Protection of mice from lethal *E. coli* infection by chimeric human bactericidal/permeability-increasing protein and IgG1 Fc gene delivery

Running title: Protection of mice from lethal *E. coli* infection by chimeric human BPI<sub>23</sub>-Fcγ1 gene delivery

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Abbreviation: Bactericidal/permeability-increasing protein: BPI; Functional N-terminus (1 to 199 amino acidic residues) of human BPI: BPI23; Chimeric human BPI23 and IgG1 Fc: BPI23-Fcγ1; Adeno-associated virus serotype 2: AAV2; Recombinant AAV2 containing chimeric BPI23-Fcγ1 gene: rAAV2-BPI23-Fcγ1; Gram-negative bacterium: GNB; Minimal lethal dose: MLD.
Abstract

To evaluate the potentiality of applying gene therapy to bacterial infections, especially for preventing infection in high-risk patients, we investigated protection of mice from challenge of lethal *E. coli* infection by adeno-associated virus serotype 2 (AAV2) mediated gene transfer of a chimeric BPI$_{23}$-Fc$\gamma$1 gene which consisted of human bactericidal/permeability-increasing protein gene encoding functional N-terminus (1 to 199 amino acidic residues) of human BPI and Fc$\gamma$1 gene encoding Fc segment of human immunoglobulin G1. Here we show the target protein that was expressed and secreted into the serum of the gene-transferred mice showed activity of neutralizing endotoxin, killing *E. coli* and mediating opsonization. After lethal *E. coli* infection, the count of bacteria, and the level of endotoxin and proinflammatory cytokines in the gene-transferred mice were decreased. The survival rate of BPI$_{23}$-Fc$\gamma$1 gene transferred mice markedly increased, especially when in conjunction with antibiotics. Our data suggested that AAV2-mediated chimeric BPI$_{23}$-Fc$\gamma$1 gene delivery could potentially be used clinically for the protection and treatment of gram-negative bacteria infection in high-risk individuals.

Keywords: BPI$_{23}$-Fc$\gamma$1, AAV2, GNB, gene delivery, gene therapy
Introduction

Gram-negative bacteria (GNB) infections are common in clinic, and cases developing to sepsis and subsequent endotoxic shock with a high mortality, being up to over 100,000 deaths in the United States annually, have increased in recent decades. The increase can be attributed to an escalating number of patients who are undergoing cancer therapy, immunosuppressive therapy, invasive surgical procedures and HIV infection, who are at high-risk patients of developing sepsis. In addition, GNB lyses may excessively release endotoxin and cause endotoxemia during antibiotic therapy. Various novel sepsis therapies currently under development and evaluation in clinical trials, including anticoagulant therapy, neutralization of lipopolysaccharide (LPS) and cytokine therapies, have not progressed essentially.

Bactericidal/permeability-increasing protein (BPI) is a 55-60 kD human neutrophil granule-associated defense molecule specific for gram-negative bacteria, found in 1978. BPI has specific effect of neutralizing endotoxin and directly killing GNB, but no adverse effect on eukaryotic cells. It was demonstrated that the N-terminus of BPI is identical to natural BPI in the effect on LPS and GNB. Recent studies with animal model of sepsis and endotoxemia and clinical trials of treating septic patients suggested that the recombinant N-terminus of BPI (rBPI21) was a promising therapeutic agent. However, rBPI21 has relatively low efficacy and short half-life in vivo, and administration of rBPI21 in large dosage is very expensive and difficult to maintain an optimal therapeutic level. In addition, recombinant BPI21 and conventional antibiotics are principally suited to the treatments of existing bacterial infection rather than prevention of high-risk patients of developing sepsis.

In order to prolong and improve the activity of recombinant BPI21 for clinical therapy of GNB infection, we applied similar strategy (CAP18-Ig fusion protein) of Warran and colleagues to design and express a recombinant chimeric BPI23-Fcγ1 protein that consisted
of functional N-terminus (1 to 199 amino acidic residues) of human BPI and the Fc segment of human IgG1. It has been demonstrated that the chimeric BPI\textsubscript{23}-Fc\textsubscript{γ1} protein has the effect of neutralizing endotoxin, directly killing GNB (including drug-resistant GNB), as well as mediating opsonization.\textsuperscript{3} Based on our preliminary work, we have sought to develop BPI\textsubscript{23}-Fc\textsubscript{γ1} transgene-based modality and to evaluate its potential in preventing clinical high-risk patients infected by GNB and accordingly in reducing the morality of sepsis caused by GNB. In this study, the chimeric BPI\textsubscript{23}-Fc\textsubscript{γ1} gene was re-constructed within a recombinant adeno-associated virus serotype 2 vector as rAAV2-BPI\textsubscript{23}-Fc\textsubscript{γ1}, and subsequently delivered and expressed both \textit{in vitro} and \textit{in vivo}. The protective efficacy of chimeric BPI\textsubscript{23}-Fc\textsubscript{γ1} gene delivery mediated by AAV2 to lethal \textit{E. coli} infection in the gene-transferred mice was fully characterized.

