

Article

Protection of Immuno-Compromised Mice from Lethal Infection of *Klebsiella pneumonia* by rAAV2-BPI23-Fcγ1 Gene Transfer

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In previous research, chimerical BPI23-Fcγ1 gene which consisted of human bactericidal/permeability increasing protein (BPI) gene of encoding the functional N terminus (amino acid residues 1 to 199) of human BPI and Fcγ1 gene of encoding the Fc segment of human immunoglobulin G1 was successfully reconstructed within a recombinant adeno-associated virus serotype 2 (rAAV2) vector as rAAV2-BPI23-Fcγ1. Here, to evaluate the potentiality of applying gene therapy to gram negative bacterial (GNB) infection in high-risk patients, we investigated protection of immuno-compromised mice and immunocompetent mice from challenge with minimal lethal dose (MLD) *Klebsiella pneumonia* infection after rAAV2-BPI23-Fcγ1 gene transferred. The results showed that the survival rate of rAAV2-BPI23-Fcγ1 transferred immunocompetent mice as well as immuno-compromised mice (40.0% and 44.4%, respectively) were significantly higher than that of corresponding control mice (6.7% and 4.4%, respectively); the bacteria counting, level of endotoxin and proinflammatory cytokines in the rAAV2-BPI23-Fcγ1 transferred immuno-compromised mice were markedly lower than that of rAAV2-EGFP and rAAV2-Null transferred immuno-compromised mice. Our data suggest that rAAV2-BPI23-Fcγ1 gene transferring offered immuno-compromised mice with resistance against GNB infection, so it is quite potential in preventing GNB infection of clinical high-risk patients. *Cellular & Molecular Immunology*. 2008;5(6):439-445.

Key Words: rAAV2, BPI, immuno-compromised mouse, *Klebsiella pneumonia*, gene transfer

Introduction

Gram negative bacteria (GNB) infections are common in clinics. Sepsis and subsequent endotoxin shock have been increasing in recent years. The situation is explained by an increased population of patients receiving anti-cancer chemotherapy, immunosuppressive medicine after organ transplantation and invasive surgical procedures. These patients are classified as a high-risk group susceptible to sepsis (1-4). The death rate of GNB infection patients will be significant decreased if a prophylaxis therapy is clinically available.

Bactericidal/permeability-increasing protein (BPI) is a 55

kDa cationic bactericidal protein, present in many mammalian polymorphonuclear neutrophil leukocytes, such as human, bovine, pig, etc. (5). BPI has the effect of neutralizing endotoxin and directly killing GNB, but has no adverse effect on eukaryotic cells (6, 7). The phase II/III trial of rBPI₂₃ and rBPI₂₁ demonstrated that administration of rBPI has effect on GNB infected patient (8, 9). But rBPI₅₅ and rBPI₂₁ have low efficacy and short half-life (less than 45 minutes) *in vivo*, and administration of them in large dosage is very expensive (10, 11). It is difficult to use in clinical therapy. In order to improve the activity and prolong the half-life of rBPI, we designed and expressed a recombinant human BPI₂₃-Fcγ1 fusion protein (12). It was demonstrated that BPI₂₃-Fcγ1 fusion protein has the effect of neutralizing endotoxin, directly killing GNB (including some drug-resistant GNB), opsonization, as well as prolonged half-life *in vivo* which protect the mice from minimal lethal

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dose (MLD) *E. coli* infection (13). Based on the above study, the protective efficacy of chimeric BPI₂₃-Fcγ1 gene delivery mediated by rAAV2 against lethal *E. coli* infection in the gene-transferred mice was studied. At the 2ND week of gene delivery, BPI₂₃-Fcγ1 fusion protein was expressed and secreted into blood circulation in rAAV2-BPI₂₃-Fcγ1 gene transferred mice. After MLD *E. coli* and LPS challenge, the survival rate of rAAV2-BPI₂₃-Fcγ1 gene transferred mice (35%-40%) was significantly higher than that of control mice (0%-6.7%) (14).

