Chicken avian β-defensin 8 modulates immune response via the mitogen-activated protein kinase signaling pathways in a chicken macrophage cell line

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Abstract

Background: Defensins are antimicrobial peptides composed of three conserved disulfide bridges, a β-sheet, and both hydrophobic and cationic amino acids. In this study, we aimed to demonstrate the immunomodulation role of avian β-defensin 8 (AvBD8) in a chicken macrophage cell line.

Results: Chicken AvBD8 stimulated the expression of proinflammatory cytokines (interleukin (IL)-1β, interferon-γ, and IL-12p40) and chemokines (CCL4, CXCL13, and CCL20) in macrophages. Furthermore, by western blotting and immunocytochemistry, we confirmed that AvBD8 activated the mitogen-activated protein kinase (MAPK) signaling pathway via extracellular regulated kinases 1/2 (ERK1/2) and p38 signaling molecules.

Conclusion: Overall, AvBD8 regulates host immune system as not only an antimicrobial peptide, but also an immunomodulator by activating the MAPK signaling pathway and inducing the expression of proinflammatory cytokines and chemokines.

Background

Antimicrobial peptides (AMPs) are known to play an important role in innate immunity by killing bacteria, viruses, and fungi [1](Reddy, 2004 #92). As a first line of defense, AMPs kill the invading bacteria mainly by disrupting the bacterial cell membrane, but they might also interfere with DNA, protein, and cell wall syntheses and protein folding [2]. Because most of the AMPs have cationic properties, they can attach to negatively charged components in the bacterial cell membrane, such as lipopolysaccharide and lipotechoic acid. In addition to their direct killing effect, AMPs can modulate the immune response. They inhibit lipopolysaccharide-induced pro-inflammatory cytokine production, induce pro-inflammatory cytokine production, and promote wound healing [3-5]. Owing to their various mechanisms of action, broad spectrum antimicrobial activities, and immunomodulation effect, AMPs have been actively studied as alternative antibiotic and immune therapy agents.

The defensin peptides are cysteine-rich antimicrobial peptides composed of three conserved disulfide bridges, a β-sheet, and both hydrophobic and cationic amino acids. Defensins are classified into the following three categories based on their structure: α-, β-, and θ-defensins [6, 7] and are found in mammals, vertebrates, and Old World monkeys, respectively [8, 9]. Defensins exhibit antimicrobial activity against pathogens, including Gram negative and Gram positive bacteria, fungi, and certain enveloped viruses such as human immunodeficiency virus [10] [11-13]. In addition to the antimicrobial activity, defensins also play a role in immunomodulation. Defensins have monocyte-chemotactic activity in human neutrophils [14], T lymphocytes [15], and immature dendritic cells [16]. Especially, β-defensins are well known as an immunomodulator. For example, human β-defensin 3 (hBD-3) induces the expression of costimulatory molecules on immune cells [17]. Furthermore, hBD-3 has chemotactic effects in immune cells via chemokine receptors 2 and 6 [18, 19]. hBD-3 also shows anti-inflammatory activity by targeting the Toll-like receptor (TLR) signaling pathways [20].

Avian species express only β-defensins, named as avian β-defensins (AvBDs). To date, approximately 14 AvBDs (AvBD1-AvBD14) have been identified in chicken, turkey, duck, ostrich, and king penguin [21-26]. Most of these β-defensins exhibit antimicrobial activity against various pathogens [8, 21, 25, 27-29]. Several studies have reported the expression of AvBDs against pathogen infections [30-32]. However, details of immune regulation mechanisms of AvBDs are limited. In this study, chicken macrophage cell line was used because macrophages have important roles in immune system including phagocytosis, antigen presenting cells, and wound healing [33]. Therefore, we examined the immunomodulating mechanism of AvBD8 in a chicken macrophage cell line.

Materials And Methods

Reagents and antibodies

Rabbit phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204, #4370) and rabbit phospho-p38 MAPK (Thr180/Tyr182, #4631) monoclonal antibodies were purchased from Cell Signaling (Danvers, MA, USA). Mouse anti-chicken GAPDH antibody was purchased from Thermo Fisher Scientific (AM4300; Waltham, MA, USA). Alexa Fluor 488 goat anti-rabbit IgG (H + L) secondary antibody was purchased from Invitrogen (A-11008, Carlsbad, CA, USA). Anti-rabbit IgG (H+L) HRP-conjugated antibody was purchased from Promega (W4011; Madison, WI, USA). Goat anti-mouse IgG HRP-conjugated antibody (A16078) was purchased from Thermo Fisher Scientific. In addition, HRP-conjugated rabbit anti-6-His antibody (A190-114P) was purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA) and 4',6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (Rockford, IL, USA). RIPA lysis and extraction buffers were purchased from Thermo Fisher Scientific.