\textbf{Materials and Methods}

\textbf{Construction and production of rAAV2-BPI\textsubscript{23}-Fc\textsubscript{γ1}}

The BPI gene fragment encoding the signal peptide and the functional N-terminus (1 to 199 amino acid residue) of human BPI, named as BPI\textsubscript{23}, was generated by RT-PCR using the primers (P1: 5’-CTGGTACCATGAGAGAGAACATGGCCA-3’ and P2: 5’-GCAAGCTTCTATTTTGGTCATTACTGGCAG-3’) from the mRNA of HL-60 cell line (ATCC CCL-240, Manassas, VA, USA). The Fc\textsubscript{γ1} gene fragment encoding the Fc fragment of human immunoglobulin G1, was generated by RT-PCR using the primers (P3: 5’-GTAAGCTTCTACATGCCCACCGTGCCCAG-3’ and P4: 5’-TCGTCGACGGATCTTATTATCCCGAGACAGGGGAG-3’) from the mRNA of human peripheral blood lymphocytes derived from an healthy volunteer. BPI\textsubscript{23} and Fc\textsubscript{γ1} DNA fragments were then digested with Kpn I/Hind III and Hind III/Sal I, respectively, and were
then co-ligated into the Kpn I/Sal I sites of the pSNAV vector (AGTC Gene Technology Co. Ltd., Beijing, China), designed as the pSNAV-BPI\textsubscript{23}-Fc\textgamma\textgreek{l} expression vector in which, chimeric BPI\textsubscript{23}-Fc\textgamma\textgreek{l} gene controlled by the CMV promoter and SV40 polyA was flanked by AAV serotype 2 inverted terminal repeats.

rAAV2-BPI\textsubscript{23}-Fc\textgamma\textgreek{l} viruses were prepared by AGTC Gene Technology Co. Ltd., complying with the guideline of SFDA and GMP facility, according to the protocols as described.\textsuperscript{36, 37} Briefly, BHK-21 cells (ATCC CCL-10) were transfected with the pSNAV-BPI\textsubscript{23}-Fc\textgamma\textgreek{l} plasmid DNA using Metafectene (Biontex Laboratories GmbH, Munich, Germany) and selected by G418. rAAV2-BPI\textsubscript{23}-Fc\textgamma\textgreek{l} viruses were rescued and produced by infecting the G418-resistant BHK-21 clones containing BPI\textsubscript{23}-Fc\textgamma\textgreek{l} gene with recombinant HSV1-rc/\DeltaUL2 helper viruses (AGTC Gene Technology Co. Ltd.).\textsuperscript{37} The rAAV2-BPI\textsubscript{23}-Fc\textgamma\textgreek{l} viruses were purified and diluted to $1 \times 10^{12}$ vector genomes (v. g.)/ml used for the study.

**Verification and expression of rAAV2-BPI\textsubscript{23}-Fc\textgamma\textgreek{l} in CHO cells**

rAAV2-BPI\textsubscript{23}-Fc\textgamma\textgreek{l} virus with a multiplicity of infection (MOI) of $5 \times 10^4$, $1 \times 10^5$ and $5 \times 10^5$ v. g./cell were used to infect CHO-K1 cells (ATCC CCL-61). The infected cells were incubated for 48-72 hours in serum-free DMEM/F12 medium under the condition of 37°C and 5% CO\textsubscript{2}. The supernatants were analysed for the presence of secreted BPI\textsubscript{23}-Fc\textgamma\textgreek{l} protein by Dot-blot and Western blot analysis using HRP-conjugated goat anti-human IgG antibody (Sigma, St Louis, MO, USA) and chemiluminescent substrate (Pierce Biotech Inc., Rockford, IL, USA).

**Mouse models of gene transfer**
5-6 week old female Balb/c mice (provided by the Laboratory Animal Centre of The Academy of Military Medical Sciences, Beijing, China) were used for developing mouse models of gene transfer by AAV2. rAAV2-BPI23-Fcγ1 gene transferred mice were administered a 100µl injection containing $1 \times 10^{11}$ v. g. of rAAV2-BPI23-Fcγ1 through the quadriceps muscles of right hind leg; rAAV2-EGFP gene transferred mice were administered a 100µl injection containing $1 \times 10^{11}$ v. g. of rAAV2-EGFP virus (AGTC Gene Technology Co. Ltd.); PBS control mice were administered a 100µl injection of PBS. All experiments with gene-transferred mice described below were performed at an interval of two weeks after these injections were administered.