In this study, we established immuno-compromised mice model to simulate high-risk patients. We tried to compare the protection of immuno-compromised mice with immuno-competent mice from lethal *Klebsiella pneumonia* infection by transferring chimeric BPI₂₃-Fcγ1 gene through rAAV2 vector, which will help us to infer whether transfer of the rAAV2-BPI₂₃-Fcγ1 can protect clinical high-risk patients from GNB infection.

Materials and Methods

Immuno-compromised mice model

BALB/c mice (5-6 weeks, with weight of 16 ± 2 g) provided by Laboratory Animal Center, Military Academy of Medical Sciences (China) were divided into two groups. Group 1 (n = 5) were injected with hydrocortisone (0.2 mg/200 μl/mouse, NO 04050910, purchased from Jinyao Co., Tianjin, China) and cyclophosphamide (1 mg/200 μl/mice, NO 0502221 Hengrui Co., Jiangsu, China) every second day for total three times (15, 16) as immuno-compromised mouse. Group 2 (n = 5) were injected with only PBS as immunocompetent mice. At the 8th day after first injection, the body weight, spleen weight and spleen/body ratio were recorded and lymphocyte function was analyzed.

The analysis of T cell subsets by flow cytometry

Peripheral blood (100 μl each mouse) was incubated with 10 μl CD3-PE monoclonal antibody (Biotech Company), CD4-FITC or CD8-FITC monoclonal antibody (Biotech Company), mouse IgG isotype control (Biotech Company) at room temperature for 20 min. Then 2 ml RBC-lysing solution was added into each tube. Five minutes later, after centrifuged at 1000 rpm for 10 minutes, the pellet was resuspended into 0.5 ml of cold PBS for flow cytometry.

Lymphocyte proliferation

Mice were killed and single-cell suspensions of splenocytes were prepared. Cells were resuspended as 5×10^6 /ml in RPMI 1640 (Hyclone), 10% fetal calf serum, 2 mmol/L L-glutamine, 50 pg/ml streptomycin, and 100 U/ml penicillin. Cells were incubated in 96-well plate. Negative control well contains 5 μl RPMI 1640 medium only, while positive control wells contained 5 μl LPS (100 μg/ml) and 5 μl ConA (100 μg/ml). The cells were cultured at 37°C with 5% CO₂ for 48 h. Then 15 μl MTT (5 mg/ml, Sigma) was added and then incubated for another 4 hours. The formazan crystals were dissolved with DMSO solution (Sigma) and the

absorption was determined by an ELISA reader at 570 nm. A stimulation index (SI) was used to evaluate lymphocyte proliferation. The SI of T lymphocyte was employed to define the ratio of OD₅₇₀ mean value of the ConA stimulated cells to that of the control (unstimulated). The SI of B lymphocyte was employed to define the ratio of OD₅₇₀ mean value of the LPS stimulated cells to that of the control (unstimulated).

Phagocyte assay

Mouse of each group was intraperitoneally injected with 0.5 ml staphylococcus solution (3×10^9 /ml). Forty-eight hours later, a second intraperitoneal injection of 1 ml yeast solution was administered. Fifteen minutes later, celiac liquid was aspirated for Wright's stain and microscopy. Percentage of phagocytes and phagocyte index were applied to evaluate phagocyte function.

Minimal lethal dose of Klebsiella pneumonia to immuno-competent mice or immuno-compromised mice

Klebsiella pneumonia (ATCC700603), provided by Beijing Tiantan Biological Products Co., Ltd. was diluted into 5×10^6 CFU/ml, 2×10^6 CFU/ml, 1×10^6 CFU/ml and 2×10^5 CFU/ml with PBS containing 2% (w/v) dried yeast. Each group of immunocompetent mice were intraperitoneally injected with one of four concentrations of the bacteria (0.5 ml/mouse) respectively. Four concentrations of bacteria as 2×10^6 CFU/ml, 1×10^6 CFU/ml, 2×10^5 CFU/ml and 1×10^5 CFU/ml were prepared to challenge immuno-compromised mice. Each group of immuno-compromised mice were intraperitoneally injected with one of four dosages of the bacteria (0.5 ml/mouse) respectively. The minimal dose that caused a mortality of 90%-100% in 72 h was defined as minimal lethal dose (MLD) of *Klebsiella pneumonia*.

