Improved expression of recombinant fusion defensin gene plasmids packed with chitosan-derived nanoparticles and effect on antibacteria and mouse immunity

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Abstract. In order to develop a secure and competent technique to express the human immune gene for fighting infections, we cloned and expressed the BD2/3 using VR1020 (a eukaryotic expression plasmid). BD2/3 contains human β-defensin 2 (BD2) and human BD3. To explore safe and effective DNA delivery molecules *in vitro* and *in vivo*, the fusion genes of BD2/3 were used as an immune-labelled gene to verify transfection effectivness of modified chitosan (CS). Plasmid of VR1020-BD2/3 was packed with biomaterials: CS, average molecular weight: 25000D; polyethylene glycol-O-chitosan-polyethylenimine (PEG-O-CS-PEI); liposomes (LP); polyamine cationic liposomes (PCL); polyamine cationic liposomes of protamine (PCL-protamine) by ionotropic gelation. We observed that BD2/3 fusion gene showed high bioactivity *in vitro* and *in vivo*. The BD2/3 fusion protein inhibited the proliferation of bacteria (*S. aureus, S. pneumoniae, P. aeruginosa* and *E. coli).* The Kunming mice were immune to these nanoparticles and we analyzed their delivery efficiency and gene expression effect. BD2/3 results in multiple changes of innate and required immune system of mice. BD2/3 increases expression of IgG, IgG1, IgG2a, IL-2, IL-6, IFN-γ, as well as of lymphocytes and monocytes. Following challenge with virulent *E. coli*, $CD4^+$ and $CD8^+$ positive T-cell counts were highly elevated in the BD2/3 immunized mice, resulting in higher survival rates of mice. These results indicate that nanoparticles containing modified CS and BD2/3 are potentially safe and effective drugs *in vivo* to improve the immunity against bacterial infection and enhance innate immunity and adaptive immunity against infectious diseases.

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Introduction

The increasing discovery of multiple antibiotic-resistant bacteria requires urgent development of novel and effective antibacterial reagents (1-3). Natural defensins have attracted attention for their multiple functions against antibiotic resistant bacteria (4,5). Extensive studies have shown that defensins can, not only kill or inhibit the growth of diverse pathogens directly, but also boost specific immune responses that provide highly effective immunity against pathogens (5,6).

Human β-defensin 2 (BD2) and BD3 are major members of defensin family of antimicrobial peptides (AMP). In addition to their direct bactericidal action, they have been demonstrated to modulate the innate and adaptive immune responses (4,5,7). BD2 acts primarily by its chemotactic ability to recruit memory T cells and immature dendritic cells, which are key immunological molecules of the immune system during infection or injuries (8). BD2 can also induce mast cell migration, degranulation and histamine release (9). BD3 has been reported to be chemotactic for monocytes and macrophages, which plays an important role in combating infection (10,11). However, single use of BD2 and BD3 as new antimicrobial drugs has to face the deficiency of low bioactivity and short half-time *in vivo* due to their rapid degradation rate.

Recently, some strategies have been used to overcome the problems of weak bioactivity and half-life period *in vivo*, such

as fusion of multiple AMPs (12) and the application of gene delivery systems (11,13). The design of multivalent AMPs usually involves conjugation of several AMP monomers together via linkers, and have been reported to display more novel and stronger bioactivities than the original monomeric forms (14-16). For example, vancomycin, in the form of a component of fusion multivalent antibiotics, has been shown to be more effective against microbes (14) and fusion multivalent indolicidin shows higher bioactivity against multidrug-resistant (MDR) strains (17). These results indicate that fusion of multiple AMPs provides a novel approach for creation of stronger antibiotics to improve the resistance of hosts against infectious disease.

Chitosan (CS) and liposomes (LP) have both been used as gene delivery systems because of their non-toxic, biocompatible properties and efficient DNA loading rates (18,19). However, the nanoparticles containing entrapped genes in CS and LP are rapidly cleared in the blood and are degraded by protease *in vivo*. To address these problems, LP have been conjugated with amino groups to improve cellular phagocytosis and to promote lysosomal escape due to the positive charge (20). CS has been modified with polyethylene glycol (PEG) and polyethylenimine (PEI) to prolong blood circulation time and enhance escape of DNA from lysosomes (21,22). Such advances in the application of modified CS gene carriers to enhance DNA transfection efficiency *in vivo* have encouraged further research.

Until now, little information has been available on the synergetic antibacterial effects between BD2 and BD3 *in vitro* and *in vivo*. Therefore, the present experiment was carried out to compare the delivery efficiency and expression effect of the fusion gene of BD2 and BD3 that were entrapped with different LP, CS and its derived package molecules with the aim of developing a safe, effective and biocompatible material to improve the bioactivity of fusion antibacterial peptide gene *in vivo* against bacterial infection in animals.

Materials and methods

General. The study was approved by the Ethics Committee of Sichuan University (Chengdu, China).