**Minimal lethal dose (MLD) of endotoxin or E. coli to Balb/c mice**

LPS (Sigma) was diluted to 9.0µg/ml, 7.5µg/ml, 6.0µg/ml and 4.5µg/ml with PBS containing 60mg/ml D-galactosamine (Sigma). 100µl of the selected dose of LPS was intraperitoneally injected into 4 separate groups of mice. The minimal dose that caused a mortality of 90-100% within 48 hours was determined as the MLD of LPS to Balb/c mice.

_E. coli_ O111:B4 (CMCC (B) No. 44101-9, Beijing, China) was diluted to $2.5 \times 10^5$, $5 \times 10^4$, $5 \times 10^3$, and $2.5 \times 10^2$ CFU/0.5ml with autoclaved PBS buffer containing 5% (w/v) dried yeast. 0.5ml of the selected dose of _E. coli_ for each mouse was intraperitoneally injected into 4 separate groups of mice. The minimal dose that caused a mortality of 90-100% within 48 hours was determined as the MLD of _E. coli_ O111:B4 to Balb/c mice.

**RT-PCR**

mRNA was extracted from rAAV2-BPI23-Fcγ1 injected mouse muscles by the Oligotex Direct mRNA Kit (Qiagen, Hilden, Germany). RT-PCR was performed according to the manufacture’s instruction for Access RT-PCR System (Promega, Madison, WI, USA) in
order to detect BPI23-Fcγ1 gene expression at mRNA level. The specific primers used in RT-PCR are P1 and P4 described above.

160 **Immunohistochemical and histopathological observation**

The paraffin-embedded sections of the injected mouse muscles were prepared and analysed by standard immunohistochemical staining with HRP-conjugated mouse anti-human IgG Fc (Zymed Laboratories, Inc., San Francisco, CA, USA) and diamino-benzidine (DAB) (Boster Biotech, Wuhan, China). The paraffin-embedded sections of the main tissues of liver, small intestine, spleen and kidney at 24 hours post lethal *E. coli* challenge were prepared and examined by standard Haematoxylin and Eosin (H&E) staining.

**Enzyme linked immunosorbent assay (ELISA)**

A modified enzyme linked immunosorbent assay (ELISA) was performed as follows for examining the secreted BPI23-Fcγ1 protein in mouse sera. 100µl serum of rAVV2-BPI23-Fcγ1 gene transferred mice was fully absorbed by nitrocellulose membrane for 15 min, while 100µl serum of rAVV2-EGFP gene transferred mice and 100µl serum of PBS control mice were used as controls. The nitrocellulose membranes were dried in air for 20 min and laid on naked microtiter plates; these were tested by a biotinylated antibody against human BPI according to the protocol of Human BPI ELISA Kit (HyCult Biotechnology b.v., Uden, Netherlands).

Proinflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in serum samples were detected by ELISA according to the Kit’s instruction (R&D Systems Inc., Minneapolis, MN, USA).

**Limulus amebocyte lysate (LAL) assay**
Serum samples were diluted in pyrogen-free water, and then tested according to the instruction of Limulus Amebocyte Lysate Kit (Shyihua Corp., Shanghai, China).

**Bacterial-count assay**

In vitro: 100µl of serum or anti-coagulated blood from rAAV2-BPI\textsubscript{23}-Fc\gamma\textsubscript{1} gene transferred mice or rAAV2-EGFP gene transferred mice was mixed with 100µl of *E. coli* (1×10\textsuperscript{3} CFU/ml) respectively and incubated at room temperature for 15 min, then plated on two LB agar plates. Bacterial counts were performed after overnight incubation at 37°C from three individual experiments.

In vivo: After *E. coli* attack, serum samples and homogenated samples from spleens and livers were serially diluted for bacterial count, then dilutions samples were inoculated respectively on two LB agar plates, and incubated at 37°C for 24 hours. Clones were counted and the average from two plates was calculated in order to determine the bacterial count.

**Statistical Analysis**

Data were presented as mean ±SD. Chi-square test was performed for the survival rate comparison. Differences among groups were analysed by Independent-samples t-test. (α=0.05, two-sided; *P* value less than 0.05 were considered to be statistically significant).

**Results**

**Construction and verification of rAAV2-BPI\textsubscript{23}-Fc\gamma\textsubscript{1}**

We constructed the pSNAV-BPI\textsubscript{23}-Fc\gamma\textsubscript{1} expression vector and successfully produced recombinant AAV2-BPI\textsubscript{23}-Fc\gamma\textsubscript{1} virus as mentioned in methods. The expression cassettes of chimeric BPI\textsubscript{23}-Fc\gamma\textsubscript{1} gene controlled by CMV promoter and SV40 polyA is flanked by AAV2
inverted terminal repeats (Figure 1). The rAAV2-BPI23-Fcγ1 virus was purified and diluted to 1×10^{12} vector genomes (v. g.)/ml used for the study.