Survival rate of mice after MLD Klebsiella pneumonia challenge

rAAV2-BPI₂₃-Fcγ1 gene (constructed by AGTC Gene Technology Company Ltd., Beijing, China) transferred mouse was injected with 100 μl solution containing 1×10^{11} v. g. of rAAV2-BPI₂₃-Fcγ1 through the quadriceps muscles of right hind leg; rAAV2-EGFP gene transferred mouse was injected with 100 μl solution containing 1×10^{11} v. g. of rAAV2-EGFP. rAAV2-Null gene transferred mouse was injected with 100 μl solution containing 1×10^{11} v. g. of rAAV2-Null.

One week after these injections, the mice in test group were given immuno-suppressive drugs. By the end of the 2nd week, the immuno-compromised mice were challenged with MLD of *Klebsiella pneumonia* (1×10^5 CFU/mouse) and the mice in control group were challenged with MLD *Klebsiella pneumonia* (5×10^5 CFU/mouse) respectively, and then the mortality was recorded in 72 h.

Blood cultivation and colony counting

Blood samples of immuno-compromised mice were collected from orbital bulb at 6, 9, 12 and 18 hours after MLD *Klebsiella pneumonia* challenge. Serum was diluted (1:10) by

Table 1. Body weight, spleen weight and spleen/body ratio

Groups	Body weight (g)	Spleen weight (g)	Spleen/body ratio
Immunocompetent (n = 5)	15.9 ± 0.98	0.060 ± 0.006	0.38 ± 0.04
Immuno-compromised (n = 5)	15.1 ± 1.42	0.033 ± 0.003*	0.22 ± 0.03*

Data were shown as mean ± SD of five independent replicates with similar results. There was no significant difference in body weight between the two groups. * $p < 0.01$ compared with immunocompetent mice.

normal saline (NS) and then dilution sample (50 µl) was inoculated on Luria-Bertani media and followed by incubation at 37°C for 16 h. Livers and spleens of the test animals were collected and triturated by steel wire screen (200 meshes). Then 50 µl homogenate (diluted by NS 1:100) was inoculated on Luria-Bertani media and incubated at 37°C for 16 hours. Bacterial colony formation units (CFU) were then counted.

Limulus amoebocyte lysate (LAL) assay

Serum sample was diluted (1:10) in pyrogen-free water and LAL assay was performed according to the procedures recommended by Amebocyte Lysate Kit (YiHua Co., Shanghai, China).

Proinflammatory cytokine assay

Interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in serum sample was detected by ELISA according to procedures

from manufacturer (R&D Systems Inc., Minneapolis, MN, USA).

Statistical analysis

Data were represented as mean ± standard deviation (SD) of more than three separate replicate experiments. Chi-square test was performed for survival rate comparison. Differences among groups were analysed by independent-samples *t* test. ($\alpha = 0.05$, two-sided). The value of $p < 0.05$ were considered to be statistically significant.

Results

The physical profile of immuno-compromised mice

There was no significant difference on body weight between the two groups. The spleen weight and spleen/body weight ratio of mice in immuno-compromised group are lower than that of mice in immunocompetent group (Table 1). Short-

