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Effects of Human Parvovirus B19 on Expression of Defensins and Toll-Like Receptors

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Abstract

Both cell-mediated and humoral immunity have been widely investigated for the roles in pathogenesis of human parvovirus B19 (B19) infection. However, little is known about the effects of B19 infection on innate immunity. In the current study, expression of α-human neutrophil peptides (HNP) 1-3, α-human defensin (HD) 5, HD6, β-human defensin (hBD)-1, hBD-3, toll-like receptor (TLR) 4, TLR5, TLR7 and TLR9 in B19-nonstructural protein (NS)-1 or B19-viral protein (VP)-2 transfected COS-7 cells was investigated by reverse transcription (RT)-PCR or by western blots. Significantly increased HNP1-3, HD5, HD6, hBD1 and hBD3 mRNA levels were detected at both 24 h and 20 days post-transfection in COS-7 cells transfected with pEGFP-NS1. In pEGFP-VP2-transfected COS-7 cells, significantly increased HNP1-3, HD5, HD6, hBD-1 and hBD-3 mRNA expression levels were observed on day 20, albeit only hBD3 mRNA increased significantly at 24 h post-transfection. Additionally, TLR4, TLR5 and TLR7 proteins decreased significantly in COS-7 cells transfected with pEGFP-NS1 or pEGFP-VP2 at 48 h but significantly increased on day 20. Notably, only TLR9 protein increased significantly in the cells transfected with pEGFP-NS1 on day 20. No significant variation of TLRs was observed in cells transfected with pEGFP-NS1K334E, a single substitution mutantation of B19-NS1 protein without original cytotoxicity, at both 48 h and on day 20. These novel findings revealed the different effects of B19-NS1 and VP2 on the stimulation of defensins and TLRs and could provide a clue in understanding the roles of B19-NS1 and VP2 on innate immunity.

Key Words: human parvovirus B19 (B19), non-structural protein (NS1), viral protein 2 (VP2), Defensin, toll-like receptor (TLR)

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Introduction

Human Parvovirus B19 (B19) was first discovered in 1975 (8) and has contributed to a wide spectrum of illnesses, such as erythema infectiosum, arthropathies, cardio-vasculitis, vasculitis, hepatitis and neuron disorders (48). B19 is a small, non-enveloped virus containing a single-stranded DNA genome of 5,600 nucleotides encoding two capsid proteins, VP1 and VP2, and a nonstructural protein, NS1 (48). The molecular weight of the VP2 protein is 58 kDa, which is the major capsid protein of B19 and which comprises 95% of the capsid. The B19-NS1 protein is known as a transactivator of the promoters of the B19 viral p6 and various cellular genes (24) and is involved in DNA replication, cell cycle arrest and initiation of apoptosis in erythroid and epithelial cells (16, 26). B19-NS1 contains a well-conserved nucleoside triphosphate (NTP)-binding motif that is crucial for its cytotoxicity. However, the activities of B19-NS1 can be abolished by a single amino acid substitution within the NTP-binding domain, which is known as NS1K334E (25-27, 35). Significantly higher mitochondria-dependent apoptosis was detected in COS-7 cells transfected with pEGFP-NS1 compared to those transfected with the control pEGFP plasmid whereas significantly reduced apoptosis was observed in COS-7 cells transfected with pEGFP-NS1K334E compared to those transfected with pEGFP-NS1 (16, 17, 35, 38).

The innate immune system, including defensins and toll-like receptors (TLRs), provides the first line of host defense against a variety of pathogens (43, 46, 47). Defensins are endogenous, small, cysteine-rich antimicrobial peptides that can be classified into three distinct classes, α, β, and θ-defensins, in which only α- and β-defensins are expressed in humans. Both α- and β-defensins are composed of short cationic peptides containing six conserved cysteine residues (10, 20). The four human α-defensins originally isolated from neutrophil are named as human neutrophil peptides (HNP) 1 through to 4, HD-5 and HD-6. Human β-defensins are named as HβD-1 through to 4 and were originally isolated from human plasma (HβD-1) and psoriatic scales (HβD-2 and HβD-3). In contrast, HβD-4 has not yet been isolated, but has been identified solely by genomics (9, 14, 19, 29). Moreover, the production of defensins can be induced by cytokines or TLR activation (13, 14, 19). TLRs are evolutionarily conserved innate receptors expressed in various immune and non-immune cells of the mammalian host. TLRs play a crucial role in defending against pathogenic microbial infection through the induction of inflammatory cytokines and type I interferons. Furthermore, TLRs also play roles in pattern pathogen-specific humoral and cellular adaptive immune responses (22, 33).

B19 is known to play crucial roles in the development of autoimmune disorders (21, 23, 30, 36, 37) and has been associated with various TLRs such as TLR-4, -5, -7 and -9 (12, 39, 41). Additionally, several TLRs have been demonstrated to play a role in the production of defensins (1, 13, 14, 19). However, the effects of B19 viral proteins on expression of defensins and TLRs are rarely described. The present study intended to investigate the expression of epithelial associated defensins (HNP1-3, HD5, HD6, hBD-1 and hBD-3) and TLRs (TLR4, TLR5, TLR7 and TLR9) in B19-NS1- or -VP2-transfected COS-7 epithelial cells to clarify the effects of B19 on the expression of defensins and TLRs.

Materials and Methods

Plasmids and Oligonucleotides

The pEGFP-NS1 (expressing the EGFP-NS1 fusion protein) and pEGFP-NS1K334E (expressing EGFP-NS1K334E) constructs were expressed as described previously (35). The VP2 open reading frame (ORF) was obtained from the B19 genome (plasmid pYT104-C) by polymerase chain reaction (PCR) amplification using primers 5'-CGGAATTCCATGA CTTCAGTTAATTCTG CAGAAGCC-3' and 5'-GT CGACCAATGGGTGCACACGGCTTTT-3' containing EcoRI and SalI recognition sequences for subsequent cloning to pEGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA). PCR was performed in mixtures containing 0.2 μM primers mixtures, 1.25 μM dNTP mixture, 1.5 μM $MgCl_2$, 10 ng template, and 2.5U DNA polymerase (Takara, Tokyo, Japan). The ligation product pEGFP-VP2 (EGFP-VP2) was then transformed into *Escherichia coli* DH5α competent cells, which were obtained from Life Technologies (Carlsbad, CA, USA). Restriction enzyme digestion, PCR and DNA sequencing analysis were used to verify the plasmid.