Firstly, we verified the integrity of our vectors in vitro. About 60% confluent CHO-K1 (ATCC CCL-61) cells were infected by rAAV2-BPI23-Fcγ1 virus at various multiplicity of infection (MOI) and then cultured in serum-free medium for 48 hours. Dot-blot analysis demonstrated the presence of secreted BPI23-Fcγ1 protein in the conditioned medium in a virus load-dependent manner (Figure 2a). Western blot analysis demonstrated the presence of a 48 kD band in DTT-deoxidized and a 96 kD band in non-deoxidized, the expected size of BPI23-Fcγ1 protein (Figure 2b). It also was found that the secreted BPI23-Fcγ1 protein in the conditioned medium of CHO-K1 cells infected by rAAV2-BPI23-Fcγ1 virus had high efficacy of killing GNB (including drug-resistant GNB), neutralizing endotoxin, and mediating opsonization in vitro as well as of protecting mice from lethal E. coli infection in vivo (data not shown).

**BPI23-Fcγ1 expression in vivo**

The injected muscles were examined by RT-PCR and immunohistochemical staining to identify target gene expression at mRNA and protein levels in rAAV2-BPI23-Fcγ1 gene transferred mice two weeks after intramuscular administration. An expected 1.4 kb-sized band was found by RT-PCR using the primers (5’-CTGGTACCATGAGAGAACTGGC-3’ and 5’-TCGTCGACGGATCCTTATTTACCCGGAGACAGGGAG-3’) for rAAV2-BPI23-Fcγ1 gene transferred mice.

DAB positive staining was observed in rAAV2-BPI23-Fcγ1 gene transferred mice, but not in rAAV2-EGFP gene transferred mice and PBS control mice (Figure 3). Green
fluorescence was observed in the sections of the injected muscle of rAAV2-EGFP gene transferred control mice. The results indicated that the target gene was successfully expressed in the injected muscles of rAAV2-BPI\textsubscript{23}-Fc\textgammal gene transferred mice 2 weeks after injection.

Detection of secreted BPI\textsubscript{23}-Fc\textgammal in serum

Serum samples were collected 2 weeks after administration of vector genomes. The following experiments were performed at the same time.

Firstly, the serum was analysed by a modified enzyme linked immunosorbent assay (ELISA) to detect the secreted BPI\textsubscript{23}-Fc\textgammal protein in serum. The OD\textsubscript{450} value for the serum from rAAV2-BPI\textsubscript{23}-Fc\textgammal gene transferred mice was 0.849±0.164 (n=3), while that for the serum from rAAV2-EGFP gene transferred control mice and PBS control mice was 0.283±0.026 (n=3) and 0.290±0.020 (n=3) respectively. There was statistically significant difference between rAAV2-BPI\textsubscript{23}-Fc\textgammal gene transferred mice and the control mice (P<0.05) but not between rAAV2-EGFP gene transferred control mice and PBS control mice (t =0.23, P=0.832). It was proved that there was secreted BPI\textsubscript{23}-Fc\textgammal protein in the serum of rAAV2-BPI\textsubscript{23}-Fc\textgammal gene transferred mice.

The mice sera were precipitated with Ammonium sulfate solution. The intactness of BPI\textsubscript{23}-Fc\textgammal protein in the precipitated sera was confirmed with Western-blot. The result was showed in revised paper as Figure 4. The explanations are as follows: (1) An expected 96 kD band was showed on the film in the rAAV2-BPI\textsubscript{23}-Fc\textgammal transferred mice, but not in the rAAV2-EGFP control mice. (2) A heavily stained 140 kD band was showed on the film both in rAAV2-BPI\textsubscript{23}-Fc\textgammal transferred mice and in the control mice, which proved that anti-human-IgG antibody labelled by the HRP does cross-reacted with the mouse IgG.
Detection of BPI_{23}-Fcγ1 protein activity \textit{in vitro}

To assess the endotoxin-neutralizing activity of secreted BPI_{23}-Fcγ1 protein in serum, 50µl serum was incubated with 50µl endotoxin solution (0.25EU/ml) diluted with pyrogen-free water for 30 min at 37°C, and then the mixture was tested by limulus amebocyte lysate (LAL) assay. LPS standard in pyrogen-free water (0.125EU/ml LPS standard) showed OD_{545} as 0.320±0.03 (n=3). However, the same quantity of LPS in the dilution contains 50% rAAV2-EGFP control mouse serum showed OD_{545} as 0.287±0.021 (n=3), which is lower than the standard. The result proved, as reported by literatures, that some components (high-density lipoprotein, Cathelicidin) in mouse serum are able to neutralize LPS. Nevertheless, the LPS sample containing serum from rAAV2-BPI_{23}-Fcγ1 gene transfer mice showed OD_{545} as 0.173±0.021 (n=3), that is significantly lower than 0.287±0.021 (n=3) from control group containing normal mouse serum. So we believe that the target gene expression creates BPI_{23}-Fcγ1 protein to neutralize LPS though there are some non-specific LPS neutralizing activities in normal mouse serum.