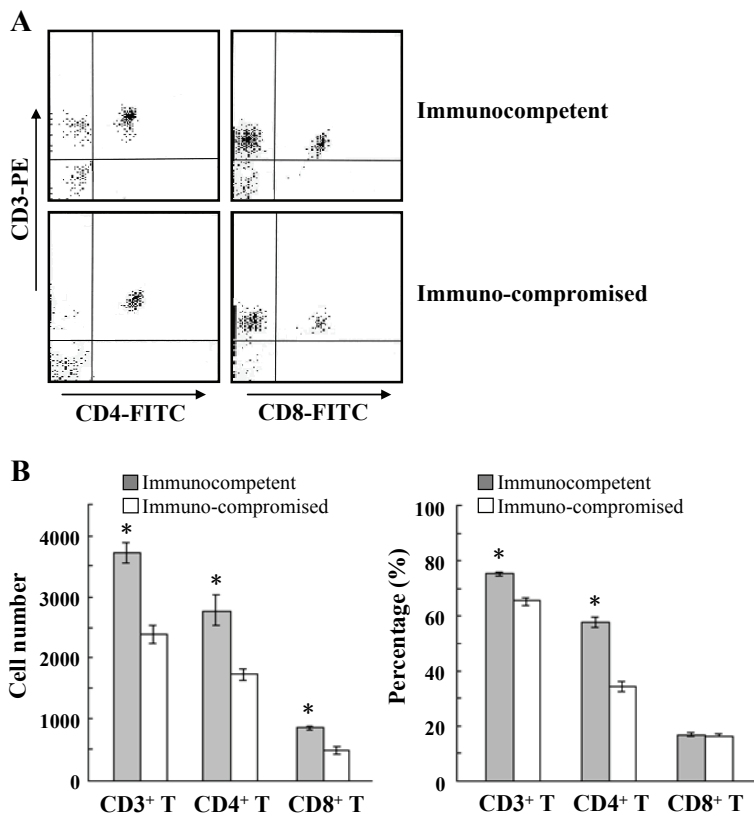


Figure 1. Comparison of the total number and percentage of the CD4⁺/CD8⁺/CD3⁺ T lymphocytes between the immunocompetent and immuno-compromised mice. Immuno-compromised mice were injected with hydrocortisone (0.2 mg/200 µl/mouse) and cyclophosphamide (1 mg/200 µl/mouse,) every second day, total three times. Immuno-competent mice were injected with only PBS. (A) The percentage of CD3⁺/CD4⁺/CD8⁺ T cell in immunocompetent, immuno-compromised or negative control mice were analyzed by flow cytometry at the 8th day after first injection. (B) The total number and percentage of the CD3⁺/CD4⁺/CD8⁺ T cell of immuno-compromised mice were calculated from five independent replicates with similar results. Data were shown as mean ± SD. * $p < 0.05$ compared with immuno-compromised mice.

Table 2. Lymphocytes proliferation and phagocytes function

Groups	SI of T cell	SI of B cell	Percentage of phagocytosis	Phagocytic index
Immunocompetent mice (n = 5)	2.59 ± 0.23	3.83 ± 0.41	56.0 ± 8.51	0.95 ± 0.13
Immuno-compromised mice (n = 5)	1.29 ± 0.19*	1.56 ± 0.17*	30.5 ± 4.85*	0.41 ± 0.11*

Data were shown as mean ± SD of five independent replicates with similar results. * $p < 0.01$ compared with immunocompetent mice.

term administration of immunosuppressive drugs had little impact on the body weight of mice but significantly decreased spleen mass. The immunosuppressive drugs also inhibited lymphocyte response to Con A and LPS as shown by poor response of T lymphocytes and B lymphocytes (Table 2). Likewise, the percentage of phagocyte and phagocytic index of immuno-compromised mice in test group were significant lower than that of mice in control group (Table 2). The total number and percentage of the CD3⁺/CD4⁺/CD8⁺ T lymphocytes of immuno-compromised mice in test group were much lower than that of mice in the control group ($p < 0.05$) (Figure 1). These results demonstrated that the animal model of immuno-compromised mice well simulated immunosuppressive situation *in vivo*.

MLD *Klebsiella pneumonia* in immunocompetent and immuno-compromised mice

The mortality of each group in immunocompetent and immuno-compromised mice after challenge with different doses of *Klebsiella pneumonia* was shown in Table 3. When immunocompetent mice were infected with *Klebsiella pneumonia*, all animals in the 1st and the 2nd group died in 36 h. The mortality of the 3rd and 4th group was 90% and 40% respectively in 72 h. Therefore, the MLD *Klebsiella pneumonia* to immunocompetent mice was defined as 5×10^5 CFU/mouse. When *Klebsiella pneumonia* infected immuno-compromised mice, animals of the 1st and 2nd group died in 48 h. The mortality of the 3rd and the 4th group was 95% and 55% respectively in 72 h. Therefore, the MLD *Klebsiella pneumonia* to immuno-compromised mice was defined as 1

$\times 10^5$ CFU/mouse.