Cell Culture and Transfection

COS-7 cells are known to be potentially useful for identifying B19 viral structural and non-structural proteins (6, 16, 27). COS-7 cells were originally obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) at 37°C and 5% CO_2 in an incubator. A total of 1×10^6 cells were grown to 70% confluency in 100 mm^2 culture plates before transfection. The transfection reaction was performed by using Lipofectamine Plus reagents (Invitrogene, CA, USA) with 2 μg of each plasmid, pEGFP-C1, pEGFP-NS1, pEGFP-

Table 1. Sequences of primers and predicted product sizes

Gene type	Sequence	Length (bp)
B19-NS1	F: 5'-GGGGGGCCAGGGTTAAACCCCAGA-3'	678
	R: 5'-CTTTAACACATGCTGCCCCACCAA-3'	
B19-VP2	F: 5'-TCTCCCGCAGCGAGTAGCT GCC-3'	570
	R: 5'-GTTGACTGCAGCC CTCTAA-3'	
HNP1-3	F: 5'-ATGAGGA CCCTCGCCATCCT-3'	275
	R: 5'-TCAGCAGCAGAATGC CCAG-3'	
HD4	F: 5'-ATGAGGATTATCGCCCTCC-3'	294
	R: 5'-TTAATCGACACGCGTGC-3'	
HD5	F: 5'-ATGAGGACCATCGCCATCCT-3'	284
	R: 5'-TCAGCAGCA GAATGCCCAG-3'	
HD6	F: 5'ATGAGAACCCTCACCATCC-3'	303
	R: 5'-TCAGAGGCAGCAGAATC-3'	
hBD1	F: 5'-TCAGCGACAGCAGAGTCTG-3'	206
	R: 5'-TCACT TGCAGCACTTGGCC-3'	
hBD3	F: 5'-ATGAGGATCCATTA TCTTC-3'	191
	R: 5'-TCGGCAGCATTTTCGGCC-3'	
hBD5	F: 5'-ATGGCCCTGATCAGGAAGAC-3'	237
	R: 5'-TCAGATCCTCTGTCTGC-3'	
hBD6	F: 5'-ATGAGGACTTT CCTC-3'	198
	R: 5'-TTAATCTATAATGCTCCCA-3'	
GAPDH	F: 5'-CCATGGCA CCGTCAAGGCTGA-3'	533
	R: 5'-TTGGCA GTGGGGACACGGAA-3'	

B19: human parvovirus B19; NS1: nonstructural protein 1; VP2: viral particle 2; HNP: Human Neutrophil Peptide; hBD: human β-defensin; F: forward primer; R: reverse primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp: base pair.

NS1K334E or pEGFP-VP2 according to the manufacture's instruction. The transfection efficiency is approximately 50-70%. The transfected cells were then treated with neomycin (800 μg/ml) before subsequent experiments were performed. Expression of EGFP alone, EGFP-NS1, EGFP-NS1K334E or EGFP-VP2 fusion proteins was examined by fluorescence microscopy, western blot analysis, flow cytometric analysis and RT-PCR.

Fluorescence Microscopy

EGFP expression in transfected cells was observed with a Zeiss Axioplan-2 epifluorescence microscope equipped with a fluorescence filter. Digital images of the cells were recorded by using a spot camera system.

Semiquantitative RT-PCR and Real-Time PCR Analysis

All studies were carried out in a designated PCR-clean area and the reactions were performed as described elsewhere (15, 42). RNA was extracted from infected cells using Trizol reagent (Invitrogene) according to the manufacture's instruction. Total RNA was isolated from COS-7, and COS-7 cells expressing EGFP or the EGFP-NS1, EGFP-NS1K334E or EGFP-VP2 fusion proteins. RNA samples were resuspended in diethyl pyrocarbonate (DEPC)-treated water, quantified and then stored at -80°C until use. The first-strand cDNA for RT-PCR was synthesized from total RNA (2 μg) using the Promega RT-PCR system (Promega, Madison, WI, USA). The primers used in this study are shown in Table 1. Amplification was performed in a 50-μl reaction volume containing 1× reaction buffer (Promega), 1.5 μM of $MgCl_2$, 200 μM of dNTPs, 1 μM of each primer and 2.5 units of Taq DNA polymerase (Promega), using a Perkin-Elmer Gene Amp PCR system 2400. The RT-PCR-derived DNA fragments obtained by 30 PCR cycles were subjected to electrophoresis on a 1.7% agarose gel. Real-time PCR was performed on the generated cDNA product in the IQ5 sequence detection system using SYBR Green (Bio-Rad, Laboratories, Inc., Hercules, CA, USA). GAPDH mRNA level was used as an internal control for normalizing the mRNA levels in control and experimental samples. Dilution curves confirmed the linear dependence of the threshold cycle number on the concentration of template RNAs. The relative quantitation of each

mRNA in control and experimental samples was obtained using the standard curve method.

Immunoblotting

COS-7 cells transfected with different plasmids were lysed in an aliquot volume of 600 µl PRO-PREP™ solution (iNtRON Biotech, Gyeonggi-do, Korea) for 30 min on ice. The cell lysates were then centrifuged at 15,000 rpm for 10 min at 4°C and the supernatant was isolated and stored at -80°C until use. Protein concentration was determined according to the method described by Bradford (2). Thirty µg of each protein sample were applied and separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100-120 V for 1.5 h before being transferred to nitrocellulose membranes. The membranes were cut into strips and soaked in 5% nonfat dry milk in PBS for 30 min at room temperature to saturate irrelevant protein binding sites. Antibodies against actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), EGFP (Eugene, OR, USA), TLR4, TLR5, TLR7 and TLR9 (Abcam, Cambridge, UK) were diluted in PBS with 2.5% BSA and incubated for 1.5 h with gentle agitation at room temperature. The antigen-antibody was then reacted with an horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and detected with Pierce's Supersignal West Dura HRP Detection Kit (Pierce Biotechnology Inc., Rockford, IL, USA). Quantified results were performed by densitometry (Appraise, Beckman-Coulter, Brea, CA, USA).

Statistical Analysis

All the statistical analyses were performed using the SPSS 10.0 software (SPSS Inc, Chicago, IL, USA). Three independent experiments were repeated. Statistical analyses were performed using one-way ANOVA and the Bonferroni's Multiple Comparison Test. $P < 0.05$ was considered statistically significant.