Construction of prokaryotic expression plasmid of fusion gene of BD2/3. To construct a fusion gene of BD2/3 (GenBank: AF071216, AF301470), six oligodeoxynucleotide fragments were designed and synthesized by Sangon Biotech Co., Ltd., Shanghai, China (Table I). The fused gene of BD2/3 was prepared by overlap extension PCR (23). The stop codon of BD3 and the start codon of BD2 were deleted, and restriction sites *Bam*HI, *Eco*RI and *Bgl*II were added to both ends. The fused BD2/BD3 genes were connected by linker sequence Ser-Ser-Gly-Ser-Gly-Ser. The fusion gene BD2/3 was digested with *Bam*HI and *Eco*RI, and then cloned into the pGEX-4T-1 expression plasmid (Pharmacia Biotech; GE Healthcare, Chicago, IL, USA), a prokaryotic expression vector containing the Tac promoter and a glutathione S transferase (GST) tag sequence for fusion protein expression. Sequence of the resulting plasmid was confirmed by double restriction enzyme digestions and sequencing, and designated as pGB2B3.

Construction of eukaryotic expression plasmids for fusion BD2/3. The recombinant plasmid pGB2B3 was digested with *Bam*HI and *Bgl*II, and then the target fragments BD2/3 were ligated to VR1020 expression plasmid (Vical, San Diego, CA, USA). It is a eukaryotic expression vector containing human CMV promoter and a tissue plasminogen activator (TPA) signal sequence for secretion. The correct recombinant plasmid was screened by double restriction enzyme digestions, plasmid PCR and sequencing (data not shown) and then named VRB2B3.

Bioactivity assay in vitro of fusion BD2/3 protein expressed by E. coli. *E. coli* (DH5α) cells transformed with pGB2B3 and pGEX-4T-1 were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to express the fusion BD2/3 protein which was purified on a GST affinity column (Amersham Biosciences; GE Healthcare). The bioactivity of the fusion protein was measured by inhibition of 4 standard pathogen strains (*E. coli* ATCC25922, *S. aureus* ATCC 26112, *S. pneumoniae* ATCC49619 and *P. aeruginosa* ATCC10211).

Minimum inhibition concentration (MIC), minimal bactericide concentration (MBC) of fusion BD2/3 protein expressed by E. coli. Broth dilution methods were carried out to determine the MIC of fusion BD2/3 protein against bacterial cultures of $5x10^5$ CFU/ml (24). MBCs were determined by transferring 100 µl samples from clear wells onto agar plates without antibiotics. The MBC was the lowest concentration at which there was no visible microbial growth.

Large-scale preparation of recombinant VRB2B3. A single colony of *E. coli* containing the recombinant VRB2B3 plasmid was inoculated in Luria Bertani (LB) broth with kanamycin (100 mg/ml), with shaking at 37˚C overnight. Plasmid DNA was extracted following large-scale alkaline lysis and precipitation by the spermine method (19), then suspended in sterile saline water and stored at 20˚C until use.

Preparation of LP. Lecithin, cholesterol, octadecylamine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and dimethyldistearylammonium bromide (DDAB) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

A mixture of 3 mg lecithin, 1.7 mg cholesterol and 0.5 mg octadecylamine (30:17:5 by weight) in 10 ml chloroform was added to a 250 ml round bottom flask and evaporated under vacuum in a rotary evaporator at 37˚C, forming a thin film on the inner surface. The 20 ml of ddH_2O was added at 37˚C and the flask was shaken with intermittent sonication in a bath sonicator.

Preparation of nanoparticles using LP modified CS. Five different delivery systems (CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine) with and without entrapped VRB2B3 (VRB2B3-CS, VRB2B3-PEG-O-CS-PEI, VRB2B3-LP, VRB2B3-PCL, VR B2B3-PCL-protamine) were prepared by the ionotropic gelation method (26). Briefly, biomaterials (CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine) were diluted, respectively, by buffer CH_3COOH/CH_3COONa (pH 5.5) containing triphosphate and heated for 10 min at 65˚C with

Table I. Primers of BD3 and BD2.

Primer	Sequence (5'-3')
P1	GCCATGGGAATCATAAACACATTACAGAAATATTATTGCAGAGTCAGAGGCGGC
P2	AGAGTCAGAGGCGGCCGGTGTGCTGTGCTGAGCTGCCTTCCAAAGGAGGAACAGATCGGCAAG
P3	GAACAGATCGGCAAGTGCTCGACGCGTGGCCGAAAATGCTGCCGAAGAAAGAAAAGCAGCGGT
P4	GAAAGAAAAGCAGCGGTAGCGGAAGTGGTATAGGCGATCCTGTTACCTGCCTAAAGAGTGGAGCC
P5	TGCCTAAAGAGTGGAGCCATATGTCATCCAGTCTTTTGCCCTCGTCGGTATAAACAAATTGGAACCTG
P6	AAACAAATTGGAACCTGCGGTCTCCCTGGAACAAAATGCTGCAAAAAGCCATAAAGATCTGAATTCCG

BD, β-defensin.

mild magnetic stirring. Then, the solution of plasmid was added slowly to the solution of biomaterial drop by drop, and the mixed solution was remixed and left for 5 min. The average diameter and zeta potential of the polymeric micelles were detected by Zetasizer 3000 HS/IHPL (Malvern Instruments Ltd., Malvern, UK).

