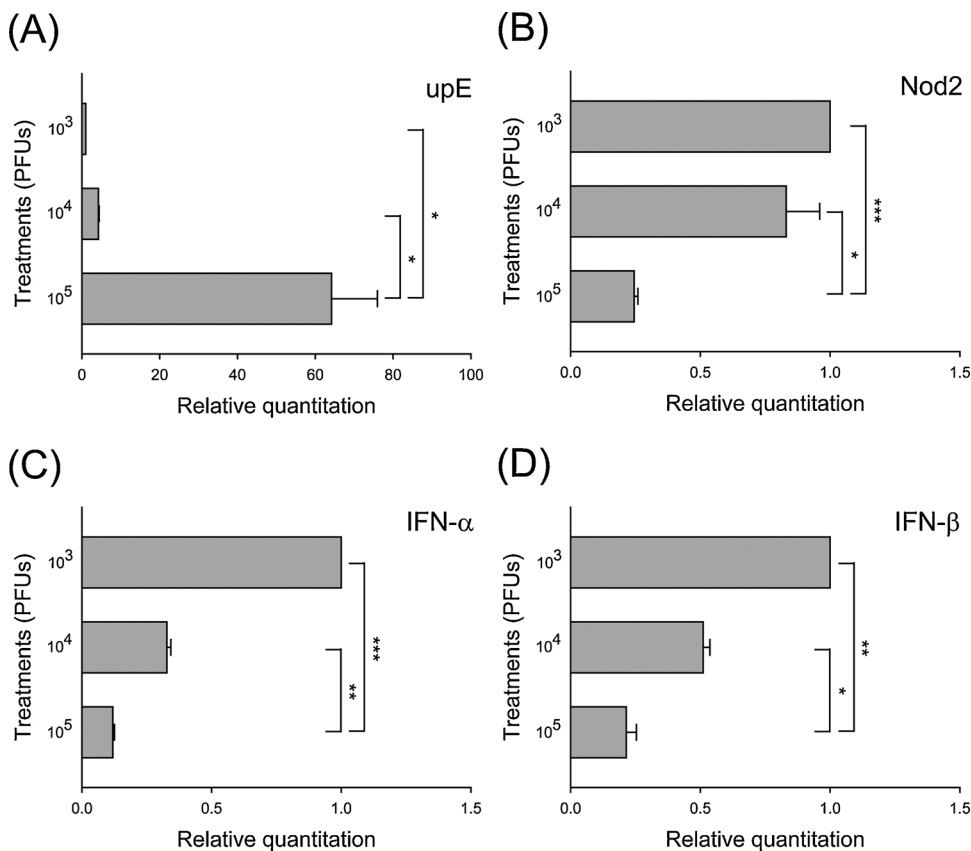


Fig. 1. MERS-CoV infection of THP-1 cells inhibits the expression of genes involved in innate antiviral responses. (A) Viral RNA levels in THP-1 cells treated with the indicated doses of MERS-CoV at 24 h post-infection were determined by quantitative real-time RT-PCR of the upE gene and normalized to that of the internal control gene, hACTB. (B–D) Cells were treated with the indicated doses of MERS-CoV and the expression levels of (B) Nod2, (C) IFN-α, and (D) IFN-β at 24 h post-infection relative to that of hACTB were analyzed by quantitative real-time RT-PCR in duplicate. The resulting values normalized to that of hACTB using the level in the cells treated with 10³ PFU of MERS-CoV as a reference basal expression value are shown as means ± SD. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

3.4. CCR2- and Nod2-mediated signaling activated by HBD 2 induces differentiation and polarization of THP-1 macrophage-like cells

Macrophages can differentiate into M1 (classically activated) or M2 (alternatively activated) macrophages. Classical M1-type macrophages are key effector cells for the elimination of pathogens, virus-infected cells, and malignant cells, while M2-type macrophages exhibit anti-inflammatory and tissue repair activities (Gordon and Martinez, 2010). NF-κB signaling is an intracellular proinflammatory pathway (Hoesel and Schmid, 2013) and activates M1-type macrophage differentiation (Saijo and Glass, 2011). Consequently, we analyzed the expression of marker genes of M1 and M2 macrophages in S RBD-HBD 2-treated THP-1 cells by quantitative real-time RT-PCR (Fig. 6). The expression of CD16 and CD80 was significantly (*p* < 0.05) enhanced by the HBD 2-conjugate, suggesting differentiation into M1-type macrophage cells. By contrast, there was no significant difference in the expression of M2 marker genes (CD163 and CD206) between cells treated with S RBD and S RBD-HBD 2 (data not shown).