Results

Expression of α- and β- Defensins mRNA

The plasmids pEGFP-C1, pEGFP-NS1, pEGFP-NS1K334E, and pEGFP-VP2 were obtained or constructed as described in Materials and Methods. The COS-7 epithelial cells were individually transfected with each of these plasmids. The expression of EGFP and the EGFP-NS1, EGFP-NS1K334E and EGFP-VP2 fusion proteins was observed under fluorescence microscope and confirmed by western blot (Fig. 1A). The mRNA expression of B19-NS1 and VP2 mRNA was confirmed by RT-PCR (Fig. 1B). To clarify the influence of B19 viral proteins on defensins expression, the HNP1-3, HD5, HD6, hBD-1 and hBD-3 mRNA were examined in COS-7 cells singly transfected with pEGFP-C1, pEGFP-NS1, pEGFP-NS1K334E and pEGFP-VP2. Significantly increased expression levels of HNP1-3, HD5, HD6, hBD1 and hBD3 mRNA were detected in COS-7 cells transfected with pEGFP-NS1 compared to those transfected with the pEGFP control plasmid at 24 h post-transfection (Figs. 1C, 1D, 1E, 1F and 1G). Similar results were observed in COS-7 cells transfected with pEGFP-NS1 at day 20 post-transfection (Fig. 2). Similarly, significantly increased HNP1-3, hBD1 and hBD3 mRNA levels were observed in COS-7 cells transfected with pEGFP-NS1K334E at 24 h post-transfection (Figs. 1C, 1D, 1E, 1F and 1G) and the significantly increased HNP1-3, HD5 and HD6 mRNA levels on day 20 (Fig. 2). In pEGFP-VP2-transfected COS-7 cells, hBD3 mRNA was increased significantly at 24 h (Figs. 1C, 1D, 1E, 1F and 1G) compared to those transfected with pEGFP; significantly increased HNP1-3, HD5, HD6, hBD-1 and hBD-3 mRNA expression was detected in COS-7 cells on day 20 compared to those transfected with pEGFP (Fig. 2).

Expression of TLRs Proteins

Since defensin production can be induced by TLR activation, expression of TLR molecules was examined. Expression of TLR-4, TLR-5, TLR-7 and TLR-9 in COS-7 cells transfected with pEGFP, pEGFP-NS1, pEGFP-NS1K334E and pEGFP-VP2 was analyzed by western blots at 48 h and on day 20 after tranfection. As shown in Fig. 3, significantly decreased expression amounts of TLR4, TLR5 and TLR7 proteins were detected in cells transfected with pEGFP-NS1 at 48 h compared to those transfected with pEGFP. Similar results were observed in COS-7 cells transfected with pEGFP-VP2 at 48 h relative to cells transfected with pEGFP (Figs. 3A, 3B and 3C). In contrast, significantly increased expression of TLR4, TLR5 and TLR7 proteins was found in COS-7 cells transfected with pEGFP-NS1 or pEGFP-VP2 on day 20 compared to those transfected with pEGFP (Figs. 4A, 4B and 4C). Notably, only TLR9 protein increased significantly in COS-7 cells transfected with pEGFP-NS1 on day 20 (Figs. 3D and 4D). No significant variations of TLR4, 5, 7 or 9 were observed in COS-7 cells transfected with pEGFP-NS1K334E at both 48 h and on day 20.

Discussion

Parvovirus B19 is a significant human pathogen connected to a wide spectrum of disorders, including erythema infectiosum in immunocompetent children,