Polyamine cationic liposomes (PCL) were prepared using 30 mg DOPE, 10 mg cholesterol and 10 mg DDAB per round bottom flask and were used to produce PCL as described above. PCL/protamine formulations were prepared by adding protamine to the PCL ($V_{protamine}$:V_{PCL} = 1.5:1). CS (95% deacylated, MW = 150 kDa) was supplied by Chengdu Organic Chemistry Institute of China Academy of Science; polyethyleneglycol-O-chitosan-polyethyleneimine (PEG-O-CS-PEI) was provided by the College of Chemistry of Sichuan University (25).

Agarose gel electrophoresis assay of nanoparticles. The DNA binding ability of biomaterials (CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine) were evaluated by agarose gel electrophoresis. The nanoparticle solutions of plasmid DNA with biomaterials (CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine) copolymer were loaded into individual wells of 0.7% agarose gel, electrophoresed at 100 V for 45 min and stained with 0.01% gold-view. The plasmid migration pattern was revealed under UV irradiation.

Transfection and efficiency analysis of fusion BD2/3 gene in eukaryotic cells in vitro. 293 cells (human embryonic kidney cells; ATCC no. CRL-1573TM) were purchased from the Chinese Academy of Science Cell bank (Shanghai, China). 293 cells were cultured in 6 well plates ($1.5x10^6$ cells/well) for 24 h and grown in 2 ml Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 4.0 mM L-glutamine, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.), and maintained at 37˚C in a 5% CO_2 humidified incubator (Sanyo Electric Co., Ltd., Tokyo, Japan) until the cell confluency of 293 achieved 80%. The complexes of nanoparticles containing CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine each containing 5 μg VRB2B3 plasmid were added into each well to transfect cells, respectively. Then, the transfected cells were incubated for 48 h and the supernatants were collected for antibacterial bioactivity detection (*E. coli* and *S. Pneumoniae*) of fusion BD2/3.

Animal inoculation and challenge with nanoparticles. Sixty female 3-week-old healthy Kunming mice (Animal Center of Sichuan University) were randomly divided into 6 groups (5 treatment groups and an untreated control). Mice in the treatment groups were injected intramuscularly (quadriceps) with 150 μg plasmids, respectively, in VRB2B3-LP (A group), VRB2B3-CS (B group), VRB2B3-PEG-O-CS-PEI (C group), VRB2B3-PCL (D group) or VRB2B3-PCL-protamine (E group). Controls received 150 μg blank plasmid VR1020-CS. Blood samples were collected by tail vein 0, 7, 14 and 21 days post-injection (p.i.). Two weeks after injection, all the experimental mice were challenged intraperitoneally (i.p.) with 0.2 ml $3x10^9$ CFU/ml virulent EPEC *E. coli* strain O139:K88 (Center of Animal Disease Control of Sichuan Province). Mice were euthanized at 28 days post-challenge.

The care and use of experimental mice fully complied with Chinese animal welfare laws, guidelines and regulations.

Immunological assays of immunized mice

Assay of IgG, IgG1and IgG2a by sandwich ELISA. Total serum IgG, IgG1 and IgG2a were measured by mouse Ig ELISA quantitation kits (Bethyl Laboratories, Montgomery, TX, USA) according to manufacturer's instructions. Capture antibody-coated 96-well plates were incubated with 100 μl serially diluted sera samples and standards for 1 h at ambient temperature. HRP-conjugated goat anti-mouse secondary antibodies IgG, IgG1 and IgG2a were added to the wells in triplicate and incubated for 1 h at 37˚C. Absorbance was measured in a multi-functional microplate reader 680 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Assay of IL-2, IL- 6 and IFN-γ by sandwich ELISA. Serum IL-2, IL-6 and IFN-γ were assayed by mouse IL-2, IL-6 and IFN-γ ELISA kits (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The OD_{450} values of the samples were measured in triplicate by a microplate reader 680 (Bio-Rad Laboratories, Inc.).

Assay of immune cell quantity in the peripheral blood. The immune cells of mice were counted using an automatic Excell™ 22 blood cell counter (Denam Co., New York, NY, USA).