Cloning of AvBD8

The primers were designed using DNASTAR (DNASTAR Incorporation, Madison, WI, USA) to amplify the mature sequence of AvBD8 from *Gallus gallus* avian beta-defensin 8 (AvBD8) mRNA sequence (NM_001001781.1). The AvBD8 coding sequence was amplified using total RNA derived from the intestinal mucosal layer of White Leghorn chickens, kindly provided by the Animal Biosciences and Biotechnology Laboratory (Beltsville, MD, USA) of the USDA Agricultural Research Service. The PCR product was amplified using the following specific primers: forward, 5'-CGGAATTCAACAACGAGGCACAGTGTG-3' and reverse, 5'- CCAAGCTTGTCGTACACAGTCCG-3' (the EcoRI and HindIII restriction enzyme sites are underlined) with DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific). The PCR amplification was carried out under the following conditions: a pre-denaturation step at 95°C for 5 min, a denaturation step at 95°C for 30 s, an annealing step at 55°C for 30 s, an extension step at 72°C for 30 s for 35 cycles, and a final extension at 72°C for 5 min. The PCR products were purified using the FavorPrep™ GEL/PCR purification kit (Favorgen, Ping-Tung, Taiwan), cloned into the pCR2.1-TOPO vector (Invitrogen), and transformed using *Escherichia coli* TOP 10 competent cells (Invitrogen) according to the manufacturer's protocol. Through blue–white screening, positive clones were picked out and cultured overnight in Luria–

Bertani (LB) broth (with 100 μg/mL ampicillin). Plasmids were extracted using the FavorPrep™ plasmid DNA extraction mini kit (Favorgen) and sequenced by Genotech (Daejeon, South Korea). The AvBD8/pCR2.1-TOPO vector was digested with the restriction enzymes EcoRI and HindIII (Thermo Fisher Scientific, USA). The protein expression vector pET32a (Novagen, Madison, WI, USA) was also digested with the same restriction enzymes. The digested fragments were purified by agarose gel electrophoresis using the FavorPrep™ GEL/PCR purification kit (Favorgen) and ligated using T4 DNA ligase (Invitrogen). The ligated vector and insert were transformed into One Shot BL21 (DE3) chemically competent *E. coli* (Invitrogen) and sequenced.

Production of recombinant AvBD8 protein

Recombinant AvBD8 protein was produced as previously described for chicken IL-26 [34]. Briefly, the positive clones of AvBD8/pET32a were incubated at 37°C overnight in a shaking incubator at 225 rpm in LB broth with 100 μg/mL ampicillin. The bacterial culture was then induced for recombinant protein expression with 1 mM isopropyl-β-D-thiogalactopyranoside (USB Corporation, Cleveland, OH, USA) for 4 h at 37°C, and then centrifuged at 5000 × *g* for 15 min. The AvBD8 recombinant protein was extracted with B-PER bacterial protein extraction reagent (Thermo Fisher Scientific) and purified using HisPur cobalt resin (Thermo Fisher Scientific). The recombinant AvBD8 protein was eluted using 250 mM imidazole and analyzed by sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and western blotting using HRP-conjugated rabbit anti-6-His antibody (Bethyl Laboratories). The purified recombinant protein was dialyzed using SnakeSkin™ dialysis tubing (Thermo Fisher Scientific) in phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C with stirring and analyzed by SDS-PAGE and western blotting. Endotoxins in recombinant AvBD8 were evaluated using Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific) according to manufacturer's protocol.

Cell culture and recombinant protein treatment

Chicken macrophage cell line HD11 [35] was cultured in complete RPMI 1640 medium (Thermo Fisher Scientific) containing 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) in a humidified 5% CO_2 atmosphere at 41°C. The cells (1.0×10^6/well) were incubated in a 12-well plate containing 1 mL of culture medium and treated with 100 ng/mL (final concentration) recombinant AvBD8 protein and incubated for 0, 0.5, 1, 2, and 4 h in a humidified 5% CO_2 atmosphere at 41°C.

Quantitative real-time polymerase chain reaction

HD11 cells were washed with ice-cold PBS, and then total RNA was extracted from the cells using TRIzol™ reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. cDNA was synthesized from the total RNA using the RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific) according to

the manufacturer's protocol. To analyze the cytokine gene expression, primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1) and qRT-PCR was performed using FastStart Essential DNA Green Master (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions, in the LightCycler96 system (Roche). The chicken glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as the control to normalize RNA quantity. The relative quantification of gene-specific expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with the *GAPDH* gene expression level [36]. All qRT-PCRs were performed in triplicate.

Western blotting

HD11 cells (5.0×10^6/well) were incubated in a 6-well plate (Thermo Scientific) containing 2 mL of culture medium and stimulated with 100 ng/mL recombinant AvBD8 proteins for 0, 15, 30, 60, and 90 min in a humidified 5% CO_2 atmosphere at 41°C. After incubation, the cells were washed with ice-cold PBS and the proteins were extracted from the cells using RIPA lysis and extraction buffers according to the manufacturer's protocol. Halt™ phosphatase inhibitor cocktail (Thermo Fisher Scientific) was added to the cell lysate. The cell protein concentration was measured using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's protocol. Protein samples were mixed with 4× sample buffer (200 mM Tris-Cl [pH 6.8], 20% β-mercaptoethanol, 8% SDS, 0.4% bromophenol blue, and 40% glycerol) and heated to 100°C for 5 min. The protein samples (30 μg) were electrophoresed on 12% Tris–glycine SDS polyacrylamide gels. The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Rydalmere, Australia) using the Mini-PROTEAN® electrophoresis system (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk (Thermo Fisher Scientific) in PBS (pH 7.4) containing 0.05% Tween-20 (Sigma-Aldrich, MO, USA) (PBST). Antibodies were prepared with 2% skim milk in PBST (Phosphate Buffered Saline with Tween 20). The membranes were washed with PBST and treated with anti-rabbit IgG (H+L), HRP-conjugated antibody (Promega). The membranes were then developed using Western Lightning ECL Plus (Thermo Fisher Scientific) for Hyperfilm (GE Healthcare).