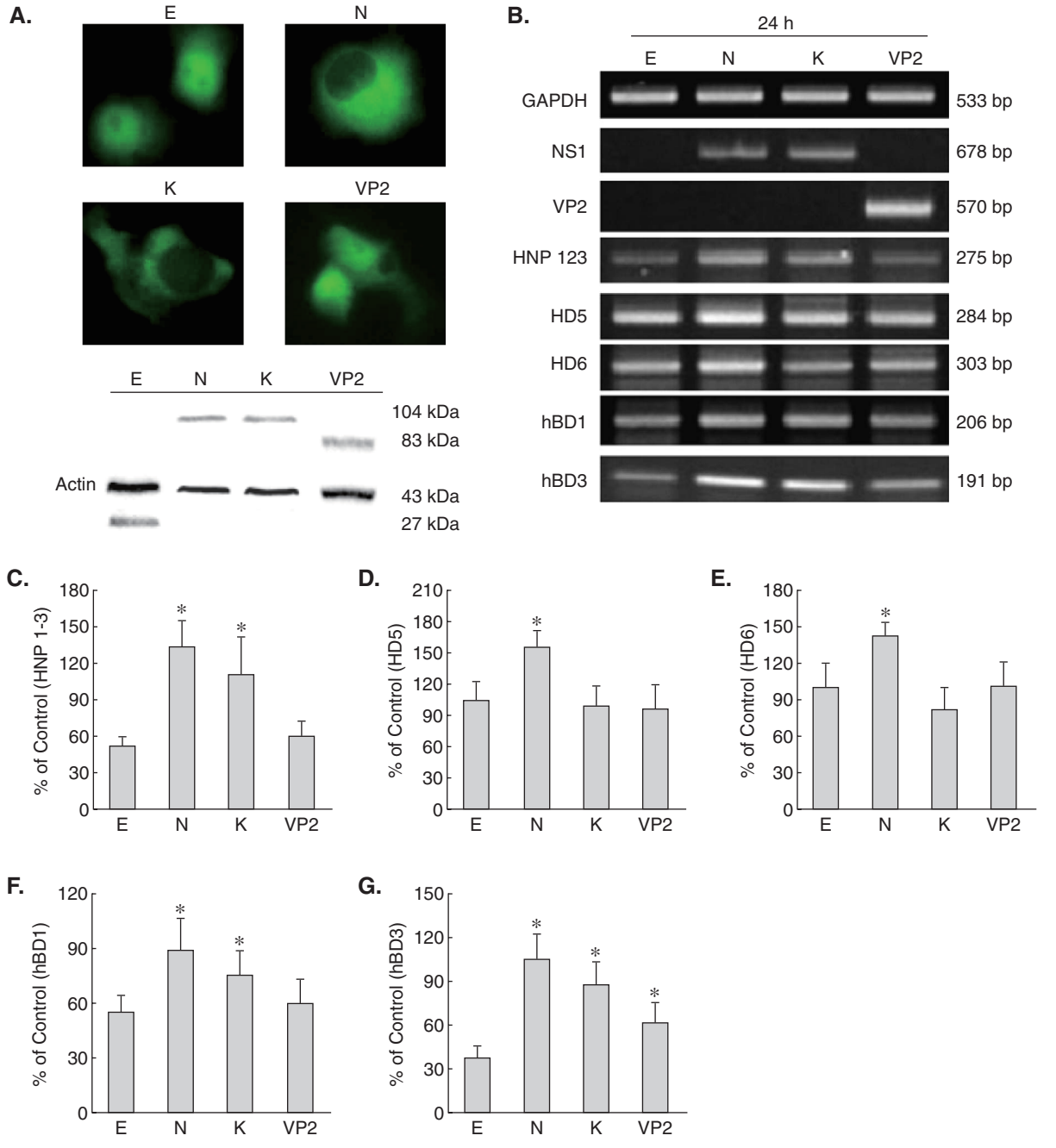

Fig. 1. Effects of B19-NS1, B19-NS1K334E and B19-VP2 on mRNA levels of HNP1-3, HD5, HD6, hBD1 and hBD3 in COS-7 cells at 24 h post-transfection. (A) COS-7 cells were observed under fluorescence microscope after transfection with pEGFP, pEGFP-NS1, pEGFP-NS1K334E and pEGFP-VP2, respectively, and cell lysates were reacted with an antibody directed against EGFP. (B) Total RNA was purified at 24 h after transfection and the presence of GAPDH, NS1, NS1K334E, VP2, HNP1-3, HD5, HD6, hBD1 and hBD3 mRNA was detected by RT-PCR. (C-G) Quantitative real-time PCR analysis of HNP1-3, HD5, HD6, hBD1 and hBD3. The symbols of E, N, K and VP2 indicate EGFP, EGFP-NS1, EGFP-NS1K334E and EGFP-VP2, respectively. Asterisk (*) indicates significant differences compared to E.

lethal cytopenias in immunocompromised patients, intrauterine fetal death in primary infected pregnant women and various autoimmune diseases (3). Although the immune mechanisms, such as humoral and cellular immune responses, elicited by parvovirus B19 have been widely investigated, little is known about the influences of B19 on innate immune responses. In this study, we first reported that different expression profiles of α-defensins, β-defensin and TLRs were elicited by the B19-NS1 or B19-VP2 in COS-7 cells.

Defensins are crucial mammalian anti-microbial

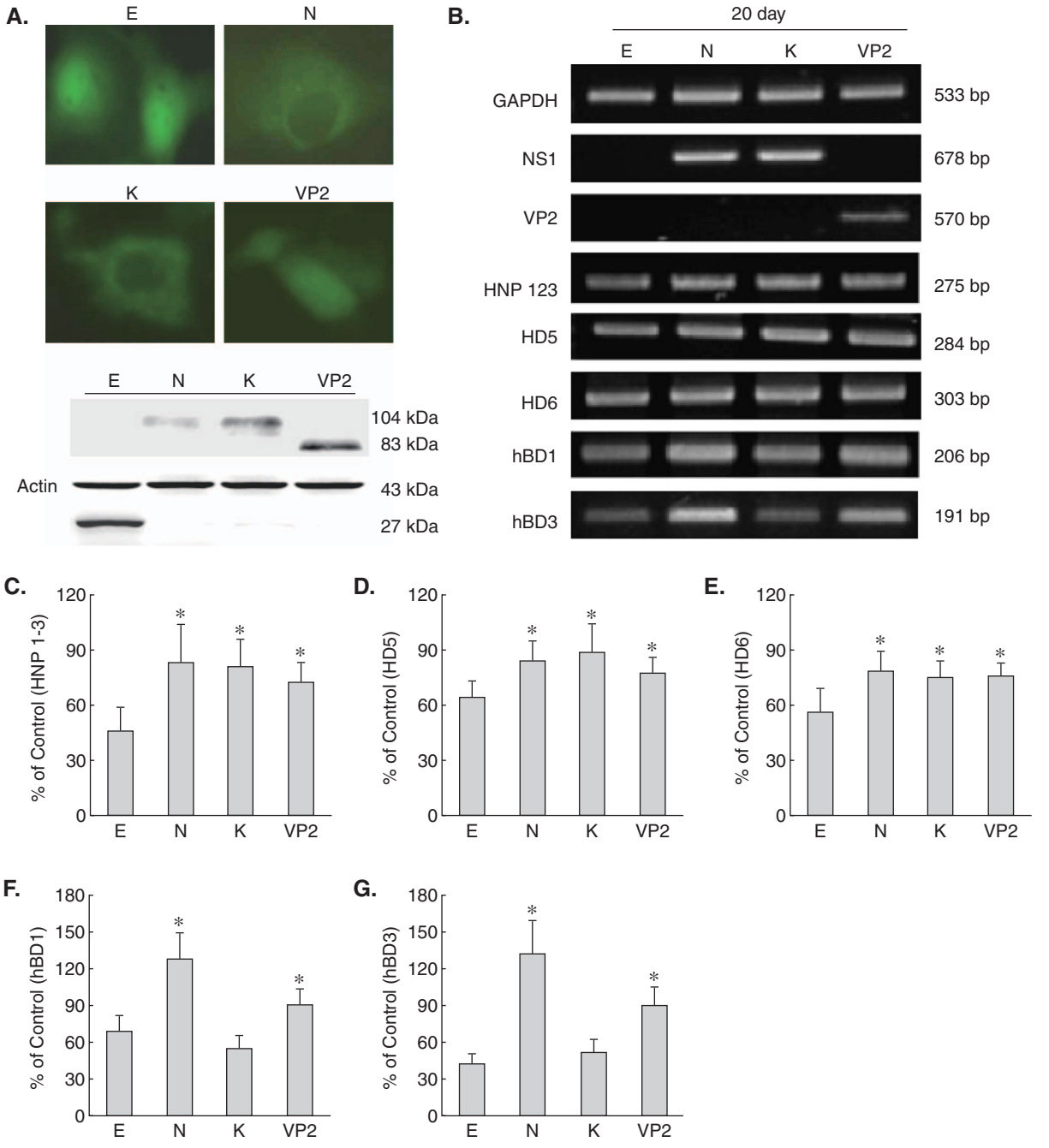

Fig. 2. Effects of B19-NS1, B19-NS1K334E and B19-VP2 on mRNA levels of HNP1-3, HD5, HD6, hBD1 and hBD3 in COS-7 cells on day 20 post-transfection. (A) COS-7 cells were observed under fluorescence microscope after transfection with pEGFP, pEGFP-NS1, pEGFP-NS1K334E and pEGFP-VP2, respectively, and cell lysates were reacted with an antibody against EGFP. (B) Total RNA was purified on day 20 after transfection and the presence of GAPDH, NS1, NS1K334E, VP2, HNP1-3, HD5, HD6, hBD1 and hBD3 mRNA was detected by RT-PCR. (C-G) Quantitative real-time PCR analysis of HNP1-3, HD5, HD6, hBD1 and hBD3. See legend to Fig. 1 for explanation of symbols used.