It demonstrated that the minimal lethal dose of *Klebsiella pneumonia* to immunocompetent mice was 5-fold higher than that to immuno-compromised mice.

Protection of rAAV2-BPI₂₃-Fcγ1 gene transferred mice from MLD *Klebsiella pneumonia* infection

rAAV2-BPI₂₃-Fcγ1 gene transferred immunocompetent mouse was challenged by MLD *Klebsiella pneumonia* (5×10^5 CFU/0.5 ml). The survival rate was recorded in 72 h after bacterial injection. Results were shown in Figure 2. The survival rate of rAAV2-BPI₂₃-Fcγ1 gene transferred immunocompetent mice (40.0%, n = 45) was significant higher than that of rAAV2-Null (6.7%, n = 30) and rAAV2-EGFP transferred immunocompetent mice (6.7%, n = 45) ($\chi^2 = 13.97$, $p < 0.01$). While the survival rate of rAAV2-BPI₂₃-Fcγ1 gene transferred immuno-compromised mice (44.4%, n = 45) was significant higher than that of rAAV2-Null (3.3%, n = 30) and rAAV2-EGFP transferred immuno-compromised mice (4.4%, n = 45) ($\chi^2 = 19.50$, $p < 0.01$).

These results indicated that rAAV2-BPI₂₃-Fcγ1 gene transferred mice were resistant to the MLD of *Klebsiella pneumonia* infection. It suggested that BPI₂₃-Fcγ1 gene delivery has potential in preventing GNB infection of clinical high-risk patients.

Biological activity of BPI₂₃-Fcγ1 protein in immuno-compromised mice

For further evaluation of biological activity of BPI₂₃-Fcγ1

Table 3. Mortality of mice after challenge with different doses of *Klebsiella pneumonia*

Groups	n	CFU	Number of death						Mortality (%)
			12 h	18 h	24 h	36 h	48 h	72 h	
Immunocompetent	I	20	2.5×10^6	12	8	0	0	0	100
	II	20	1×10^6	8	6	3	3	0	100
	III	60	5×10^5	0	12	12	24	0	90*
	IV	20	1×10^5	0	0	0	5	3	40
Immuno-compromised	I	20	1×10^6	10	8	2	0	0	100
	II	20	5×10^5	6	4	5	0	5	100
	III	60	1×10^5	0	24	18	9	6	95*
	IV	20	5×10^4	0	0	2	4	0	55

After bacteria intraperitoneal injection, the death rates of immunocompetent mice and immuno-compromised mice were calculated in the indicated time points.

*The data of group III was representative of three independent experiments with similar results.