Immunocytochemistry

Immunocytochemistry was performed using the Nunc™ Lab-Tek™ Chamber Slide (Thermo Fisher Scientific) as previously described [37]. Briefly, HD11 cells (4.0×10^4 cells/well) were cultured in a chamber slide for 30 min in a humidified 5% CO_2 atmosphere incubator at 41°C in the presence or absence of the recombinant AvBD8 proteins (100 ng/mL). The cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min, and then incubated with ice-cold methanol for 10 min at 4°C. Following overnight incubation with the anti-rabbit primary antibody at 4°C, the cells were treated with Alexa Fluor®488-conjugated secondary antibody for 1 h, and then stained with DAPI for 5 min. Finally, images were captured using EVOS® FLoid® Cell Imaging Station (Life Technologies, Carlsbad, CA, USA).

Bioactivity assay

HD11 cells (2×10^4 cells/well) were seeded and cultured in 24-well plates. After culturing overnight, the cells were incubated with the AvBD8 recombinant protein (50, 100, 200, 300, 400, 5000, and 1000 ng/mL) for 72 h in a humidified 5% CO_2 atmosphere at 41°C to analyze cell proliferation and nitric oxide (NO) production. The NO content was measured using the Griess reagent system (Promega) and cell proliferation was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocols as previously described [34].

Bioinformatics analysis

The purified plasmid was sequenced by Genotech (Daejeon, Republic of Korea). To compare the cloned chicken *AvBD8* sequence with sequences in GenBank, the data were analyzed using a Nucleotide Basic Local Alignment Search Tool (nBLAST) search (http://www.ncbi.nlm.nih.gov/BLAST/). Protein identification was performed using the Expert Protein Analysis System (https://www.expasy.org/) to determine the molecular weight. The protein structure was predicted using RaptorX (http://raptorx.uchicago.edu/) and FirstGlance in Jmol (http://www.bioinformatics.org/firstglance/fgij/).

Statistical analysis

Data are presented as mean ± standard error of mean of three independent experiments. Statistical analyses were performed using IBM SPSS software (SPSS 23.0 for Windows; IBM, Chicago, IL, USA). The results with a p-value of < 0.05 were considered statistically significant. Differences between groups were evaluated using Duncan's multiple range test.

Results

Structural analysis of chicken AvBD8

Chicken AvBD8 is composed of a signal peptide (19 aa) and mature peptide (40 aa). It also has three disulfide bonds, a typical feature of defensins (Fig. 1A). The secondary structure of mature AvBD8 is composed of one alpha-helix, three beta-sheets, and three disulfide bonds (Fig. 1B).

AvBD8 recombinant protein expression

The mature AvBD8 recombinant proteins produced by cloning mature AvBD8 sequences to pET32a expression vector. After inducing AvBD8 recombinant proteins, target proteins were purified by immobilized metal affinity chromatography (IMAC) methods using HisPur Cobalt Resin. The purified recombinant proteins were confirmed by western blotting (Fig. 1C). The molecular weight of the mature

AvBD8 protein was 4.5 kDa and that of the fusion recombinant protein of pET32a vector was 22 kDa (Trx-Tag, His-tag, thrombin, and S-Tag). Therefore, the target protein molecular weight was around 26.5 kDa.

AvBD8 stimulates Th1 cytokine and chemokine expression

Chicken HD11 cells were stimulated by 100 ng/mL AvBD8 recombinant proteins to examine the role of AvBD8. Th1 cytokine (IL-1β, IFN-γ, and IL-12p40) and chemokine (CCL4, CXCL13, and CCL20) expression was stimulated by AvBD8 (Fig. 2). The Th1 cytokine IL-1β was strongly stimulated (231.52-fold), whereas IFN-γ was moderately expressed (1.93-fold) after 2 h of incubation. Furthermore, the expression of IL-12p40 was significantly upregulated by 2.23-fold at 1 h. The expression of the chemokine CCL4 was significantly upregulated by 13.26-fold at 0.5 h and increased to 37.14-fold at 4 h. The expression of CXCL13 was significantly increased by 2.31-fold at 1 h. CCL20 was significantly upregulated by 15.20-fold at 2 h.