proteins and contribute to the innate, anti-microbial defense of the host by disrupting the bacterial cell membrane (44, 45). Defensins play different roles in various diseases and exhibit receptor-specific chemotactic activity. A recent report indicated that human α-defensins (HNP1-3 and HD-5) could inhibit HIV replication (28, 49) and block papillomavirus infection (4). In bronchoalveolar inflammation and skin diseases, such as psoriasis and mastitis, the expression of HβD-2 and HβD-3 is significantly increased (31, 32). Additionally, β-defensins have been shown to be ligands for chemokine receptor CCR6 on dendritic

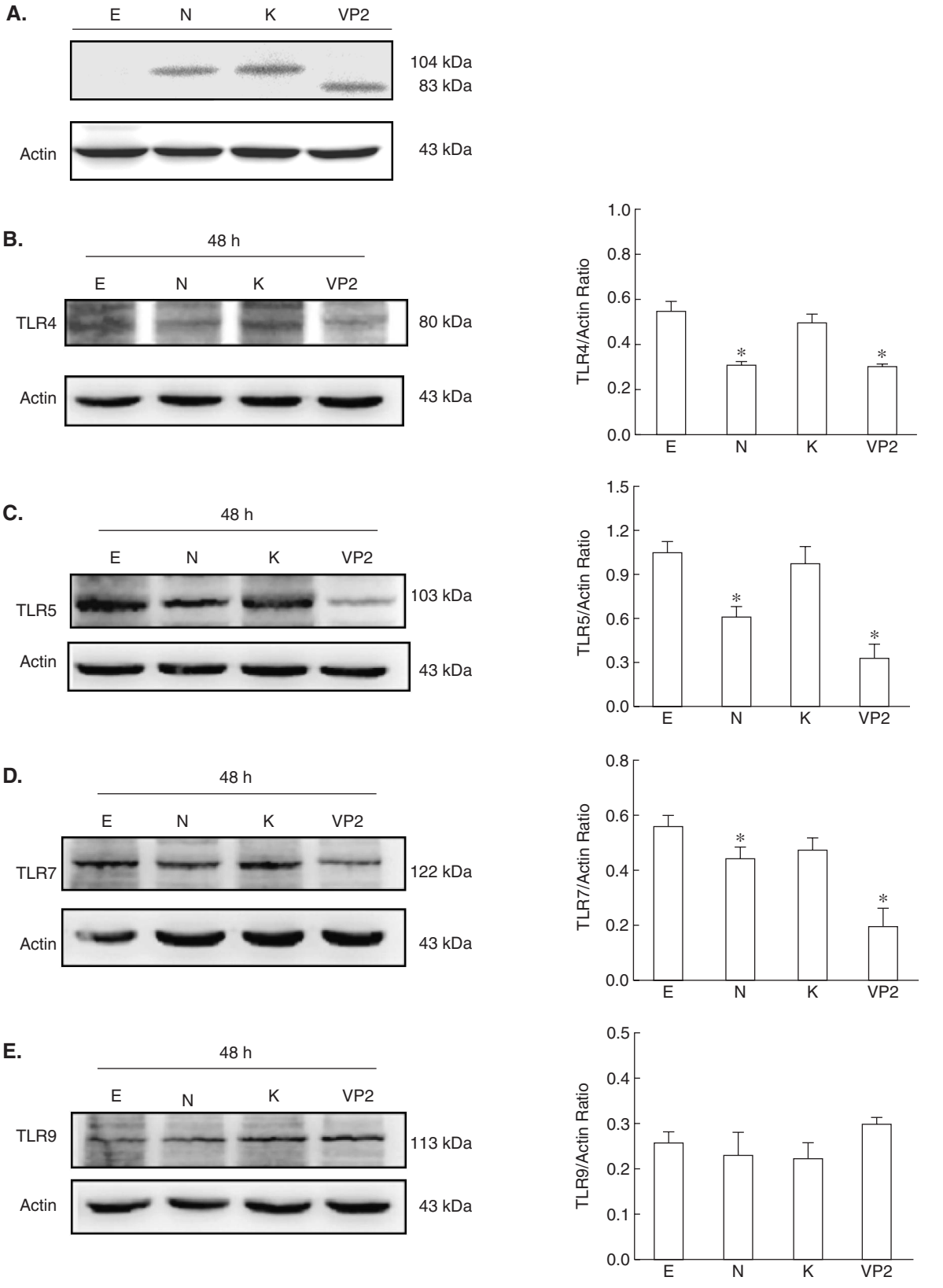

Fig. 3. Effects of B19-NS1, B19-NS1K334E and B19-VP2 on TLR expression in COS-7 cells at 48 h post-transfection. COS-7 cells were transfected with pEGFP, pEGFP-NS1, pEGFP-NS1K334E and pEGFP-VP2, respectively. Total proteins were extracted at 48 h after transfection and the expression of (A) EGFP-NS1, EGFP-NS1K334E and EGFP-VP2, (B) TLR-4, (C) TLR-5, (D) TLR-7 and (E) TLR-9 was detected by western blots. The relative protein expression levels of TLR-4, TLR-5, TLR-7 and TLR-9 were normalized on the basis of actin. See legend to Fig. 1 for explanation of symbols used.