Meanwhile, we also verified protection of mice from lethal endotoxin (LPS) attack as following. Firstly, we titrated the minimal lethal dose (MLD) of LPS (600ng /mouse) to Balb/c mice. Then, the survival rate was observed within 48 hours after lethal LPS challenge. The survival rate of rAAV2-BPI_{23}-Fcγ1 gene transferred mice (40%, n=20) was significantly higher than that of rAAV2-EGFP gene transferred control mice (5%, n=20) and PBS control mice (0%, n=20). The results also suggested that the target product in rAAV2-BPI_{23}-Fcγ1 gene transferred mice could neutralize endotoxin (LPS) and protect mice from the challenge of lethal endotoxemia.
We further assess the bactericidal activity and the opsonization effect of secreted BPI23-Fcγ1 in serum to *E. coli* O111:B4 (CMCC (B) No. 44101-9). As shown in Table 1, bacterial count from rAAV2-BPI23-Fcγ1 gene transferred mice (21.33±2.08, 13.67±3.06) was significantly lower than that from rAAV2-EGFP gene transferred mice (33.33±4.93, 34.67±5.51) either in serum (*P* =0.018) or anti-coagulated blood (*P* =0.002). Bacterial count from anti-coagulated blood in rAAV2-BPI23-Fcγ1 gene transferred mice (13.67±3.06) was significantly lower than that from serum in rAAV2-BPI23-Fcγ1 gene transferred mice (21.33±2.08) (*P*=0.037). But there was no difference between bacterial count from serum and anti-coagulated blood in the rAAV2-EGFP gene transferred control mice (*P* =0.770). These results suggested that while the secreted chimeric BPI23-Fcγ1 protein alone could kill *E. coli*, the capacity to kill *E. coli* could be improved significantly in presence of phagocytes, in contrast, phagocytes alone were unable to kill *E. coli* under the experimental conditions.

**Protection of mice from lethal *E. coli* infection**

To titrate the minimal lethal dose (MLD) of *E. coli* O111:B4 to Balb/c mice, different dose of *E. coli* O111:B4 was administered intraperitoneally to 4 groups of Balb/c mice (n=10). The infected mice began to die 18 hours after infection and the mortality rate of the group injected with the dose of 2.5×10⁴, 5×10³, 2.5×10⁴ and 5×10³ CFU/0.5ml *E. coli* O111:B4 within 48 hours was 100%(10/10), 100%(10/10), 92.5±5% (37/40) and 60%(10/10) respectively. Hereby, 2.5×10⁴ CFU/0.5ml was determined as the MLD of *E. coli* O111:B4 to Balb/c mice.

To prove protection of mice from challenge of lethal *E. coli* infection, Balb/c mice were attacked by MLD of *E. coli* O111:B4 intraperitoneally two weeks after transferred with a 100μl injection containing 1×10¹¹v.g. Of rAAV2-BPI23-Fcγ1 virus intramuscularly, while the mice transferred with a 100μl injection containing 1×10¹¹v.g. / 100μl of rAAV2-EGFP virus...
and injected 100µl PBS as controls. Then, the survival rate was observed within 48 hours after MLD of *E. coli* infection, and the reproducible results were obtained (Table 2). The survival rate of rAAV2-BPI23-Fcγ1 gene transferred mice (37.50%) was significantly higher than that of rAAV2-EGFP gene transferred mice (2.50%) and PBS control mice (4.17%), while there was no statistically significant difference between that of rAAV2-EGFP gene transferred control mice and that of PBS control mice. The results demonstrated that rAAV2-mediated BPI23-Fcγ1 gene transfer protected mice from the challenge of lethal *E. coli* O111:B4 infection.

Furthermore, data in Table 2 also showed that the survival ratio of rAAV2-BPI23-Fcγ1 gene transferred mice administered by the minimal antibiotic (25µg cefuroxime/per mice) was markedly increased to 67.50%. Survival ratio of cefuroxime group and rAAV2-EGFP plus cefuroxime group was 5.00% and 7.50% respectively. This indicates the potential for the co-operative use of rAAV2-mediated BPI23-Fcγ1 gene delivery and the minimal dose of antibiotics in the clinical scenario, to improve the therapy of GNB infections.