To identify the signaling pathways involved in HBD 2-mediated activation and polarization of macrophages, we evaluated the effect of a CCR2 inhibitor, RS 102,895, and Nod2 inhibitor, GSK 717, on cells treated with S RBD with or without HBD 2 conjugation. Expression of the M1-type macrophage marker genes, CD16 and CD86, was markedly downregulated by the CCR2 and Nod2 inhibitors compared to the control (Fig. 6A and B). Interestingly, expression of CD14, a marker of early activation of macrophages, was stably upregulated in both S RBD alone- and S RBD-HBD 2-treated THP-1 cells, while CD14 expression was markedly downregulated by pretreatment with a CCR2 or Nod2 inhibitor, although the CCR2 inhibitor did not completely reverse the HBD 2-mediated enhanced expression of CD14 (Fig. 6C). These results demonstrate that CCR2-mediated activation of Nod2 signaling pathway by S RBD-HBD 2 is associated with the activation and M1 polarization of macrophage-like THP-1 cells.

4. Discussion

Innate immunity is the first line of defense against exogenous and endogenous threats, including pathogen infection and tissue damage. Innate immunity not only precedes the Ag-specific adaptive immune response but also enables a long-lasting memory response by innate Ag-presenting cells (APCs), which interact with adaptive immune cells. Innate immune cells, such as macrophages and mast cells, recruited into infected sites produce a wide range of cytokines that regulate the balance between pro- and anti-inflammatory responses and so maintain immunological homeostasis (Gallenga et al., 2019). Cytokines have pleiotropic effects on the functions of immune cells and immune responses that constitute the host defense against infectious agents. For instance, members of the IL-1 family, which are produced by macrophages and mast cells, are important regulators of the innate immune response and play a role in inflammatory processes (Varvara et al., 2018). Several IL-1 family cytokines are pro-inflammatory, while others, including IL-37, IL-38, IL-1Ra, and IL-36Ra, are anti-inflammatory (Kritas et al., 2018). In addition, IL-1 indirectly participates in T-lymphocyte-mediated immunity by inducing helper type 2 T-cell polarization and the formation of Abs by plasma cells by producing IL-6 (Gallenga et al., 2019). These findings suggest that further studies on modulation of the balance between pro- and anti-inflammatory cytokines and network among innate immune cells, such as M1/M2 macrophage polarization, would facilitate the development of novel therapeutic approaches for immunological disorders.

Macrophages, DCs, neutrophils, natural killer cells, and innate lymphoid cells play major roles in pathogen recognition through specialized receptors such as PRRs (Akira et al., 2006; Shim and Lee, 2015; Seo et al., 2013). Viral infection activates danger signals which are transmitted via PRRs, including TLRs, nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene 1-like receptors (RLRs), C-type lectin receptors, cytosolic DNA sensors, and inflammasome signaling cascades. Cross-talk between PRRs and