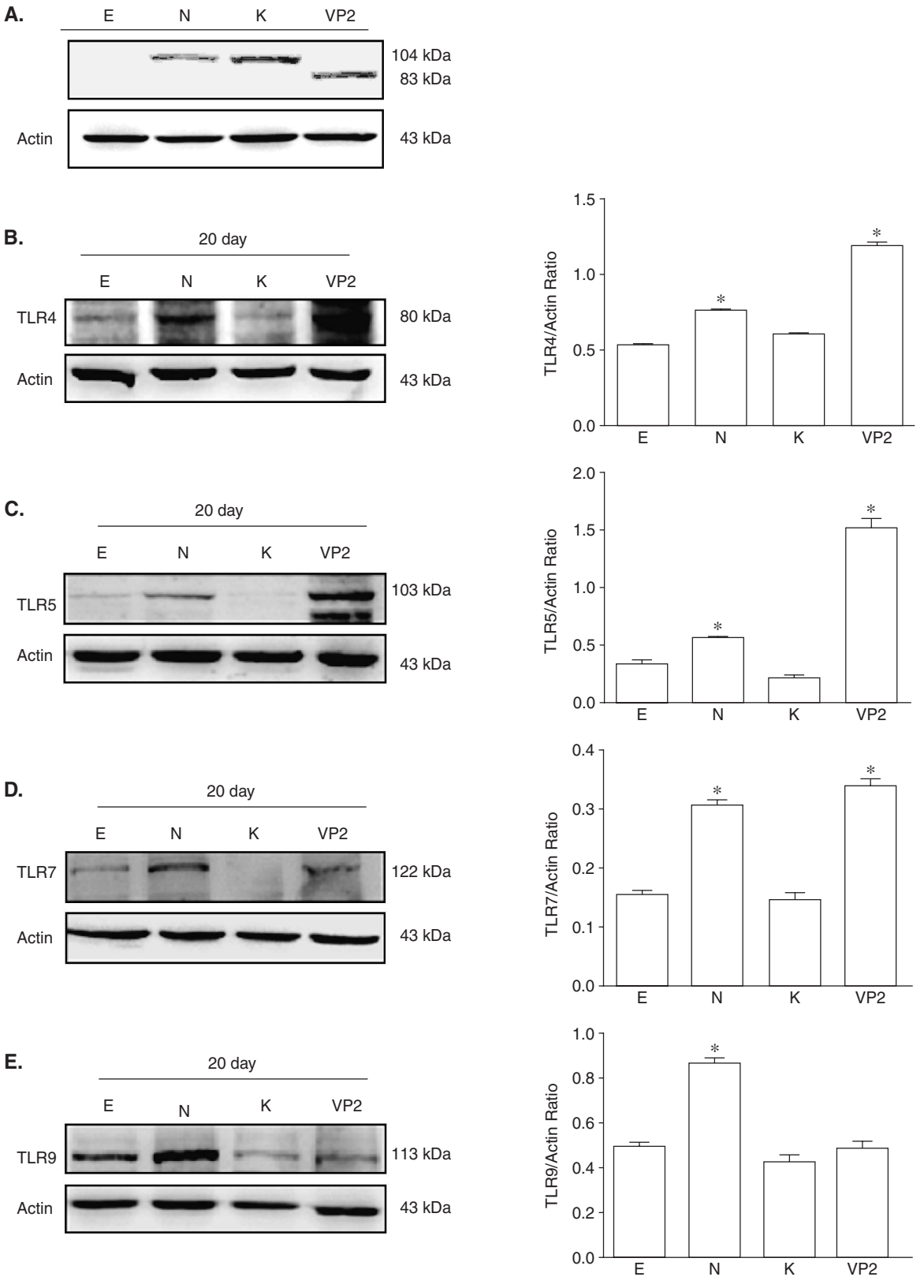

Fig. 4. Effects of B19-NS1, B19-NS1K334E and B19-VP2 on TLR expression in COS-7 cells on day 20 post-transfection. COS-7 cells were transfected with pEGFP, pEGFP-NS1, pEGFP-NS1K334E and pEGFP-VP2, respectively. Total proteins were extracted on day 20 after transfection and the expression of (A) EGFP-NS1, EGFP-NS1K334E and EGFP-VP2, (B) TLR-4, (C) TLR-5, (D) TLR-7 and (E) TLR-9 was detected by western blots. The relative protein expression levels of TLR-4, TLR-5, TLR-7 and TLR-9 were normalized on the basis of actin. See legend to Fig. 1 for explanation of symbols used.

cells and T cells (34). Indeed, hBD2 has been demonstrated to be equipotent to CCL20 in stimulating migration of human intestinal Caco2 and T84 cells and non-transformed IEC6 cells (40). However, no study of the profile of defensins in B19 infection has been reported. In the current study, we first reported that HNP1-3, HD5, HD6, hBD1 and hBD3 mRNA levels increased significantly in COS-7 epithelial cells at 24 h and on day 20 after the transfection of pEGFP-NS1. In comparison, the defensins profile was different in COS-7 cells transfected with pEGFP-NS1K334E, the mutant form of B19-NS1 without the cytotoxicity (27, 35). We found that HNP1-3, hBD1 and hBD3 mRNA increased significantly at 24 h and HNP1-3, HD5 and HD6 mRNA increased significantly on day 20 in COS-7 cells transfected with pEGFP-NS1K334E. Additionally, only hBD3 mRNA increased significantly at 24 h and HNP1-3, HD5, HD6, hBD-1 and hBD-3 mRNA increased significantly 20 days post-transfection in pEGFP-VP2-transfected COS-7 cells. Although the underlying mechanisms need further investigation, these findings imply that different B19 proteins elicit distinct defensins profiles and the cytotoxicity property of B19-NS1 might play a role in the induction of defensin expression.

Several TLRs have been demonstrated to play a role in the production of defensins (1, 13, 14, 19). Alpha defensin can be induced by stimulating TLR2 or TLR5 signaling in $CD56^{+}CD3^{-}$ NK cells or in $CD56^{+}CD3^{+}$ T lymphocytes (13). In fact, NK cells directly recognize pathogen *via* TLRs and secrete α-defensins as a protective cytotoxic pathway against microorganisms (5). A previous study has indicated that β-defensins are expressed in response to TLR activation and bind to CCR6 on immature (dentric cells) DCs. Interestingly, murine beta-defensin 2 (mBD-2) could act directly on immature DCs as a ligand for TLR4 (45). Additionally, many studies also indicated that human β-defensin-2 could be induced by TLR2, TLR-3, TLR-4 and TLR9 (17, 39, 41, 42). Another study also reported that TLR3 could induce β-defensin-3 mRNA expression (11). Interestingly, our experimental results revealed that B19-NS1 and B19-VP2 attenuated the TLR4, 5 and 7 expression in COS-7 cells at 48 h but elicit them on day 20 post-transfection. Additionally, significantly increased TLR9 protein was also detected in COS-7 cells transfected with pEGFP-NS1 on day 20. Besides, our previous study has indicated increased expression and secretion of interleukin-6 in COS-7 epithelial cells transfected with human parvovirus B19 non-structural protein (NS1) (17). These findings suggest TLR-mediated defensins eliciting in B19 infection and are accord with previous studies (1, 5, 11, 13, 14, 19, 30). Moreover, we also suggest that the cytotoxicity of B19-NS1 may play crucial roles in eliciting TLRs since no significant variations of TLR4, 5, 7 and 9 were observed in COS-7 cells transfected with pEGFP-NS1K334E at both 48 h and on day 20.