AvBD8 activates MAPK signaling pathway

HD11 cells were stimulated with 100 ng/mL recombinant AvBD8 protein for 0, 15, 30, 60, 90, and 120 min to investigate whether AvBD8 is involved in the MAPK pathway. Phosphorylated (Thr^{202}/Tyr^{204}) extracellular regulated kinases 1/2 (ERK1/2) was detected at 15 min after AvBD8 protein treatment, and its level peaked at 30 min and decreased marginally until 120 min (Fig. 3). Phosphorylated (Thr^{180}/Tyr^{182})5 p38 was also detected at 30 min after AvBD8 treatment, and its level peaked at 60 min and then gradually decreased.

Furthermore, to visualize phosphorylated ERK1/2 and p38, HD11 cells were stimulated with 100 ng/mL AvBD8 protein for 1 h for ICC. The signal of phosphorylated ERK1/2 and p38 was observed in the cytoplasm (green color) after treatment with the recombinant AvBD8 protein (Fig. 4). Overall, these results suggested that chicken AvBD8 mediates immune response via the MAPK signaling pathway.

AvBD8 induces the expression of CD40

Cluster of differentiation 40 (CD40) is a protein that is expressed in antigen-presenting cells. After binding with the CD40 ligand in T cells, it activates antigen-presenting cells and triggers the downstream signals. AvBD8 significantly induced the expression of *CD40* after 4 h of incubation, with a fold change of 2.28 (Fig. 5).

Cell proliferation and nitric oxide production

Cell proliferation and NO production were measured to determine the effects of AvBD8 in chicken HD11 cell line (Additional file 1). The results showed that the proliferation of chicken HD11 cells was neither

significantly inhibited nor enhanced. Moreover, NO production was not affected by AvBD8 treatment.

Discussion

In this study, we demonstrated the immunomodulation mechanism of AvBD8 in a chicken macrophage cell line. AvBD8 stimulated pro-inflammatory cytokines and chemokines expression in chicken macrophage cell line and activated MAPK signaling pathway and CD 40 expression in chicken macrophage cell line.

α-Defensins are unique to mammals, whereas b-defensins are found ubiquitously in vertebrate species, including avian species [38, 39]. As an AMP, several AvBDs have antimicrobial activities against both Gram positive and Gram negative bacteria [40, 41]. For example, chicken AvBD2, AvBD3, AvBD4, AvBD6, AvBD7, AvBD11, and AvBD12 showed antibacterial effect against *E. coli* [31]. Furthermore, duck AvBD2 showed antibacterial effect against *M. lutues*, *E. coli*, and *R. anatipestifer* [26].

Besides antimicrobial activity, AvBDs have immunomodulatory activity. Yang et al. [42] demonstrated the role of AvBD6 and AvBD12. AvBD6 and 12 showed LPS neutralizing effect and chemotactic effect for chicken macrophages expressing chicken chemokine receptor 2. Also, AvBD12 induced migration of murine immature dendritic cells. Also, mRNA expression of AvBD2 was upregulated with Newcastle disease virus (NDV) infection in chicken embryo fibroblasts through p38 MAPK-dependent manner [43]. Yang et al. [16] demonstrated that human β-defensin-2 induced chemoattraction of CD4+ memory T cells and immature dendritic cells, by binding to CCR6. Murine β-defensin 2 induced costimulatory molecules CD40, CD80, and CD86 by acting directly on immature dendritic cells as an endogenous ligand for Toll-like receptor 4 (TLR-4) [44]. Furthermore, human β-defensin-3 has been described as an activator of antigen-presenting cells inducing CD40, CD80, and CD86 expression in a TLR-dependent manner and the subsequent activation of MyD88-dependent signaling [17]. It stimulated the cytokines, including interleukin(IL)-1α, IL-6, IL-8, CCL18, and tumor necrosis factor-α (TNF-α) in macrophages derived from peripheral blood monocytes [45]. Moreover, human β-defensin-2, -3, and -4 stimulated the expression of IL-6, IL-10, IP-10, monocyte chemoattractant protein-1, and macrophage inflammatory protein-3α in keratinocytes and induced the phosphorylation of epidermal growth factor receptor, signal transducer and activator of transcription-1 (STAT1), and STAT3, which are intracellular signaling molecules [46]. In this study, AvBD8 upregulated the expression of proinflammatory cytokines (IL-1β, IFN-γ, and IL-12p40) and chemokines (CCL4, CXCL13, and CCL20) (Fig. 2). Proinflammatory cytokines initiate the inflammatory response as a host defense mechanism against pathogens mediating the innate immune response. Chemokines induce the migration of immune cells to the site of injury and inflammation [47]. Therefore, AvBD8 regulate the innate immune system when the host is infected with pathogens, by acting not only as an antimicrobial agent but also as an immune-stimulating system.