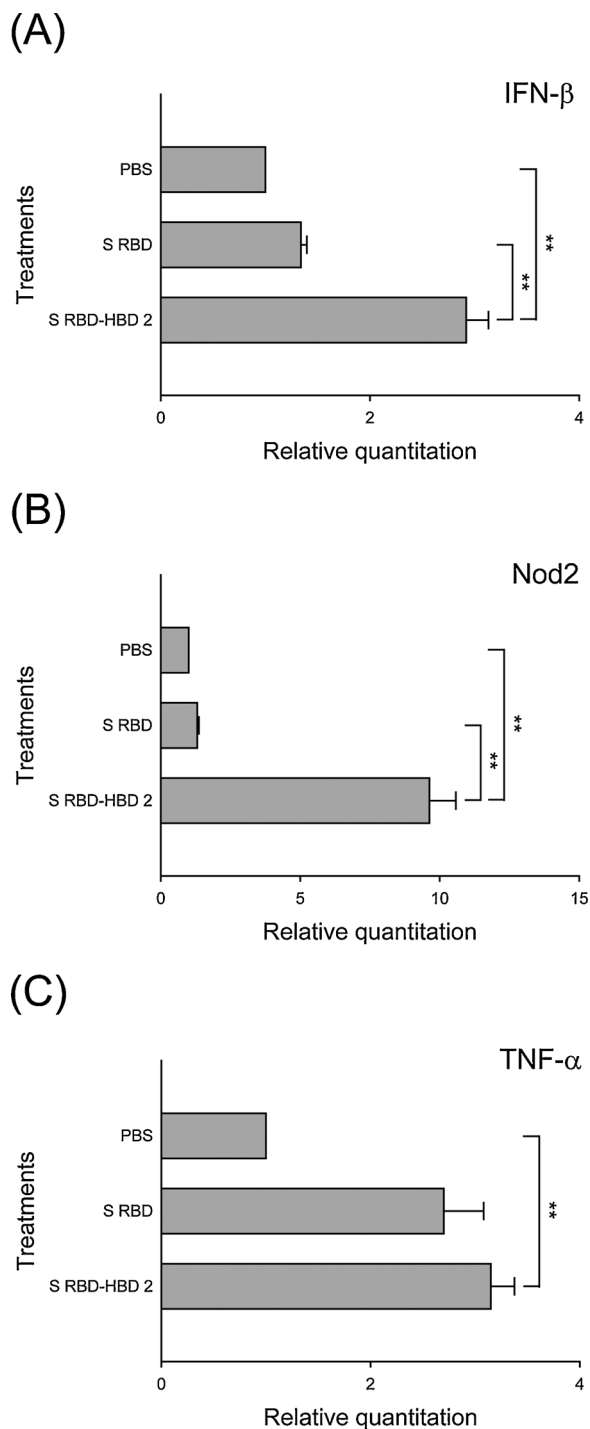


Fig. 2. HBD 2-conjugated S RBD Ag treatment of THP-1 cells stimulates the expression of genes involved in innate immunity and antiviral responses. THP-1 cells were stimulated with 1 μg/mL recombinant S-RBD or HBD 2-conjugated S RBD for 24 h and the expression of the indicated genes was analyzed by quantitative real-time RT-PCR in duplicate, with normalization to the expression of the internal control (hACTB). Expression levels relative to those of PBS-treated control cells are shown as means ± SD. ***p* < 0.01.

activation of these signaling cascades induces an antiviral immune response by upregulating the expression of antiviral cytokines, including type I IFNs. However, viruses can evade the antiviral function of IFN by inhibiting IFN production and signal transduction (Ferran and Skuse, 2017). Type I IFNs, mainly IFN-α and IFN-β, are major effector cytokines in the innate antiviral response (González-Navajas et al., 2012)