Taken together, these findings suggest that the NS1 and VP2 proteins of the B19 virus elicit different profiles of defensins and TLRs and provide a clue in understanding the effects of B19 infection on TLR-mediated expression of the defensin proteins.

Acknowledgments

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References

1. Birchler, T., Seibl, R., Büchner, K., Loeliger, S., Seger, R., Hossle, J.P., Aguzzi, A. and Lauener, R.P. Human Toll-like receptor 2 mediates induction of the antimicrobial peptide human bdefensin 2 in response to bacterial lipoprotein. *Eur. J. Immunol*. 31: 3131-3137, 2001.
2. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72: 248-254, 1976.
3. Broliden, K., Tolfvenstam, T. and Norbeck, O. Clinical aspects of parvovirus B19 infection. *J. Intern. Med*. 260: 285-304, 2006.
4. Buck, C.B., Day, P.M., Thompson, C.D., Lubkowski, J., Lu, W., Lowy, D.R. and Schiller, J.T. Human α-defensins (HNP1-3 and HD-5) block papillomavirus infection. *Proc. Natl. Acad. Sci. USA* 103: 1516-1521, 2006.
5. Chalifour, A., Jeannin, P., Gauchat, J.F., Blaecke, A., Malissard, M., N'Guyen, T., Thieblemont, N. and Delneste, Y. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers a-defensin production. *Blood* 104: 1778-1783, 2004.
6. Cohen, B.J., Field, A.M., Mori, J., Brown, K.E., Clewley, J.P., St Amand, J. and Astell, C.R. Morphology and antigenicity of recombinant B19 parvovirus capsids expressed in transfected COS-7 cells. *J. Gen. Virol*. 76: 1233-1237, 1995.
7. Corcoran, A. and Doyle, S. Advances in the biology, diagnosis and host-pathogen interactions of parvovirus B19. *J. Med. Microbiol*. 53: 459-475, 2004.
8. Cossart, Y.E., Field, A.M., Cant, B. and Widdows, D. Parvovirus-like particles in human sera. *Lancet* 1: 72-73, 1975.
9. Daher, K.A., Selsted, M.E. and Lehrer, R.I. Direct inactivation of viruses by human granulocyte defensins. *J. Virol*. 60: 1068-1074, 1986.
10. DeSmet, K. and Contreras, R. Human antimicrobial peptides: defensins, cathelicidins, and histatins. *Biotechnol. Lett*. 27: 1337-1347, 2005.
11. Duits, L.A., Nibbering, P.H., van Strijen, E., Vos, J.B., Mannesse-Lazeroms, S.P., van Sterkenburg, M.A. and Hiemstra, P.S. Rhinovirus increases human defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. *FEMS Immunol. Med. Microbiol*. 38: 59-64, 2003.
12. Fischer, M. and Ehlers, M. Toll-like receptors in autoimmunity. *Ann. N. Y. Acad. Sci*. 1143: 21-34, 2008.
13. Froy, O. Regulation of mammalian defensin expression by Toll-like receptor-dependent and independent signalling pathways. *Cell. Microbiol*. 7: 1387-1397, 2005.
14. Ganz, T. Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol*. 3: 710-720, 2003.
15. Gong, L., Zhang, B., Zhang, Y., Hu, H., Zhao, Z. and Liu, X.