The MAPK signaling pathway plays an important role in innate and adaptive immunity and is involved in various cellular functions such as inflammation, cell differentiation, cell proliferation, and cell death [48]. ERK1/2 and p38 are MAPKs that activate various transcription factors and induce the transcription of

cytokines and chemokines [48-50]. Dawn et al. [51] demonstrated that the human cationic peptide LL-37 activated the MAPK pathway. Woo et al. [3] demonstrated that chicken NK-lysin-derived cNK-2 stimulated the MAPK pathway and induced the expression of proinflammatory cytokines and chemokines. In chicken, the MAPK pathway-related genes were differentially expressed in NE-afflicted two inbred chicken lines, namely, Marek's disease resistant line 6.3 and Marek's disease-susceptible line 7.2 [52]. In the present study, AvBD8 activated the MAPK signaling pathway by phosphorylating ERK1/2 and p38 signaling molecules (Fig. 3 and Fig. 4). Therefore, we could know that chicken AvBD8 can modulate immune system by activating MAPK signaling pathway.

hBD3 is known to stimulate the expression of the costimulatory molecules CD80, CD86, and CD40 on monocytes in a TLR-dependent manner [17]. In this study, AvBD8 induced the expression of CD40 in macrophages (Fig. 5). Because CD40 can bind to the CD40 ligand in T_H cells, AvBD8 might act as a bridge between innate and adaptive immunity.

In avian species, there are several studies on the expression of β-defensins after bacterial and viral infections. Hong et al. [30] demonstrated that genetically disparate two commercial chicken lines, Ross and Cobb, afflicted with necrotic enteritis (NE) by *Eimeria maxima* and *Clostridiun perfringens* infection showed differential expression of AvBDs. Furthermore, Truong et al. [53] reported that two Fayoumi chicken lines induced with NE, M15.1 and M15.2, showed differential gene expression pattern of AvBDs. In an *in vitro* experiment, AvBD8 gene expression was highly upregulated in chicken macrophages and T- and B-cell lines with LPS (lipopolysaccharide) treatment [32]. Therefore, chicken AvBD8 have an important role in immune system when the host were infected with pathogens. Also, because receptors of AvBDs are not yet known further studies are needed to demonstrate detailed mechanism of AvBDs.

Conclusion

In conclusion, to the best of our knowledge, this is the first study to demonstrate that chicken AvBD8 modulates immune response via activation of the MAPK signaling pathway and induction of proinflammatory cytokines and chemokines in the chicken macrophage cell line. Because AvBD8 regulates immune system when the host is infected with pathogens, it can be applied as a vaccine adjuvant like NE in the future.

Declarations

Acknowledgment

None

Authors' contributions

YH and YHH designed the experiments. YH, TTP, JL performed the experiments. Performed the experiments. HSL provide White Leghorn chickens RNA samples. YH analyzed the data. YH and YHH

wrote the paper. All authors read and approved the final manuscript.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. Sequence of primers for qRT-PCR analysis of gene expression.

Figures

Primer	F/R	Nucleotide sequence (5′-3′)	Accession No.
GAPDH	F	TGCTGCCCAGAACATCATCC	NM_204305
	R	ACGGCAGGTCAGGTCAACAA	
IL-1β	F	TGCCTGCAGAAGAAGCCTCG	NM_204524
	R	CTCCGCAGCAGTTTGGTCAT	
IFN-γ	F	AACAACCTTCCTGATGGCGT	NM_205149.1
	R	TGAAGAGTTCATTCGCGGCT	
IL-12p40	F	AGATGCTGGCAACTACACCTG	NM_213571
	R	CATTTGCCCATTGGAGTCTAC	
CCL4	F	CTTCACCTACATCTCCCGGC	NM_001030360
	R	CTGTACCCAGTCGTTCTCGG	
CXCL13	F	GCCTGTGCCTGGTGCTC	XM_420474
	R	TGCCCCCTTCCCCTAAC	
CCL20	F	AGGCAGCGAAGGAGCAC	NM_204438
	R	GCAGAGAAGCCAAAATCAAAC	
CD40	F	GCCTCTGAATGCAACGACAC	NM_204665.2
	R	CCAGCGTTGTCCTCACAGAT	