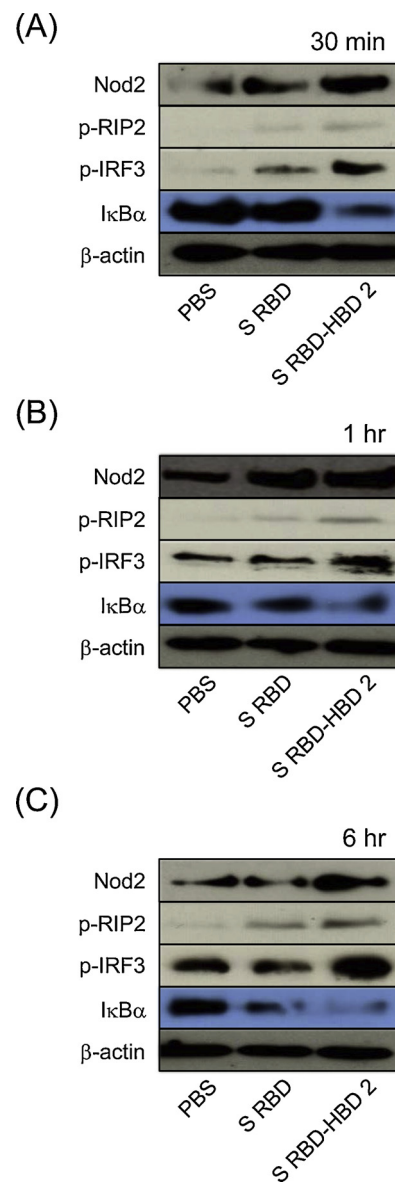


Fig. 3. HBD 2-conjugated S RBD Ag treatment of THP-1 cells stimulates the expression and activation of genes related to Nod2-mediated innate immune signaling. THP-1 cells were stimulated with 1 μg/mL recombinant S RBD or HBD 2-conjugated S RBD for the indicated periods. Cell lysates were prepared and immunoblotted with the indicated Abs. β-actin was used as the loading control.

and their encoding genes are regulated by several transcription factors, including NF-κB and IRF3 (Seth et al., 2005). Upon virus infection, IRF3 is phosphorylated, dimerizes, and enters the nucleus to upregulate the expression of type I IFN, melanoma differentiation-associated protein 5 (MDA5), and cytoplasmic retinoic acid-inducible gene I (RIG-I), leading to activation of NF-κB and IRF3 (Kato et al., 2011).

The hosts react to infection by mounting a primary response involving inflammation, followed by a pathogen-specific adaptive response. Although inflammation is a double-edged response, it is an important mechanism of protective innate immunity against infection by viruses, bacteria, fungi, prions, and parasites. Inflammatory monocyte-derived macrophages and innate immune cells are rapidly recruited to inflamed sites, where they remove harmful stimuli and induce T-cell responses by IFN-dependent mechanisms (Ginhoux et al., 2016). We previously reported that HBD 2 promotes the antiviral innate immune response in THP-1 cells and the ability of an HBD 2-conjugated