Cloning, expression and purification of human S-adenosylmethionine decarboxylase gene alpha subunit. *Chinese J. Physiol*. 50: 29-33, 2007.
16. Hsu, T.C., Wu, W.J., Chen, M.C. and Tsay, G.J. Human parvovirus B19 non-structural protein (NS1) induces apoptosis through mitochondria cell death pathway in COS-7 cells. *Scand. J. Infect. Dis*. 36: 570-577, 2004.
17. Hsu, T.C., Tzang, B.S., Huang, C.N., Lee, Y.J., Liu, G.Y., Chen, M.C. and Tsay, G.J. Increased expression and secretion of interleukin-6 in human parvovirus B19 non-structural protein (NS1) transfected COS-7 epithelial cells. *Clin. Exp. Immunol*. 144: 152-157, 2006.
18. Klenerman, P., Tolfvenstam, T., Price, D.A., Nixon, D.F., Broliden, K. and Oxenius, A. T lymphocyte responses against human parvovirus B19: small virus, big response. *Pathol. Biol. (Paris)*. 50: 317-325, 2002.
19. Klotman, M. and Chang, T.L. Defensins in innate antiviral immunity. *Nature Rev. Immunol*. 6: 447-456, 2006.
20. Kluver, E., Adermann, K. and Schulz, A. Synthesis and structure-activity relationship of β-defensins, multi-functional peptides of the immune system. *J. Pept. Sci*. 12: 243-257, 2006.
21. Lehmann, H.W., von Landenberg, P. and Modrow, S. Parvovirus B19 infection and autoimmune disease. *Autoimmun. Rev*. 2: 218-223, 2003.
22. Li, M., Zhou, Y., Feng, G. and Su, S.B. The critical role of Toll-like receptor signaling pathways in the induction and progression of autoimmune diseases. *Curr. Mol. Med*. 9: 365-374, 2009.
23. Loizou, S., Cazabon, J.K., Walport, M.J., Tait, D. and So, A.K. Similarities of specificity and cofactor dependence in serum antiphospholipid antibodies from patients with human parvovirus B19 infection and from those with systemic lupus erythematosus. *Arthritis Rheum*. 40: 103-108, 1997.
24. Mitchell, L.A. Parvovirus B19 nonstructural (NS1) protein as a transactivator of interleukin-6 synthesis: common pathway in inflammatory sequelae of human parvovirus infections? *J. Med. Virol*. 67: 267-274, 2002.
25. Momoeda, M., Wong, S., Kawase, M., Young, N.S. and Kajigaya, S. A putative nucleoside triphosphate-binding domain in the non-structural protein of B19 parvovirus is required for cytotoxicity. *J. Virol*. 68: 8443-8446, 1994.
26. Morita, E. and Sugamura, K. Human parvovirus B19-induced cell cycle arrest and apoptosis. *Springer Semin. Immunopathol*. 24: 187-199, 2002.
27. Nakashima, A., Morita, E., Saito, S. and Sugamura, K. Human Parvovirus B19 nonstructural protein transactivates the p21/WAF1 through Sp1. *Virology* 329: 493-504, 2004.
28. Nakashima, H., Yamamoto, N., Masuda, M. and Fujii, N. Defensins inhibit HIV replication *in vitro*. *AIDS* 7: 1129, 1993.
29. Oppenheim, J.J., Biragyn, A., Kwak, L.W. and Yang, D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Ann. Rheum. Dis*. 62: 17-21, 2003.
30. Romano Carratelli, C., Mazzola, N., Paolillo, R., Sorrentino, S. and Rizzo, A. Toll-like receptor-4 (TLR4) mediates human β-defensin-2 (hBD-2) induction in response to Chlamydia pneumoniae in mononuclear cells. *FEMS Immunol. Med. Microbiol*. 57: 116-124, 2009.
31. Schroder, J.M. and Harder, J. Molecules in focus human β-defensin-2, *Int. J. Biochem. Cell Biol*. 31: 645-651, 1999.
32. Selsted, M. and Ouellette, A.J. Mammalian defensins in the Antimicrobial immune response. *Nat. Immunol*. 6: 551-557, 2005.
33. Takeda, K. and Akira, S. Toll-like receptors. *Curr. Protoc. Immunol. Chapter* 14:Unit 14.12, 2007.
34. Taylor, K., Rolfe, M., Reynolds, N., Kilanowski, F., Pathania, U., Clarke, D., Yang, D., Oppenheim, J., Samuel, K., Howie, S., Barran, P., Macmillan, D., Campopiano, D. and Dorin, J. Defensin-related peptide 1 (Defr1) is allelic to Defb8 and chemoattracts immature DC and $CD4^+$ T cells independently of CCR6. *Eur. J. Immunol*. 39: 1353-1360, 2009.
35. Tzang, B.S., Chen, D.Y., Tsai, C.C., Chiang, S.Y., Lin, T.M. and Hsu, T.C. Human parvovirus B19 nonstructural protein NS1 enhanced the expression of cleavage of 70kDa U1-snRNP autoantigen. *J. Biomed. Sci*. 17: 40, 2010.
36. Tzang, B.S., Lee, Y.J., Yang, T.P., Tsay, G.J., Shi, J.U., Tsai, C.C. and Hsu, T.C. Induction of antiphospholipid antibodies and anti-phospholipid syndrome-like autoimmunity in naive mice with antibody against human Parvovirus B19 VP1 unique region protein. *Clin. Chim. Acta* 382: 31-36, 2007.
37. Tzang, B.S., Tsai, C.C., Chiu, C.C., Shi, J.U. and Hsu, T.C. Up-regulation of adhesion molecule expression and induction of TNF-α on vascular endothelial cells by antibody against human parvovirus B19 VP1 unique tegion protein. *Clin. Chim. Acta* 395: 77-83, 2008.
38. Tzang, B.S., Tsai, C.C., Tsay, G.J., Wang, M., Sun, Y.S. and Hsu, T.C. Anti-human parvovirus B19 non-structural protein antibodies in patients with rheumatoid arthritis. *Clin. Chim. Acta* 405: 76-82, 2009.
39. Tzang, B.S., Tsay, G.J., Lee, Y.J., Li, C., Sun, Y.S. and Hsu, T.C. The association of VP1 unique region protein in acute parvovirus B19 infection and antiphospholipid antibody production. *Clin. Chim. Acta* 378: 59-65, 2007.
40. Vongsa, R.A., Zimmerman, N.P. and Dwinell, M.B. CCR6 regulation of the actin cytoskeleton orchestrates human beta defensin-2- and CCL20-mediated restitution of colonic epithelial cells. *J. Biol. Chem*. 284: 10034-10045, 2009.
41. Wang, H.P., Hsu, T.C., Hsu, G.J., Li, S.L. and Tzang, B.S. Cystamine attenuates the expressions of NOS- and TLR- associated molecules in brain of NZB/W F1 mice. *Eur. J. Pharmacol*. 607: 102-106, 2009.
42. Wang, W., Zhu, G.J. and Zu, S.Y. Effects of 17β-estradiol and phytoestrogen α-zearalanol on tissue factor in plasma of ovariectomized rats and HUVECs. *Chinese J. Physiol*. 47: 67-72, 2004.
43. Yan, C.G., Ren, G.Y., Wang, F., Zhu, D.F. and Zhuang, Y. CD14-159 and -260 gene polymorphisms are associated with HBV-related cirrhotic injury in Chinese Han patients. *Chinese J. Physiol*. 51: 94-99, 2008.
44. Yang, D., Biragyn, A., Kwak, L.W. and Oppenheim, J.J. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol*. 23: 291-296, 2002.
45. Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schröder, J.M., Wang, J.M., Howard, O.M. and Oppenheim, J.J. β-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 286: 525-528, 1999.
46. Yang, D., Liu, Z.H., Tewary, P., Chen, Q., de la Rosa, G. and Oppenheim, J.J. Defensin participation in innate and adaptive immunity. *Curr. Pharm. Des*. 13: 3131-3139, 2007.
47. Yeh, J.Y., Yen, M.S., Lo, W.Y., Chao, K.C., Yuan, C.C. and Juang, C.M. Physiology and potential application of NKT cells: a minireview. *Chinese J. Physiol*. 52: 275-279, 2009.
48. Young, N.S. and Brown, K.E. Parvovirus B19. *N. Engl. J. Med*. 350: 586-597, 2004.
49. Zhang, L., Yu, W., He, T., Yu, J., Caffrey, R.E., Dalmasso, E.A., Fu, S., Pham, T., Mei, J., Ho, J.J., Zhang, W., Lopez, P. and Ho, D.D. Contribution of human α-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* 298: 995-1000, 2002.