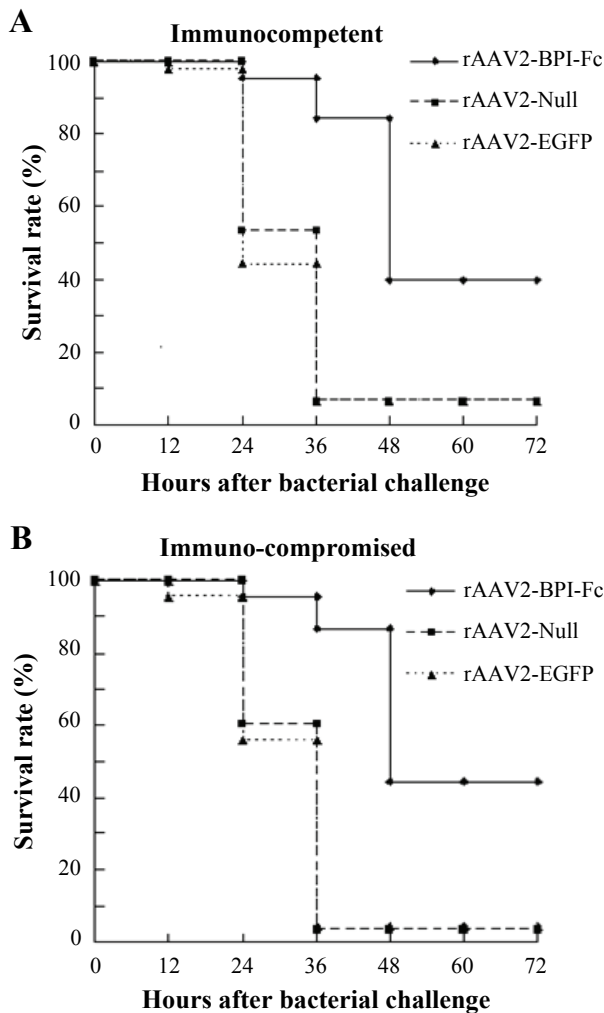


Figure 2. The survival rate of each group after MLD challenge with *Klebsiella pneumonia*. Through the quadriceps muscles of right hind leg, rAAV2-BPI23-Fcγ1 gene transferred mouse was injected with 100 μl solution containing 1×10^{11} v. g. of rAAV2-BPI23-Fcγ1; rAAV2-EGFP gene transferred mouse was injected with 100 μl solution containing 1×10^{11} v. g. of rAAV2-EGFP; rAAV2-Null gene transferred mouse was injected with 100 μl solution containing 1×10^{11} v. g. of rAAV2-Null. (A) After MLD *Klebsiella pneumonia* challenge, the survival rate of rAAV2-BPI23-Fcγ1 transferred mice ($n = 45$), rAAV2-Null transferred mice ($n = 30$) and rAAV2-EGFP transferred mice ($n = 45$) were 40.0%, 6.7% and 6.7% respectively in immunocompetent group. The survival rate of rAAV2-BPI23-Fcγ1 transferred mice was statistically higher ($p < 0.01$). (B) After MLD *Klebsiella pneumonia* challenge, the survival rates of the three groups were 44.4%, 3.3% and 4.4% respectively in immuno-compromised group. The survival rate of rAAV2-BPI23-Fcγ1 transferred mice was statistically higher than that of rAAV2-Null and rAAV2-EGFP transferred mice ($p < 0.01$). The survival rate of rAAV2-BPI23-Fcγ1 transferred mice was no statistical difference between immunocompetent and immuno-compromised groups ($p > 0.05$).

protein in BPI23-Fcγ1 gene transferred immuno-compromised mice, bacterial colony counting, level of endotoxin and