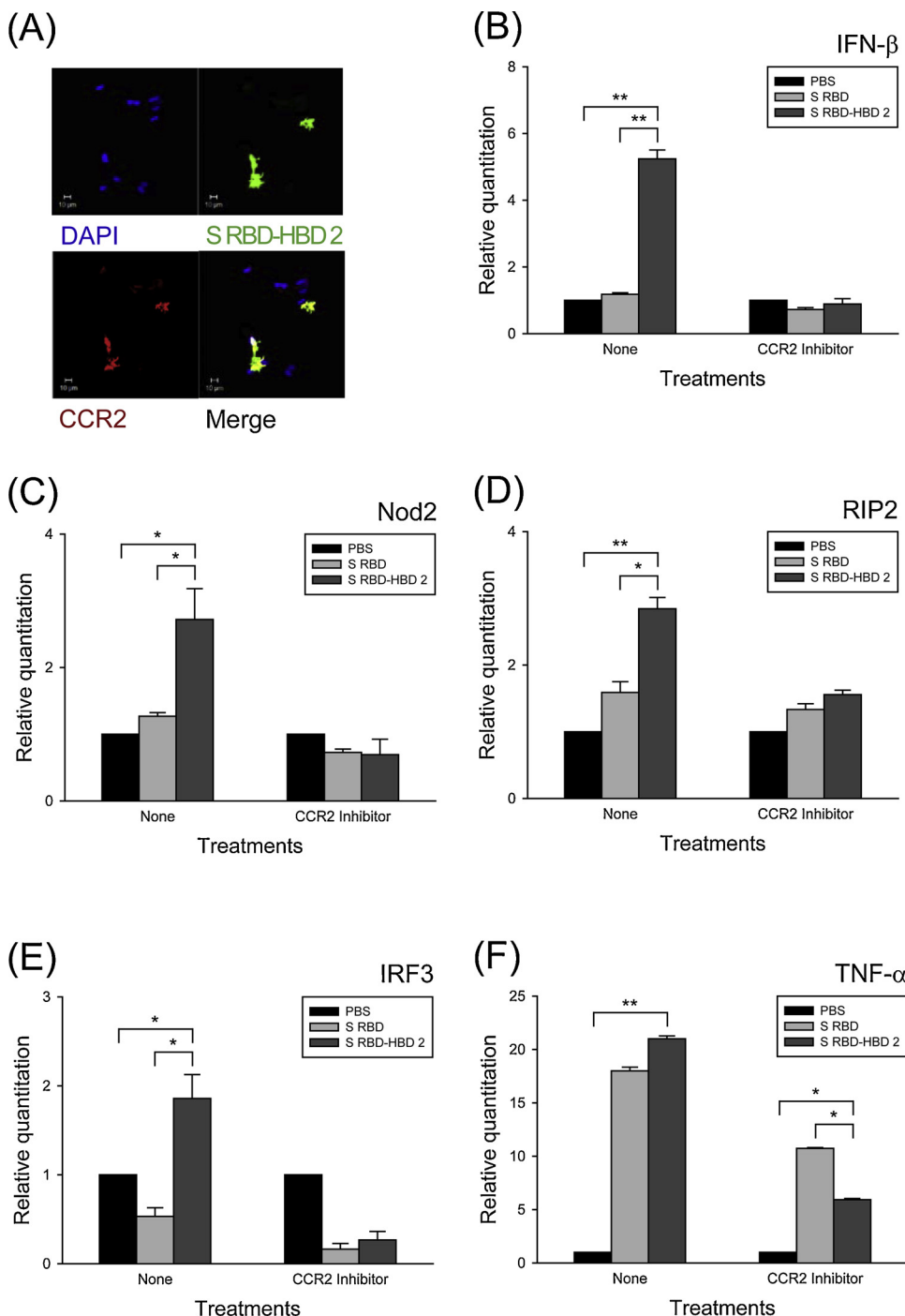


Fig. 4. HBD 2-conjugated S RBD co-localizes with CCR2 and the interaction between HBD 2-conjugated Ag and CCR2 induces Nod2 signaling. (A) THP-1 cells treated with 1 μg/mL HBD 2-conjugated S RBD were subjected to immunofluorescence assay using monoclonal Abs against S RBD and CCR2 and visualized by CLSM. DAPI-stained nuclei, blue; S RBD signal, green (Alexa Fluor 488-coupled secondary Ab); CCR2 signal, red (Alexa Fluor 555-coupled secondary Ab). Representative fields are shown at ×400 magnification. (B–F) THP-1 cells were stimulated with 1 μg/mL recombinant S RBD protein with or without HBD 2 at 6 h or 24 h after treatment with or without RS 102,895 (CCR2 antagonist) and their expression, together with that of the internal control gene hACTB, was analyzed by qRT-PCR in duplicate. Expression levels relative to those of the PBS-treated control are shown as means ± SD. **p* < 0.05 and ***p* < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

MERS-CoV Ag to elicit a greater Ag-specific and MERS-CoV neutralizing Ab response compared to HBD 2 non-conjugated Ag *in vivo* (Kim et al., 2018). Also, immunization with S RBD-HBD 2 prior to viral infection enhanced the humoral and protective immune response to MERS-CoV infection in human dipeptidyl peptidase 4 (hDPP4)-expressing mice, a model of MERS-CoV infection (data not shown). In addition, the type I IFN response, the expression of primary antiviral molecules including Nod2, a cytoplasmic viral PRR that activates IRF3, and production of IFN-β were enhanced by HBD 2 treatment of THP-1 cells. These cells are widely used as an *in vitro* model for studies of human macrophages involved in the inflammatory response and immunological homeostasis (Ginhoux et al., 2016). Here, we investigated the immunomodulatory ability of HBD 2 and the mechanism by which HBD 2 induces production of type I IFN and an inflammatory response in THP-1 cells.