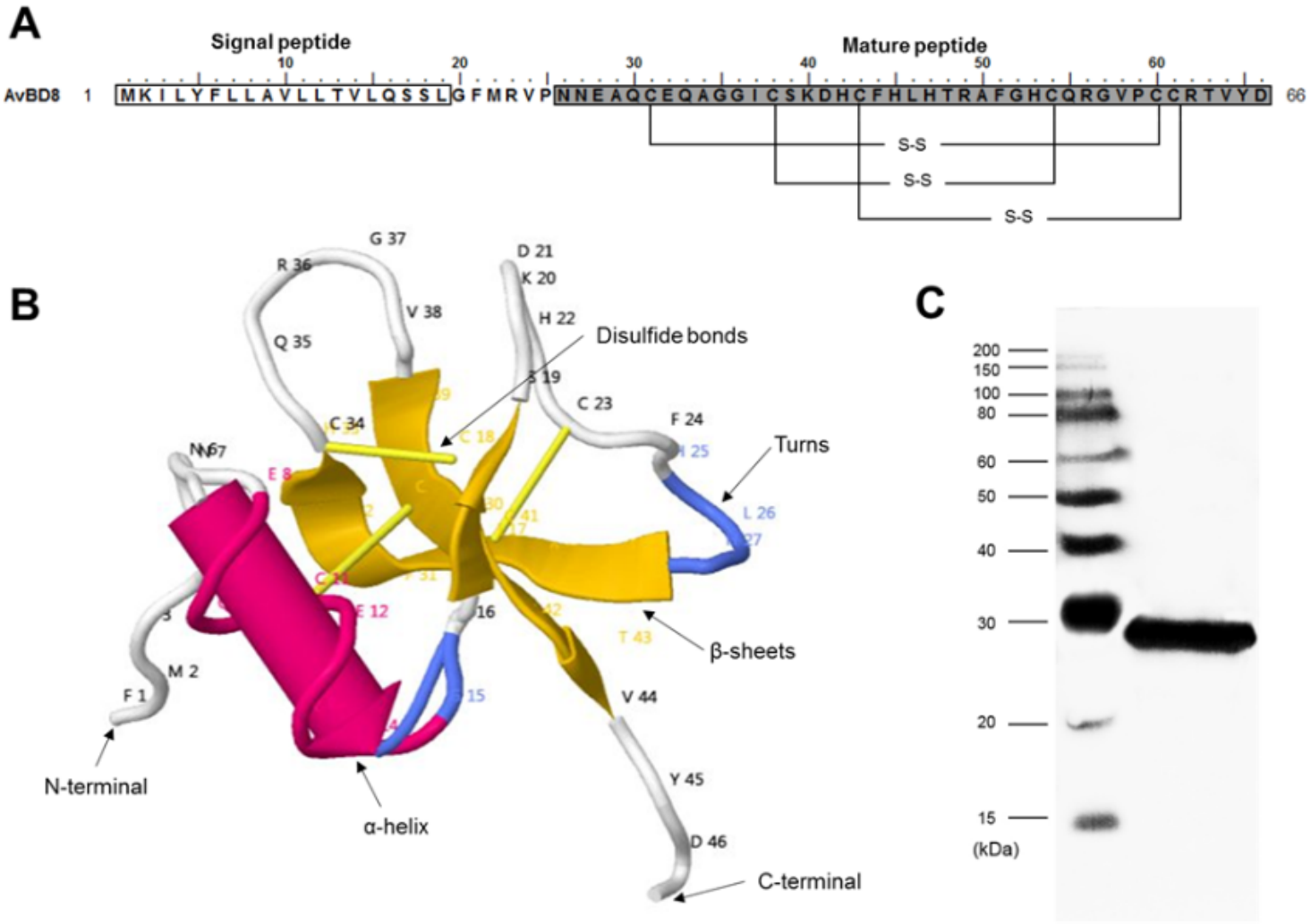

Figure 1

(A) Secondary structure of AvBD8. The white box indicates the signal peptide and gray box indicates the mature peptide. S-S indicates disulfide bonds. (B) The 3D-structure of AvBD8. The letters are

abbreviations of amino acids and the numbers beside the abbreviations indicate the position of amino acids in the protein. Alpha helices are shown as red rockets and beta strands are shown as yellow planks; blue lines indicate turns and yellow sticks indicate disulfide bonds. (C) Western blot of recombinant AvBD8 using the anti-6Histidine antibody.