Assay of CD4 and CD8 T cells by FCM. Mouse anti-mouse CD4 and CD8 mAbs, labeled with fluorescein isothiocyanate (FITC) and R-phycoerythrin (R-PE), respectively, were purchased from SouthernBiotech (Birmingham, AL, USA). A total of 2 μl of FITC-conjugated anti-CD4 and 2 μl R-PE

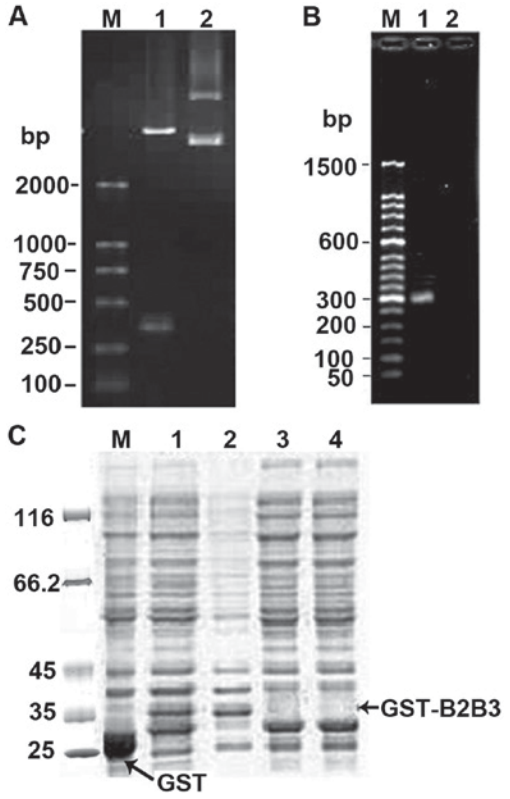

Figure 1. Electrophoretic and SDS-PAGE analysis of recombinant pGB2B3. (A) Electrophoretic identification of digested pGB2B3 recombinant (1.0% agarose gel). Lane M, DL2000 marker; lane 1, pGB2B3/BamHI+EcoRI; lane 2, pGEX-4T-1 plasmid. (B) Electrophoresis of the PCR product from pGB2B3 (1.0% agarose gel). Lane M, 50 bp marker; lane 1, PCR product of pGB2B3 plasmid; lane 2, PCR product of pGEX-4T-1 plasmid. (C) SDS-PAGE analysis of GST-tagged BD2/3 fusion protein expression. Lane M, protein MW marker; lane 1, expression products of pGEX-4T-1 induced by IPTG; lane 2, expression products of pGB2B3 induced by IPTG; lane 3, supernatant of lysed culture of pGB2B3 induced by IPTG; lane 4, pellet of lysed culture of pGB2B3 induced by IPTG; lane 5, expression products of pGB2B3 without IPTG induction. BD, β-defensin; IPTG, isopropyl-β-D-thiogalactopyranoside; GST, glutathione S transferase.

labeled anti-CD8 were added to 1.0 ml EDTA-stabilized blood and incubated for 30 min. Erythrocytes were lysed and the remaining cells were washed. Two-color-stained samples were analyzed using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cells labeled with a single conjugated mAb served as controls. For each sample, $2x10^4$ cells were analyzed by Cell-Quest™ software. The absolute numbers of each T subpopulation in peripheral blood were calculated as follows: absolute number (10^6/ml) = (% positive cells of all cells analyzed x WBC)/100.

Statistical analysis. Data from all the groups were presented as mean ± SD. Statistical analysis between groups was performed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Construction of fusion gene of BD2 and BD3. As shown in Fig. 1, a band of ~300 bp was detected by agarose gel electrophoresis following double digestion and plasmid PCR (Fig. 1A and B), indicating that fusion BD2/3 had been successfully inserted into pGEX-4T-1 vector. Sequencing of pGB2B3 confirmed this result (data not shown). SDS-PAGE analysis (Fig. 1C) revealed that GST-tagged fusion proteins were successfully induced to express mainly in the supernatant of lysed cultures.

Table II. The MIC and MBC of fusion BD2/3 antibacterial peptide.

Bacterial strain	MIC (μg/ml)	MBC (μg/ml)
S. aureus ATCC26112	2	4
S. pneumoniae ATCC49619	2	8
P. aeruginosa ATCC27853	2	8
E. coli ATCC25922	2	8

BD, β-defensin; MIC, minimum inhibition concentration; MBC, minimal bactericide concentration.

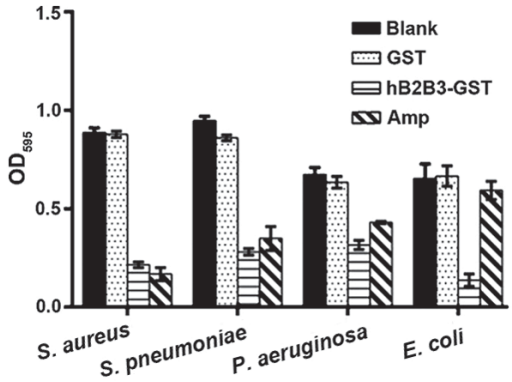

Figure 2. Antimicrobial activity of recombinant BD2/3 protein expressed by pGB2B3. BD, β-defensin.