β-Defensins exert regulatory activity in host innate and adaptive immune responses. For example, mouse β-defensin 2 activates immature DCs via TLR4, triggering a Th1 response, and human β-defensin 3 activates APCs via TLR1 and TLR2 in an NF-κB-dependent manner (Funderburg et al., 2007). Additionally, it was suggested that β-defensin is an endogenous ligand for TLR4 and shares a signal transduction pathway with other TLR4 ligands. We evaluated the possible interaction between HBD 2 and TLR4 as well as Nod2 and found that HBD 2-conjugated Ag co-localized with Nod2 (Fig. 5A) but not with TLR4 (data not shown). Moreover, inhibition of Nod2 signaling in THP-1 cells abrogated the HBD 2-mediated enhanced IFN-β expression by suppressing RIP2 and IRF3 signaling as well as TNF-α expression (Fig. 5B–E). Human and mouse β-defensins induce CCR2- and CCR6-dependent chemotaxis (Röhrli et al., 2010b) and CCR2 and CCR6 recruit

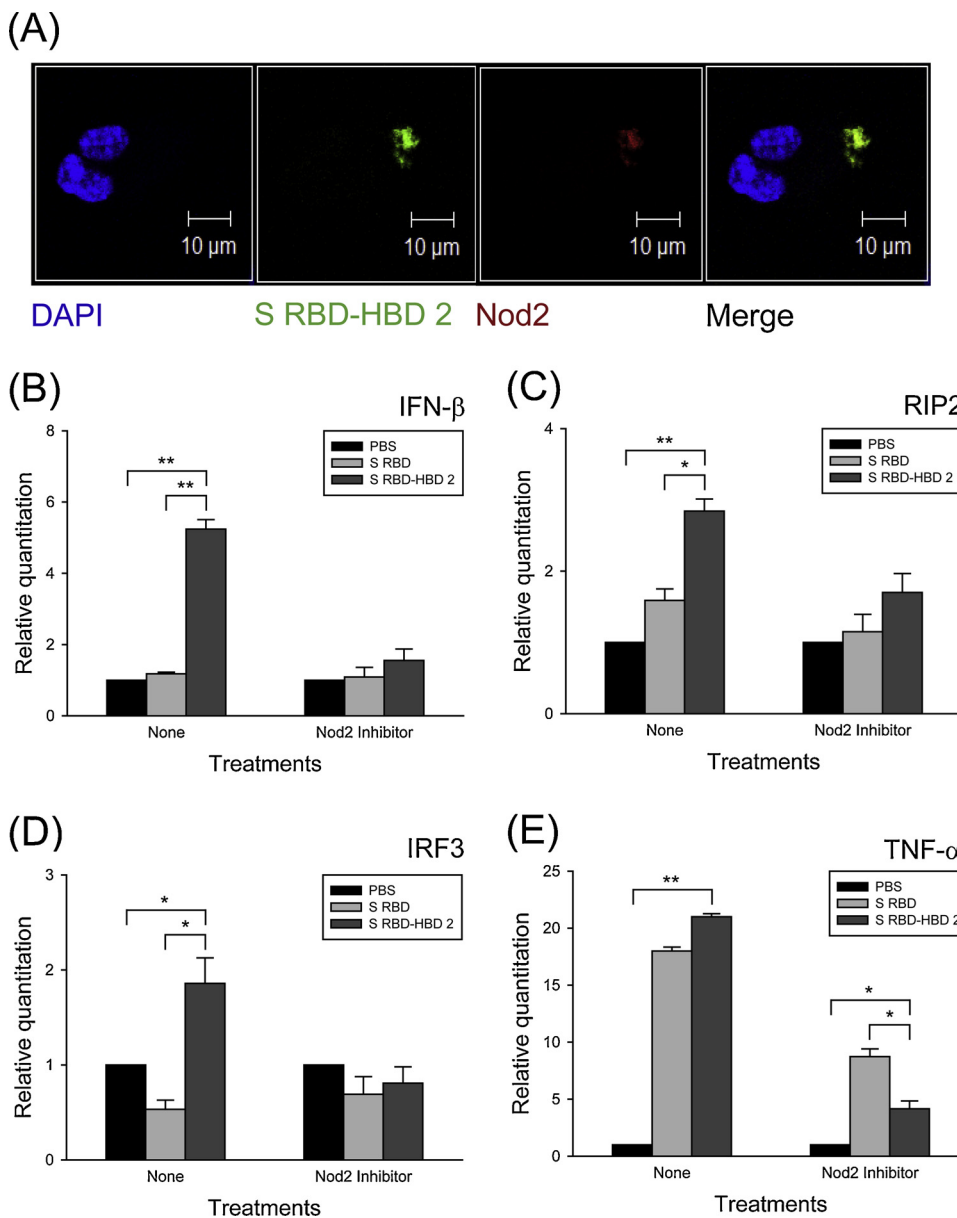


Fig. 5. HBD 2-conjugated S RBD co-localizes with Nod2 and the interaction between HBD 2-conjugated Ag and Nod2 induces signaling involved in innate antiviral immune responses. (A) THP-1 cells treated with 1 μg/mL HBD 2-conjugated S RBD were subjected to immunofluorescence assay using monoclonal Abs against S RBD and Nod2 and visualized by CLSM. DAPI-stained nuclei, blue; S RBD signal, green (Alexa Fluor 488-coupled secondary Ab); Nod2 signal, red (Alexa Fluor 555-coupled secondary Ab). Representative fields are shown at ×630 magnification. (B–E) THP-1 cells were stimulated with 1 μg/mL recombinant S RBD protein with or without HBD 2 at 6 h or 24 h after treatment with or without GSK 717 (Nod2 antagonist), and their expression, together with that of the internal control gene hACTB, was analyzed by qRT-PCR in duplicate. Expression levels relative to those of the PBS-treated control are shown as means ± SD. **p* < 0.05 and ***p* < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