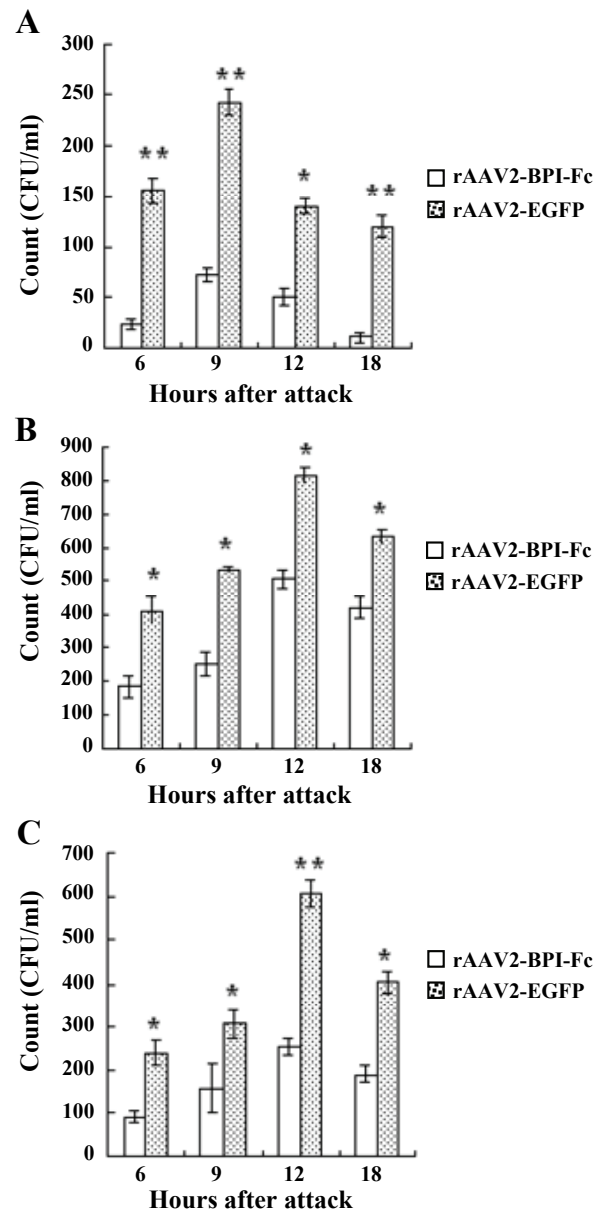


Figure 3. The quantity of bacteria in tissues. After MLD *Klebsiella pneumonia* infection, bacterial were counted in serum (A), liver (B) and spleen (C) homogenate from rAAV2-BPI23-Fcγ1 gene transferred mice or rAAV2-EGFP control mice at different time points. Data were shown as mean \pm SD of three independent replicates with similar results. * $p < 0.05$, ** $p < 0.01$ compared with rAAV2-BPI23-Fcγ1 gene transferred mice.

proinflammatory cytokines were examined after MLD *Klebsiella pneumonia* challenge. The count of bacteria in serum, spleen, and liver of BPI23-Fcγ1 gene transferred immuno-compromised mice were markedly less than that of EGFP gene transferred mice 6 hours after *Klebsiella pneumonia* challenge ($p < 0.05$). The bacterial counting reached its peak at the 9th hour in blood and 12th hour in liver and spleen (Figure 3). The target product of

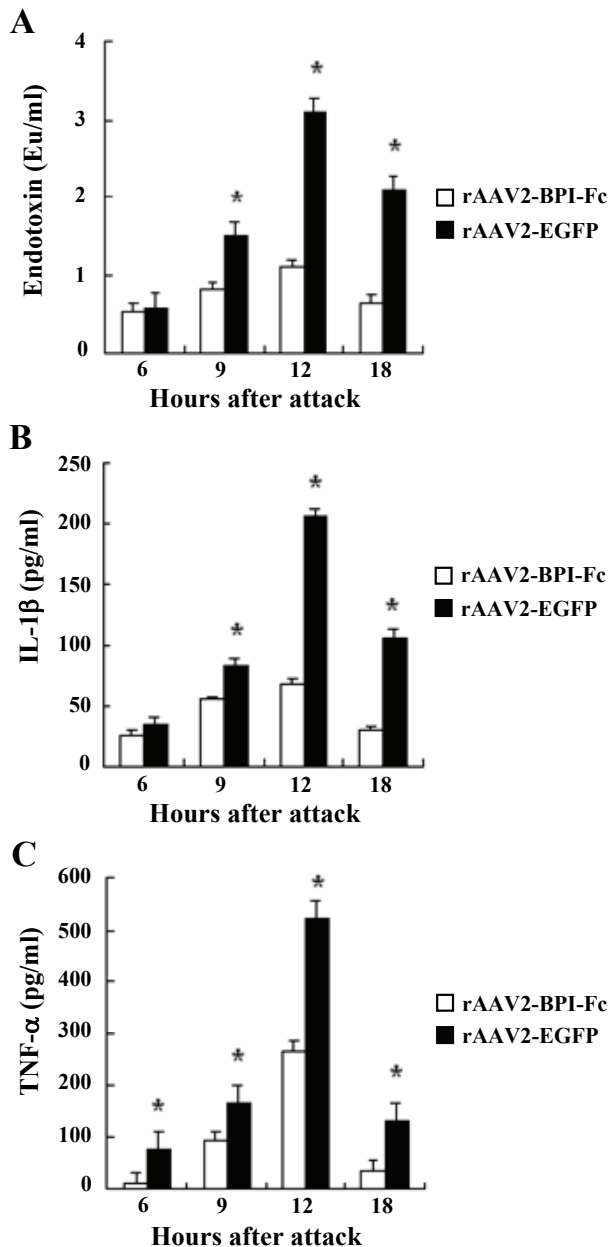


Figure 4. The level of endotoxin and pro-inflammatory cytokines in serum. After MLD *Klebsiella pneumoniae* infection, the concentration of endotoxin (A), IL-1 β (B) and TNF- α (C) in serum were analyzed by LAL (endotoxin) or ELISA (IL-1 β and TNF- α) from rAAV2-BPI₂₃-Fc γ 1 gene transferred mice or rAAV2-EGFP control mice at different time points. Data were shown as mean \pm SD of three independent replicates with similar results. * p < 0.01 compared with rAAV2-BPI₂₃-Fc γ 1 gene transferred mice.