Antimicrobial activity of fusion BD2/3 protein in vitro. Fig. 2 and the values of MIC and MBC (Table II) demonstrated that the fusion BD2/3 protein induced by IPDG significantly inhibited the proliferation of Gram-positive bacteria (*S. aureus, S. pneumoniae*) and Gram-negative bacteria (*P. aeruginosa, E. coli*) in comparison with controls and the GST group ($P<0.05$). Compared with the ampicillin control group, the fusion BD2/3 protein had higher antimicrobial activity against Gram-negative bacteria. That means the fusion BD2/3 protein can be used as an anti-bacterial infection drug.

Characterization of nanoparticles. Observation by Zetasizer 3000 HS/IHPL (Malvern Instruments Ltd., Malvern, UK) revealed that most of the liposomes/polymer VRB2/3 were spherical nanoparticles ranging from 178-381 nm with zeta potentials +11.1-22.1 mV (Table III), confirming that the DNA was fully entrapped in the nanoparticles and indicating an almost 100% package rate. Through agarose gel electrophoresis assay of nanoparticles, the condensation capability of CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine with DNA were evaluated by measuring the emitted fluorescence when adding the goldview into the nanoparticles. It was shown that

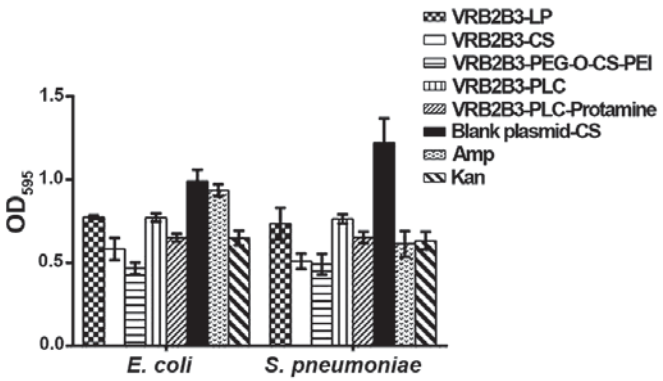

Figure 3. Comparison of the transfection efficiency of VRB2B3 among different gene delivery carriers.

Table III. Size and zeta potential of the nanoparticles.

Sample	Zeta potential (mV)	Size (nm)
VRB2B3-LP	+17.8±0.62	335±16.2
VRB2B3-CS	+15.8±0.66	230±12.3
VRB2B3-PEG-O-CS-PEI	+21.9±0.92	178±10.5
VRB2B3-PCL	+22.1±0.69	350±14.6
VRB2B3-PCL-protamine	+11.1±0.33	381±17.9

LP, liposomes; CS, chitosan; PEG-O-CS-PEI, polyethylene glycol-O-chitosan-polyethylenimine; PCL, polyamine cationic liposomes.

the complexes were positively charged when the mass ratios were 20:1, while the migration of DNA plasmid was suppressed completely when the mass ratio of CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine to DNA was 20:1. It showed that DNA plasmids were entrapped into CS, PEG-O-CS-PEI, LP, PCL and PCL-protamine successfully.

Expression efficiency of nanoparticles in vitro. Compared with those in the blank plasmid-CS control group, the antibacterial activity against *E. coli* and *S. pneumoniae* of BD2/3 significantly increased in VRB2B3-LP, VRB2B3-CS, VRB2B3-PEG-O-CS-PEI, VRB2B3-PLC and VRB2B3-PLC-protamine groups (P<0.05). Furthermore, the antibacterial ability against *E. coli* of BD2/3 in VRB2B3-PEG-O-CS-PEI group was significantly higher than those in the other groups. The antimicrobial activity against *S. pneumoniae* was also significantly raised in VRB2B3-CS and VRB2B3-PEG-O-CS-PEI groups (P<0.05), though not significantly raised between them (P>0.05) (Fig. 3). These results demonstrated that VRB2B3 was successfully transfected into 293 cells by the five gene delivery systems, LP, CS, PEG-O-CS-PEI, PC and PCL-protamine. Of these, the expression efficiency of PEG-O-CS-PEI was the highest.

Quantitation of IgG, IgG1 and IgG2a. The level of IgG significantly improved in sera of treated mice compared with that of the blank control from 21 days post-injection (p.i.), and