professional APCs to inflamed tissues and initiate an adaptive immune response (Osterholzer et al., 2005). CCR2 is expressed on various types of myeloid cells, including monocytes and neutrophils, which are crucial for innate immunity and phagocytosis (Iida et al., 2005). We found that HBD 2-conjugated Ag co-localized with CCR2 and contributed to Nod2-mediated signaling, leading to the activation of type I IFN production and an inflammatory response in THP-1 cells. Consequently, the mechanism underlying HBD 2-induced CCR2-mediated signaling should be investigated further.

Macrophages help clear infectious cells by internalizing and degrading pathogens. During infection with influenza A, the phagocytic capacity of mouse peritoneal exudate macrophages is enhanced by coculture with virus-infected epithelial cells (Fujimoto et al., 2000), and decreased phagocytic uptake of opsonized influenza A virus is correlated with decreased cell surface expression of CD16 and CD32, which are highly expressed by classically activated M1 macrophages. Also, expression of CD16 and CD32 is decreased in macrophages infected with viruses capable of replicating productively in them (Marvin et al., 2017). M1 macrophages exert a proinflammatory effect, present Ag, perform phagocytosis, produce TNF-α and IL-1β, and express CD80 and CD86 on their surface. By contrast, M2 macrophages are responsible for

tissue repair and wound healing, produce IL-10 and TGF-β, and express arginase-1 and CD163 (Gordon and Martinez, 2010). We report here that HBD 2 induces the expression of M1 markers by activating CCR2-mediated Nod2 signaling pathway in THP-1 cells (Fig. 6). Although further studies are needed, these results provide insight into the mechanism by which HBD 2 induces antiviral innate and Ag-specific adaptive immune responses.

Conflicts of interest

There are no competing financial interests in this study.