**Biological functions of secreted BPI23-Fcγ1 and resulting changes in vivo**

To further evaluate the biological activity of secreted BPI23-Fcγ1 protein in rAAV2-BPI23-Fcγ1 gene transferred mice, the levels of bacterial count, endotoxin, proinflammatory cytokines and histological alterations *in vivo* were measured after lethal *E. coli* challenge. Firstly, blood samples from orbital bulb and main viscus were simultaneously collected at 6th, 9th, 12th and 24th hour after MLD of *E. coli* O111:B4 infection, and then the serum samples from the collected blood samples and the homogenated samples from the collected integrated spleens and livers were prepared. Each serum sample and homogenated sample were detected by bacteria-counting assay. It was shown that the count of bacteria in
the serum, spleen and liver of rAAV2-BPI23-Fcγ1 gene transferred mice were markedly less than that in rAAV2-EGFP gene transferred control mice (Figure 5 a, b and c). The results demonstrated that the target product in rAAV2-BPI23-Fcγ1 gene transferred mice could kill the *E. coli* and improve the resistance of mice against the lethal *E. coli* infection.

Secondly, blood samples were collected from orbital bulb at the 6th, 12th, 18th and 24th hour after MLD of *E. coli* O111:B4 infection and serum samples of three mice from the same group were prepared and mixed together. Each 100µl of the mixed serum sample was detected by LAL assay for endotoxin and by ELISA for proinflammatory cytokines. The level of endotoxin in the serum of rAAV2-BPI23-Fcγ1 gene transferred mice reached its peak at the 12th hour and was significantly lower than that of rAAV2-EGFP gene transferred control mice with peak at 18th hour (Figure 6 a). Correspondingly, the level of IL-1β and TNF-α in the serum of rAAV2-BPI23-Fcγ1 gene transferred mice reached the peak at the 12th hour and was significantly lower than that of rAAV2-EGFP gene transferred control mice with peak at 18th hour after lethal *E. coli* infection (Figure 6 b, c). It was obvious that the markedly increasing level of endotoxin and proinflammatory cytokines in serum of rAAV2-EGFP-transferred control mice with peak at 18th hour was responsible for the death of animals (the morality up to 92.5%) occurring 18 hours after *E. coli* infection. The results suggested that rAAV2-BPI23-Fcγ1 gene transferred mice could resist the endotoxic shock caused by lethal *E. coli* infection through killing *E. coli*, neutralizing endotoxin and decreasing the level of proinflammatory cytokines.

In addition, the main viscus of the experimental mice involving liver, small intestine, spleen and kidney were examined by standard haematoxylin and eosin (H&E) staining 24 hours after challenge of lethal *E. coli* infection. In comparison, the main viscus of the survival mice protected by rAAV2-BPI23-Fcγ1 gene transfer showed only a slight congestion, while...
the main viscus of the agonal mice from rAAV2-EGFP transferred control mice showed
significant pathological alterations, such as capillary dilatation and congestion which were
consistent with what endotoxic shock should show.

Discussion

Viral delivery system has been widely used in gene therapy protocols for its high efficiency.\textsuperscript{2} Gutless viral vector is safer because of less oncogenicity and less immunogenicity,\textsuperscript{7,17} and is suitable for gene therapy of bacterial infections because it does not exaggerate the inflammatory reaction caused by infection. AAV vectors, a kind of gutless vector, are based upon a class of viruses that commonly inhabit a human host without causing any detectable pathology. In particular, AAV2 has been widely used as a gene delivery vehicle in preclinical studies and as well in early-phase clinical trials has been reported.\textsuperscript{8,9} AAV2-mediated gene delivery has a slow but long-term gene expression which reaches the peak at 2nd to 3rd week and then persists more than several months after delivery.\textsuperscript{9,14} In this study we produced rAAV2-BPI\textsubscript{23-FCγ1} virus with a high viral load, which successfully mediated BPI\textsubscript{23-FCγ1} gene transfer and expression in mouse muscle cells (Figure 3), it was further secreted into blood circulation, suggesting that AAV2-mediated BPI\textsubscript{23-FCγ1} gene delivery system may be suitable for administration to infection high-risk patients.