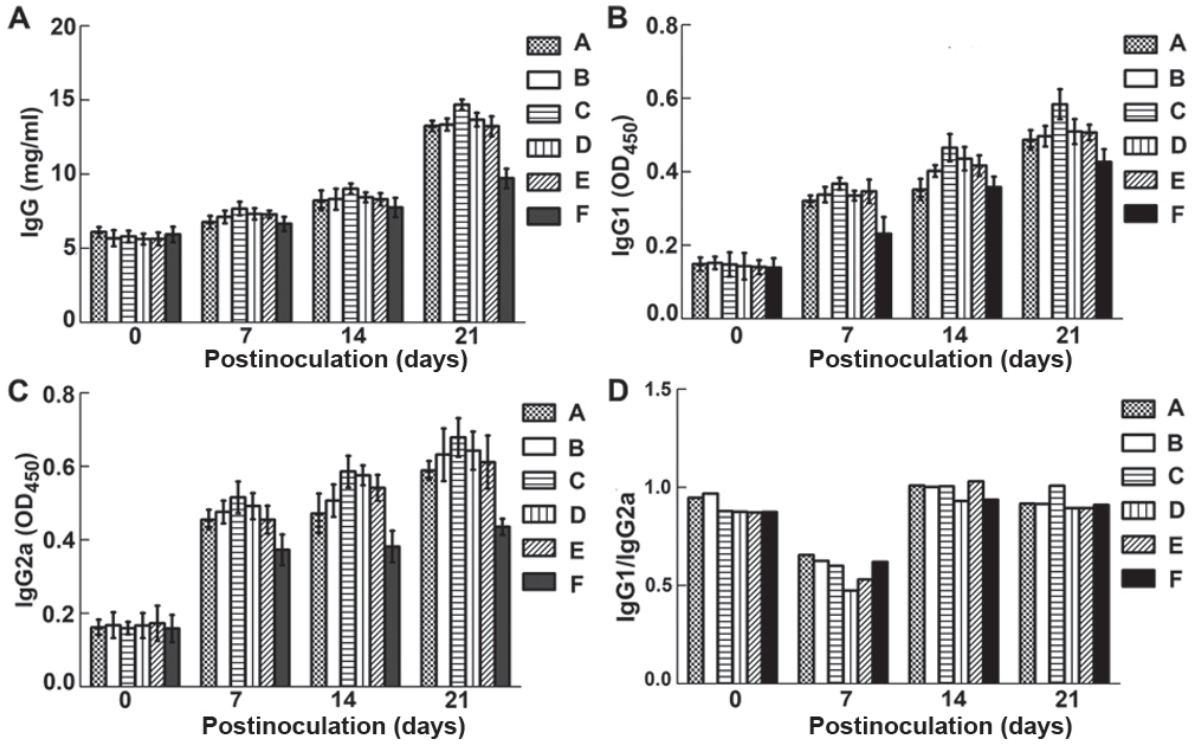

Figure 4. Levels of IgG (A), IgG1 (B) and IgG2a (C) in the sera of experimental mice. Group A, B, C, D and E were intramuscularly injected with VRB2B3-LP, VRB2B3-CS, VRB2B3-PEG-O-CS-PEI, VRB2B3-PCL and VRB2B3-PCL-protamine, respectively; group F was the blank control group. (D) IgG1/IgG2a. No obvious change was found compared with that in blank control group, which indicated Th1/Th2 homeostasis during the whole experiment. The sign (↓) indicates the challenge day with virulent *EPEC E. coli*. LP, liposomes; CS, chitosan; PEG-O-CS-PEI, polyethylene glycol-O-chitosan-polyethylenimine; PCL, polyamine cationic liposomes.

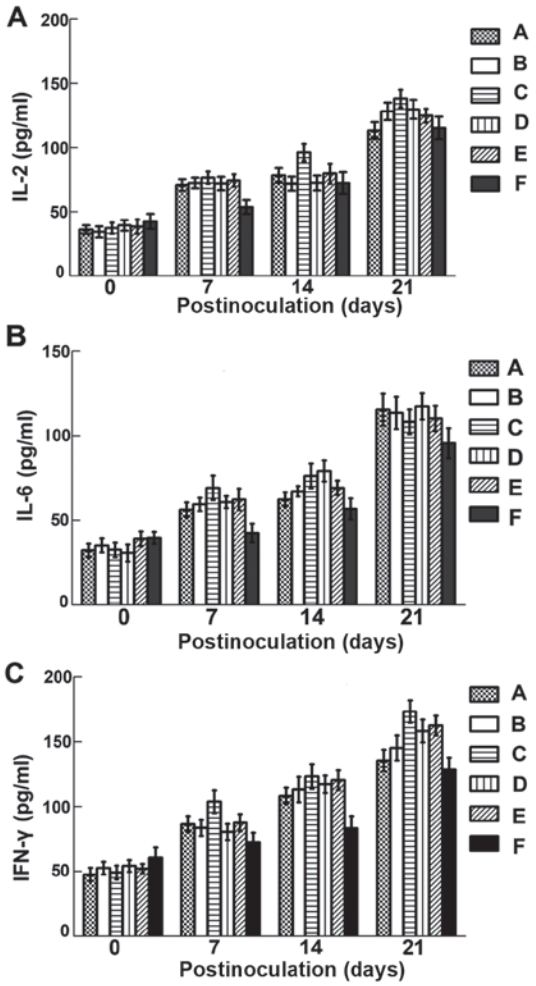

Figure 5. Changes of IL-2 (A), IL-6 (B) and IFN-γ (C) in the sera of experimental mice.