Acknowledgments

This work was supported by the National Research Council of Science & Technology (NST) grant from the Korea government (MSIP) No. CRC-16-01-KRICT and by HI15C3039 (Y.-S. Jang) through the Korea Health Industry Development Institute (KHIDI) funded by the Korean Ministry of Health and Welfare. Dr. Y.-S. Jang was supported by the Research Base Construction Fund Support Program funded by Chonbuk National University in 2019. Ms. Y. L. Yang was supported by

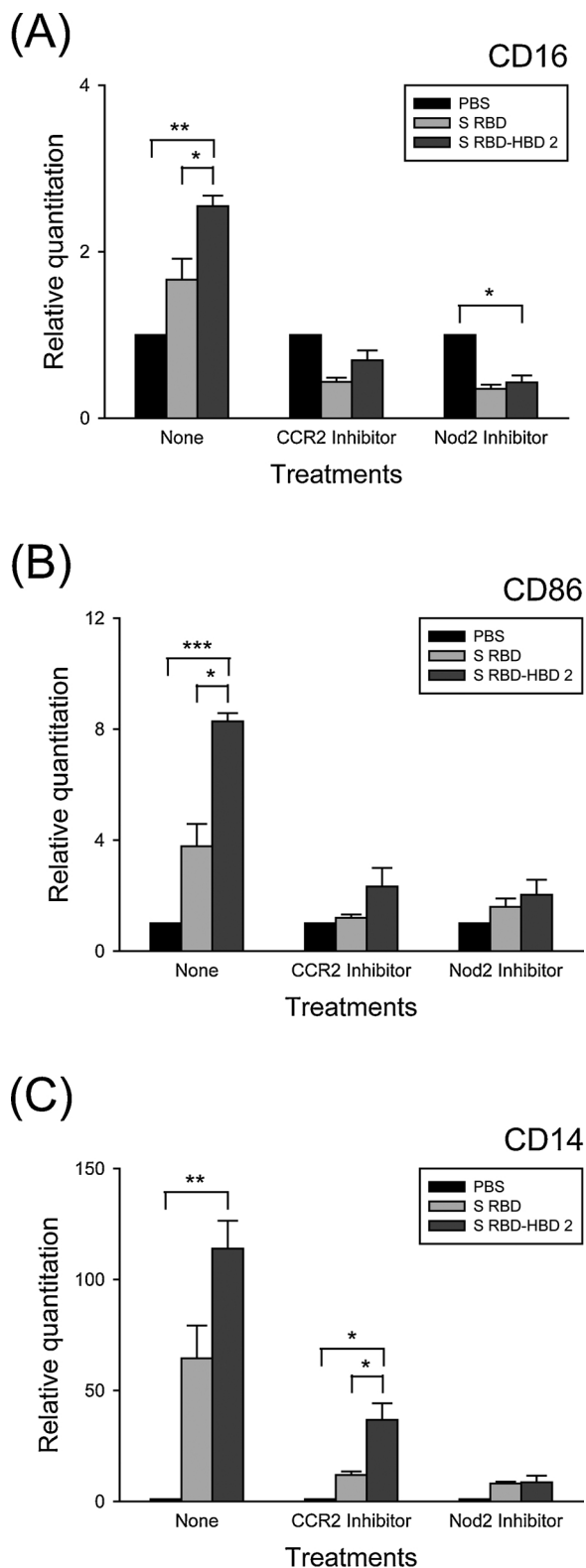


Fig. 6. HBD 2-conjugated Ag treatment induces polarized activation of THP-1 cells by regulating CCR2 and Nod2-mediated signaling. THP-1 cells were stimulated with 1 µg/mL recombinant S RBD protein with or without HBD 2 at 24 h after treatment with RS 102,895 (CCR2 antagonist) or GSK 717 (Nod2 antagonist), and their expression, together with that of the internal control gene hACTB, was analyzed by qRT-PCR in duplicate. Expression levels relative to those of the PBS-treated control are shown as means ± SD. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

the BK21 PLUS program in the Department of Bioactive Material Sciences. Confocal laser scanning microscopy was performed using the instruments installed in the Center for University-Wide Research Facilities (CURF) at Chonbuk National University.

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