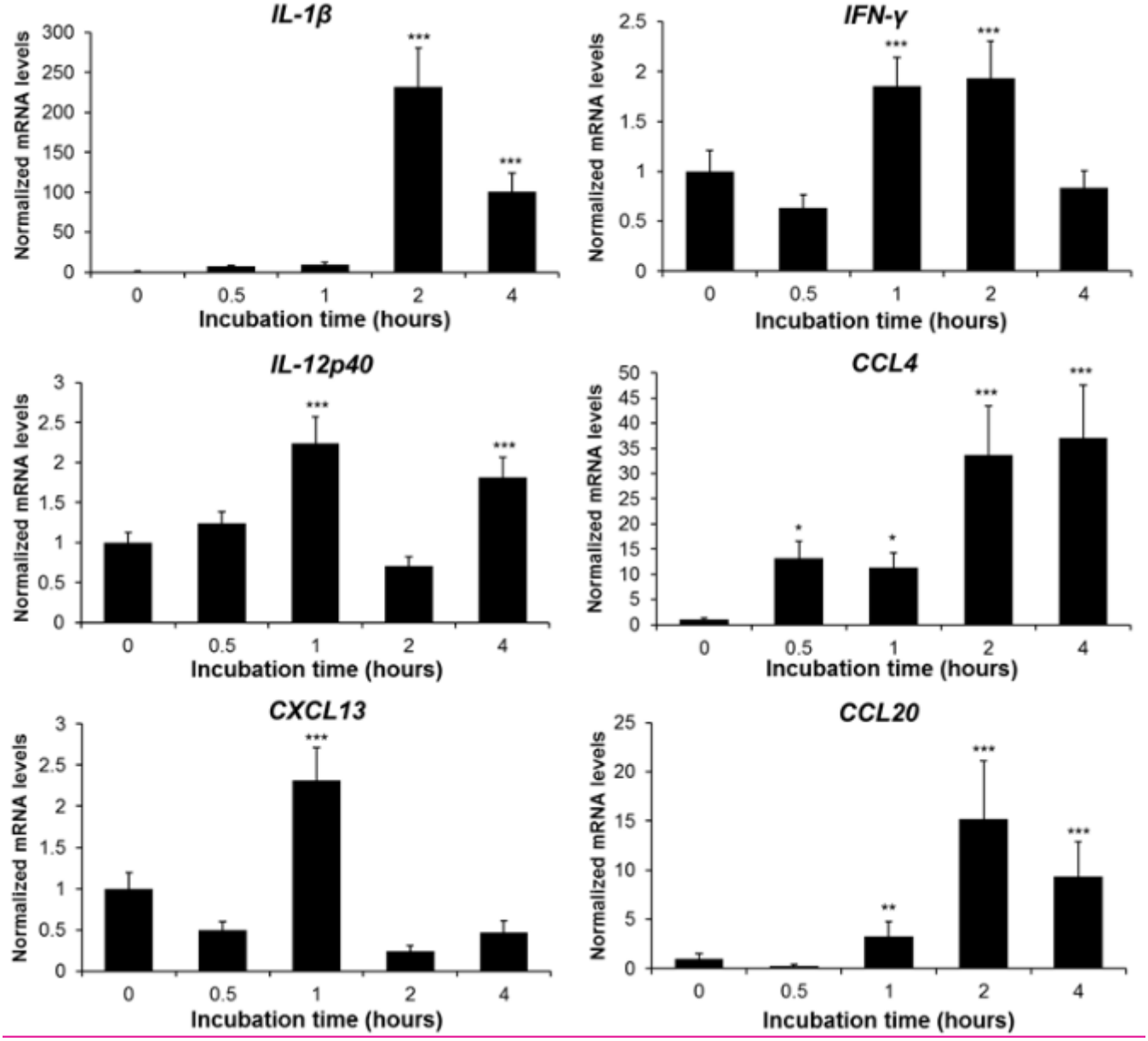

Figure 2

Effects of AvBD8 on proinflammatory cytokine and chemokine production. HD11 cells were induced by AvBD8 (100 ng/mL) for 0, 0.5, 1, 2, and 4 h and the expression of cytokines and chemokines was measured by qRT-PCR. Expression level was normalized to that of GAPDH. All culture conditions were tested in triplicate. The data are expressed as mean ± SEM and are representative of three independent experiments: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

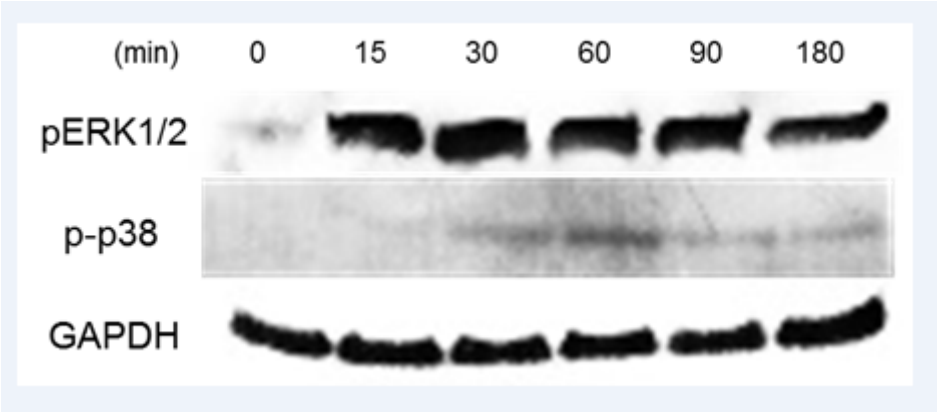

Figure 3

Western blotting of p-ERK1/2 and p-p38. HD11 cells were induced by AvBD8 (100 ng/mL) for 0, 15, 30, 60, 90, and 180 min. Total cell lysates were analyzed by western blotting using antibodies against p-ERK1/2, p-p38, and GAPDH.