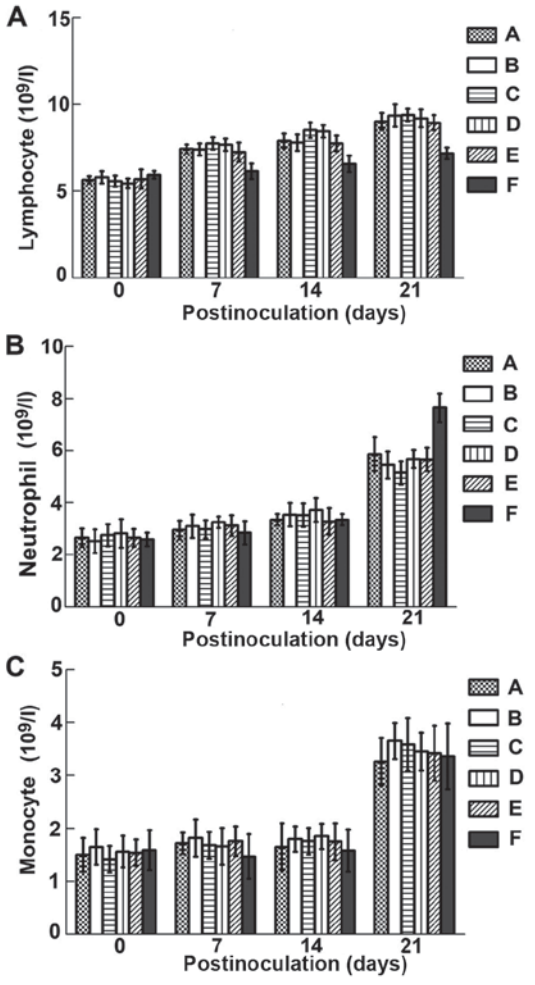

Figure 6. Effect on lymphocytes (A), neutrophils (B) and monocytes (C) in the blood of experimental mice.

IgG1 and IgG2a significantly increased from 14 days p.i. with the dominant increase in IgG2a ($P<0.05$), while there was no significant difference between treatment groups A to E, the amount of IgG, IgG1 and IgG2a in group C was higher than those in the other groups (Fig. 4). Fig. 4D shows that there was no difference in IgG1/IgG2a ratios after intramuscular administration of plasmid nanoparticles.

Cytokine levels in treated mice. Compared with those of the controls, the levels of IL-2, IL-6 and IFN-γ were significantly increased in the sera of treated mice from 7 days p.i. (Fig. 5; $P<0.05$). The levels of IL-2 and IFN-γ of group C were higher than those in group A, B, D and E, while the differences in groups A, B, D and E were not significant ($P>0.05$). After challenged with virulent bacteria, cytokine levels in treated groups were still significantly higher than those in controls except for IL-2 at 14 days post-challenge.

Effect on immune cell numbers. Fig. 6A shows that lymphocyte numbers were significantly increased in treated mice from 7 days p.i. onwards ($P<0.05$), and remained high post-challenge. As shown in Fig. 6B and C, neutrophil and monocyte counts were increased to different degrees following treatment except for neutrophils at 7 days post-challenge, but not significantly ($P>0.05$).

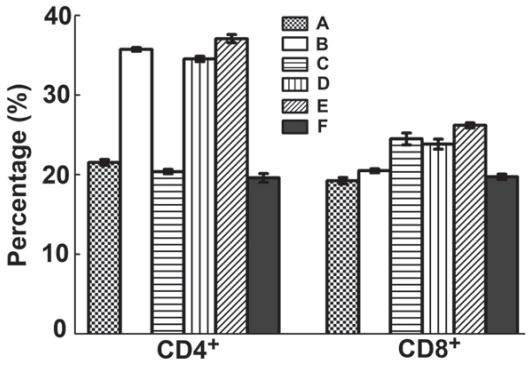

Figure 7. Changes of T cells in the peripheral blood of experimental mice.

Response to challenge

CD4 and CD8 positive T cells. After challenge, levels of $CD4^+$ Th cells of B, D and E groups were significantly increased ($P<0.05$) but it was not significant between groups ($P>0.05$). $CD8^+$ Tc cell counts were also significantly raised in group B, C, D ($P<0.05$) but again not significant between groups ($P>0.05$) (Fig. 7).

Detection of the post challenged mice. Following i.p. injection with virulent EPEC *E. coli* for 2 weeks, most mice injected

Table IV. Effect of challenge with virulent *EPEC E. coli*.

Group	Challenge number	Morbidity	Mortality	Survival rate (%)
A	10	2	2	80
B	10	1	0	100
C	10	0	0	100
D	10	1	1	90
E	10	1	1	90
F	10	10	10	0

with VRB2B3 packaged in all the tested forms survived without symptoms while the controls became lethargic and developed severe diarrhea (Table IV). Moreover, gross pathological inspection showed that the organs and tissues of surviving treated mice were normal. In contrast, control mice died of infection presented with visible lesions, including necrosis of the liver and spleen, bleeding of the stomach, and duodenal and jejunal catarrh, with *E. coli* being isolated from the affected organs by microbiological culture.