By 2004, over 700 gene therapy/transfer protocols which cover cancer, monogenic diseases, viral infections, primarily peripheral and coronary artery diseases, but not bacterial infections, had been initiated worldwide.\textsuperscript{29} Latest studies showed that adenoviral-mediated full-length BPI gene transfer could protect mice from endotoxemia but not lethal \textit{E. coli} infection.\textsuperscript{1} The half-life of recombinant BPI in \textit{vivo} was determined to be less 45 minutes, however BPI itself required 3 hours or more to kill bacteria, which might explain why BPI alone was not able to protect BPI gene transferred mice from lethal \textit{E. coli} challenge. In
comparison with BPI, chimeric BPI\textsubscript{23}-Fcγ1 protein not only had a longer half-life, but also accumulated its concentration in blood as it acted like immunoglobulin. The secreted BPI\textsubscript{23}-Fcγ1 protein in the serum of rAAV2-BPI\textsubscript{23}-Fcγ1 gene transferred mice displayed the effects of neutralizing endotoxin, killing \textit{E. coli} and mediating opsonization (Table 1). After challenge of lethal \textit{E. coli} O111:B4 infection, the count of bacteria in serum and in main viscus as well as the level of endotoxin and proinflammatory cytokines in serum of rAAV2-BPI\textsubscript{23}-Fcγ1 gene transferred mice decreased (Figure 5, 6), and the survival rate of rAAV2-BPI\textsubscript{23}-Fcγ1 gene transferred mice increased markedly, especially when combined with antibiotics (Table 2), which was similar to the previous studies on synergism of recombinant BPI and antibiotics,\textsuperscript{6, 11} implicating that the efficacy of anti-GNB infection could be maximized by delivering BPI\textsubscript{23}-Fcγ1 gene and administering minimal antibiotics in patients. In addition, corresponding to the markedly increased level of circulating endotoxin and proinflammatory cytokines, the rAAV2-EGFP control group mice had the high mortality above 90% during lethal \textit{E. coli} infection, and the agonal mice in the rAAV2-EGFP control group showed significant histological alterations, such as capillary dilatation and congestion in the main viscus, that was consistent with the clinical finding of endotoxic shock.

The results of experiment indicate AAV2-mediated BPI\textsubscript{23}-Fcγ1 gene delivery has potential in preventing clinical high-risk patients from being infected by GNB. In general, before reaching the minimal lethal dose (MLD), the invading GNB in high-risk patients may be effectively eliminated by rAAV2-mediated BPI\textsubscript{23}-Fcγ1 gene transfer. In addition, compared with traditional antibiotics, BPI\textsubscript{23}-Fcγ1 protein has the advantages of neutralizing endotoxin that can protect host from endotoxemia and endotoxic shock and of killing drug-resistance GNB with dual pathways by BPI’s direct killing and Fcγ1-mediated opsonization.\textsuperscript{11, 22} It is highlighted that chimeric BPI\textsubscript{23}-Fcγ1 protein can induce LPS-anchored phagocytosis by phagocytes so to kill GNB \textit{in vivo}. With the success of rAAV2-BPI\textsubscript{23}-Fcγ1
gene transfer modality against GNB infection in mice model, we believe that rAAV2-BPI_{23}-Fc_γ1 gene transfer can protect high-risk patients from serious GNB infection and sepsis. We also consider that BPI_{23}-Fc_γ1 gene delivery by double-stranded AAV vector or mini-Ad vector,^4^ 21, 24, 27, 31 another kinds of gutless vectors developed recently, can mediate therapeutic gene to express more quickly and strongly than single-stranded AAV vector, so it will achieve the quick and strong effect against GNB infection and will be especially potential for treatment and prophylaxis.

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**References**


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fragment of the 55-60 kD bactericidal/permeability increasing protein of human


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Tables

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mice injected by</th>
<th>Bacterial count (CFU)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
</tr>
<tr>
<td>Serum</td>
<td>rAAV2-BPI\textsubscript{23}-Fc\textsubscript{γγγγ}</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>rAAV2-EGFP</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>Anti-coagulated</td>
<td>rAAV2-BPI\textsubscript{23}-Fc\textsubscript{γγγγ}</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>blood</td>
<td>rAAV2-EGFP</td>
<td>32</td>
<td>31</td>
</tr>
</tbody>
</table>

*Sera or anti-coagulated blood were collected from the gene transferred mice in two weeks after injection. Serum from rAAV2-BPI\textsubscript{23}-Fc\textsubscript{γγγγ} gene transferred mice contains secreted BPI\textsubscript{23}-Fc\textsubscript{γγγγ} protein, with no phagocytes. Serum from rAAV2-EGFP gene transferred mice doesn’t contain BPI\textsubscript{23}-Fc\textsubscript{γγγγ} protein and phagocytes. Anti-coagulated blood of rAAV2-BPI\textsubscript{23}-Fc\textsubscript{γγγγ} gene transferred mice contains secreted BPI\textsubscript{23}-Fc\textsubscript{γγγγ} protein and phagocytes. Anti-coagulated blood of rAAV2-EGFP gene transferred mice only contains phagocytes, with no BPI\textsubscript{23}-Fc\textsubscript{γγγγ} protein.
Table 2. Protection of rAAV2-BPI\textsubscript{23}-Fcγ1 gene transferred mice from lethal *E. coli* challenge.