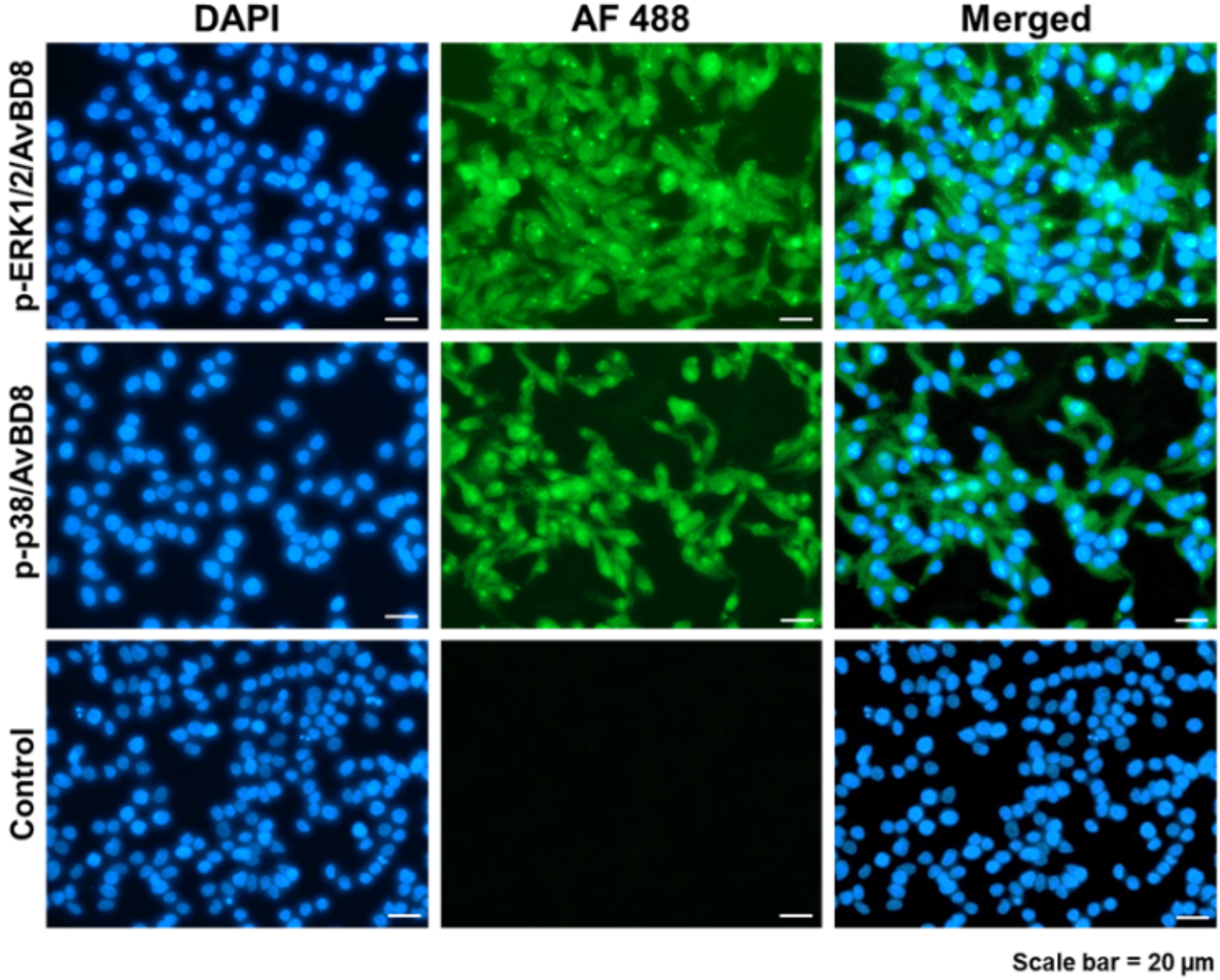

Figure 4

Immunocytochemistry analysis of p-ERK1/2 and p-p38. HD11 cells were induced by AvBD8 (100 ng/mL) for 1 h and analyzed using immunocytochemistry antibodies against p-ERK1/2 and p-p38. Both untreated and AvBD8-treated cells were incubated with the primary antibodies and Alexa Fluor 488 goat anti-rabbit IgG (H + L) secondary antibody, and then stained with DAPI (blue). Scale bar = 20 μm.

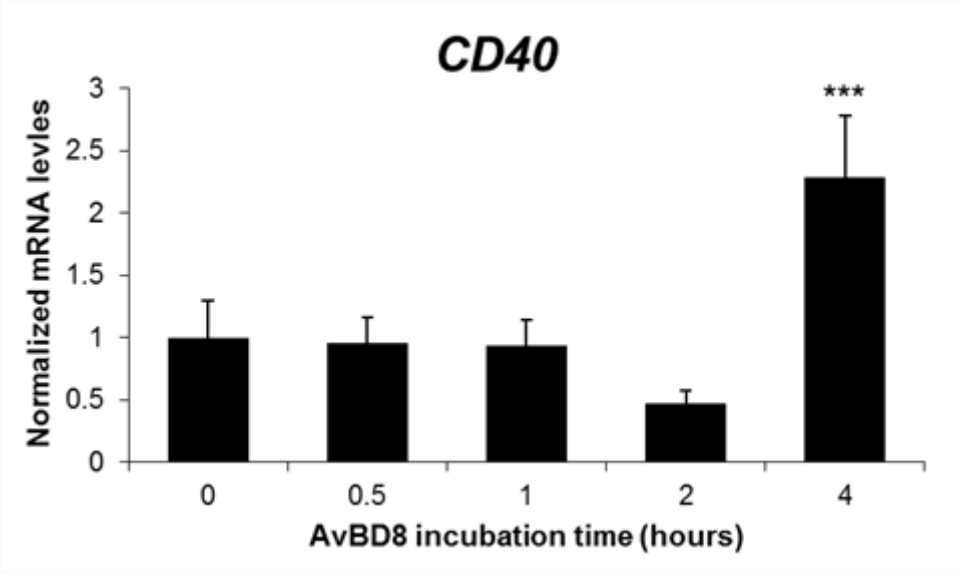

Figure 5

AvBD8 induced the expression of CD40. HD11 cells were induced with AvBD8 (100 ng/mL) for 0, 0.5, 1, 2, and 4 h and CD40 expression was measured by qRT-PCR. Expression level was normalized to that of GAPDH. All culture conditions were tested in triplicate. The data are expressed as mean ± SEM and are representative of three independent experiments: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Supplementary Files

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