Discussion

In this study, a novel fusion gene BD2/3 was successfully constructed, containing sequences from human BD-2 and BD3 genes, which individually had been found to exert significant antimicrobial ability and immunological stimulatory activity (4,5,7). Fusion gene BD2/3 contained 304 bases and encoded 93 amino acids. The molecular weight of the BD2/3 expressed fusion protein was 11.2 kDa. We proved that this novel fusion protein displayed remarkable antibacterial bioactivity *in vitro* and immunological enhancement *in vivo*. By antimicrobial assay, the results have demonstrated a significant reduction of bacterial growth *in vitro*. Most notably, the antimicrobial activity of BD2/3 fusion protein against gram negative pathogen was more potent than that of ampicillin, indicating its potential as a candidate for new antibiotic drugs.

In BD2/3 plasmid-injected mice, the levels of IgG, IgG1 and IgG2a increased considerably, so as levels of IL-2, IL-6 and IFN-γ. Moreover, $CD4^+$ Th and $CD8^+$ Tc cell counts were also significantly elevated in all treated groups after *EPEC E. coli* challenge. These observations clearly showed that BD2/3 was able to promote the specific immune response in mice, and to provide robust immune protection of mice against pathogenic infection.

When treated mice were challenged i.p. with *EPEC E. coli* at 14 days p.i., levels of all measured parameters (immunoglobulins, interleukins and immune cells) were increased to different extents, illustrating the potent enhancement of immunity by BD2/3. Immune responses are mainly regulated by the activity of two functional T helper cell types, Th1 and Th2, Th1 cells primarily promote cellular immune responses and Th2 cells generally drive humoral immunity (27), and appropriate Th2/Th1 balance is critical for maintenance of homeostasis. Many diseases are induced by the skewing of Th2/Th1 ratios (28). Dominant Th1 responses may contribute to diseases such as rheumatoid arthritis (RA) (29), multiple sclerosis (MS) (30) and type 1 diabetes (31). Atopic allergy is an example of Th2 dominance (32). The ratio of IgG1/IgG2a is usually interpreted as a reflection of different T helper cell (Th2-Th1) reactivity. Over the course of our experiments these ratios did not reveal any significant difference following treatment, indicating that BD2/3 did not influence Th2/Th1 balance. Therefore, we conclude that BD2/3 can enhance the immunity of mice to a remarkable degree and display the same biological safety as defensins in nature.

To raise the low efficiency of gene expression and to achieve stable effective gene delivery systems *in vivo*, the BD2/3 recombinant plasmid was incorporated into 5 different gene carriers: LP, CS, PEG-O-CS-PEI, PCL and PCL-protamine. Although there was no clear difference between them, except for better transfection efficiency and immune response in group C (VRB2B3-PEG-O-CS-PEI), all 5 carriers resulted in highly significant increased antibacterial bioactivity in HEK293 cells and enhancements of immunity both pre- and post-challenge. Based on these findings, it can be concluded that LP, CS, PEG-O-CS-PEI, PC and PCL-protamine are all effective gene delivery vectors *in vitro* and *in vivo*, while PEG-O-CS-PEI was the most effective and practicable. This may be attributed to the modification of PEI ligation on CS. PEI is known to improve transfection efficiency of genes *in vivo* by enhancing cellular endocytosis and escape from lysosomes, and its coupling with CS may extend the expression time of wrapped gene to prolong blood circulation time and reduce reticuloendothelial clearance. Previous studies from our laboratory have also shown that the expression efficiency of plasmids can be improved by entrapment with PEG-O-CS-PEI nanoparticles *in vitro* (20,25).

Furthermore, none of the 5 treated mouse groups displayed any systemic or local symptom and lesion, such as local injuries in injected sites, fever or loss of weight gain following intraperitoneal challenge with *EPEC E. coli*. Whereas, control mice exhibited severe gross lesions. This indicates that the BD2/3-PEG-O-CS-PEI and other nanoparticles have the potential to be applied as safe and effective gene delivery systems.

In conclusion, our results suggest that the novel fusion BD2/3 is a safe and effective molecule not only to inhibit bacteria pathogens directly but also to enhance the immunity of mice against infection. In particular, the recombinant gene packed within PEG-O-CS-PEI nanoparticles has shown promise for development as a novel effective and biocompatible delivery system for control of infection caused by antibiotic-resistant pathogens.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XW, JC, CC, ZW and RG conceived and designed the study. XW, JC, CC, HZ, SZ, JL and XL were responsible for the collection and analysis of the patient data. XW, JC, ZW and RG interpreted the data and drafted the manuscript. CC and RG revised the manuscript critically for important intellectual content. All authors read and approved the final study.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Sichuan University (Chengdu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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