rAAV2-BPI₂₃-Fc γ 1 gene in transferred immuno-compromised mice killed *Klebsiella pneumoniae* and so offered resistance to MLD *Klebsiella pneumoniae* infection.

The level of endotoxin and proinflammatory cytokine (IL-1 β , TNF- α) in the serum of BPI₂₃-Fc γ 1 gene transferred immuno-compromised mice was significantly lower than that

of EGFP transferred mice at the 9th, 12th and 18th hour after bacterial challenge (p < 0.01). The level of endotoxin and proinflammatory cytokine in the serum of both groups of mice reached its peak at the 12th hour (Figures 4A, 4B, 4C). BPI₂₃-Fc γ 1 gene transferred immuno-compromised mice were resistant to endotoxin shock caused by MLD *Klebsiella pneumoniae* infection through the mechanisms of killing *Klebsiella pneumoniae*, neutralizing endotoxin and inhibiting production of proinflammatory cytokines.

Discussion

The high-risk individuals of GNB infection are those with poor immunity resulted from chemotherapy, autoimmune disease, or immunosuppression treatment after transplantation (17, 18). However, no prophylactic method for bacterial infection occurred is available. Amazing progress in gene therapy and transfer products applied in clinical management of cancer, monogenic diseases, autoimmune diseases, HIV and bacterial infection. Viral delivery systems have been widely used in gene therapy protocols because of its high efficiency (19-22). Gutless viral vector is safe with low oncogenicity, poor immunogenicity, and does not exaggerate the inflammatory reaction (23-25). rAAV2 has been widely used as a gene delivery vehicle in pre- and early clinical trials. BPI₂₃-Fc γ 1 fusion protein has the effectiveness of killing GNB, neutralizing endotoxin, fixing complement and has a long half-life span. So we selected BPI₂₃-Fc γ 1 gene as the target gene in our test.

We prepared rAAV2-BPI₂₃-Fc γ 1 with a high viral load, which successfully mediated BPI₂₃-Fc γ 1 gene transfer and expression in mouse muscle cells. In this study, we developed immuno-compromised mice model to simulate high-risk individuals of GNB infection in clinic settings. We studied the resistance of both immuno-compromised and immunocompetent mice that were transfected with rAAV2-BPI₂₃-Fc γ 1 against MLD of *Klebsiella pneumoniae*. The MLD of *Klebsiella pneumoniae* identified in immuno-compromised mice (5×10^5 CFU/mouse) was 5-fold higher than that in immuno-compromised mice (1×10^5 CFU/mouse). The survival rate of both BPI₂₃-Fc γ 1 transferred immuno-compromised mice (40.4%) and immuno-compromised mice (44.4%) were significant higher than that in control groups (6.7% and 3.3%-4.4%) respectively after challenge with MLD *Klebsiella pneumoniae*. It suggested that transferring BPI₂₃-Fc γ 1 gene provides protection against GNB infection in both immuno-compromised and immunocompetent mice. After *Klebsiella pneumoniae* infection, the counting of bacteria in serum, liver and spleen as well as the level of endotoxin and pro-inflammatory cytokines in serum of rAAV2-BPI₂₃-Fc γ 1 gene transferred immuno-compromised mice decreased, while the survival rate increased markedly. These results prove that anti-bacteria gene transferring offers resistance to GNB infection in immuno-compromised mice. It suggests that rAAV2-BPI₂₃-Fc γ 1 gene delivery has potential clinical application especially in high-risk patients exposing to GNB infection.

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