<table>
<thead>
<tr>
<th>Mice injected by</th>
<th>N</th>
<th>Survival</th>
<th>Death</th>
<th>Survival rate (%)</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAAV2-BPI\textsubscript{23}-Fcγ1</td>
<td>40</td>
<td>15</td>
<td>25</td>
<td>37.50</td>
<td></td>
</tr>
<tr>
<td>rAAV2-EGFP</td>
<td>40</td>
<td>1</td>
<td>39</td>
<td>2.50</td>
<td>15.313*</td>
</tr>
<tr>
<td>PBS</td>
<td>45</td>
<td>2</td>
<td>43</td>
<td>4.17</td>
<td>14.462*</td>
</tr>
<tr>
<td>rAAV2-BPI\textsubscript{23}-Fcγ1/cefuroxime (25µg)</td>
<td>40</td>
<td>27</td>
<td>13</td>
<td>67.50</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime (25µg) *</td>
<td>40</td>
<td>2</td>
<td>38</td>
<td>5.00</td>
<td>33.807*</td>
</tr>
<tr>
<td>rAAV-EGFP/cefuroxime(25µg)</td>
<td>40</td>
<td>3</td>
<td>37</td>
<td>7.50</td>
<td>30.720*</td>
</tr>
</tbody>
</table>

* 100µl of 12.5, 25, 50, 100 or 200µg cefuroxime sodium was administered intramuscularly to groups of 10 Balb/c mice in 1 hour after challenge by MLD of *E. coli*. The mortality of mice in 25µg cefuroxime sodium groups at 48 hours was 91.4% (n=40). 25µg/100µl was finally defined as the minimal dose of cefuroxime sodium in experiments for synergism with antibiotics.

* p=0.000, compared with rAAV2-BPI\textsubscript{23}-Fcγ1 group

▲ p=0.000, compared with rAAV2-BPI\textsubscript{23}-Fcγ1 group

□ p=0.000, compared with rAAV2-BPI\textsubscript{23}-Fcγ1/cefuroxime group

● p=0.000, compared with rAAV2-BPI\textsubscript{23}-Fcγ1/cefuroxime group
Figure 1. The gene structure of rAAV2-BPI23-Fcy1.

Figure 2. Expression of chimeric BPI23-Fcy1 protein in CHO cells infected with rAAV2-BPI23-Fcy1 virus. Dot & western blot analysis of the conditioned medium of CHO-K1 infected by rAAV2-BPI23-Fcy1 virus at various MOI. (a) 3μl medium samples were dotted. The supernatants of CHO-K1 cells without infection were dotted as negative control (dot 1). The MOI was 5×10⁴ (dot 2), 1×10⁵ (dot 3) and 5×10⁵ v. g. /cell (dot4) respectively. 0.1μg human IgG1 was dotted as positive control (dot 5), the (b) Western blot analysis of the 10-fold concentrated medium of CHO-K1 infected with rAAV2-BPI23-Fcy1 virus at 5×10⁵ v. g. /cell (MOI). Lane 1 was prestained protein molecular weight marker, Lane 2 was DTT treated (reduced) and lane 3 was non-DTT treated (non-reduced).

Figure 3. In situ expression of BPI23-Fcy1 protein detected by Immunohistochemical staining. Immunohistochemical staining of the injected muscles from PBS control mice (slide A), rAAV2-EGFP gene transferred mice (slide B) and rAAV2-BPI23-Fcy1 gene transferred mice (slide C). Green fluorescent in the section of the injected muscles from rAAV2-EGFP transferred mice under an epifluorescent microscopy (slide D). (DAB staining, original magnification × 200).

Figure 4. The intactness of BPI23-Fcy1 protein in the murine sera was identified with Western-blot.

lane 1: the serum from rAAV-BPI23-Fcy1 mice
lane 2: the serum from rAAV-EGFP control mice
Figure 5. Bacterial counts in rAAV2-BPI23-Fcγ1 and rAAV2-EGFP gene transferred mice with lethal *E. coli* infection. According to time point, bacterial counts (CFU) were performed for serum (A), homogenated spleen (B) and liver (C) samples from two groups of mice after injection of lethal *E. coli*. ● represent rAAV2-BPI23-Fcγ1 gene transferred mice; ○ represent rAAV2-EGFP control mice.

Figure 6. The levels of endotoxin and proinflammatory cytokines in rAAV2-BPI23-Fcγ1 and rAAV2-EGFP gene transferred mice after lethal *E. coli* infection. The quantities of endotoxin (A), IL-1β (B) and TNF-α (C) in sera were measured from the time points by LAL or ELISA. ● represent rAAV2-BPI23-Fcγ1 gene transferred mice; ○ represent rAAV2-EGFP control mice.
A

The graph shows the change in endotoxin concentration (EU/ml) over time in hours after an attack, comparing two groups: rAAV2-BPI-FcyI and rAAV2-EGFP.