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RESEARCH ARTICLE

Administration of nonviral gene vector encoding rat β-defensin-2 ameliorates chronic *Pseudomonas aeruginosa* lung infection in rats

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Abstract

Background Beta-defensin-2 (BD-2) plays an important role in host defense against pathogenic microbe challenge by its direct antimicrobial activity and immunomodulatory functions. The present study aimed to determine whether genetic up-regulation of rat BD-2 (rBD-2) could ameliorate chronic *Pseudomonas aeruginosa* lung infection in rats.

Methods Plasmid-encoding rBD-2 was delivered to lungs *in vivo* using linear polyethylenimine at 48 h before challenging with seaweed alginate beads containing *P. aeruginosa*. Macroscopic and histopathological changes of the lungs, bacterial loads, inflammatory infiltration, and the levels of cytokines/chemokines [interleukin (IL)-1β, tumor necrosis factor (TNF)-α, kertinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2)] were measured at 3 and 7 days post-infection (p.i.).

Results The overexpression of rBD-2 resulted in a significant increase in animal survival rate (at 3 days p.i.), a significant decrease in bacterial loads in the lungs (at 3 and 7 days p.i.), and significantly milder lung pathology. In addition, the overexpression of rBD-2 led to increased infiltration of polymorphonuclear neutrophils (PMN), and elevated protein expression of cytokines/chemokines (IL-1β, TNF-α, KC and MIP-2) at the early stage of infection (at 3 days p.i.), at the same time as being dramatically decreased at the later stage of infection (at 7 days p.i.).

Conclusions Genetic up-regulation of rBD-2 increased animal survival rate, and reduced bacterial loads in lungs after bacterial infection. The overexpression of rBD-2 also modulated the production of several cytokines/chemokines and increased PMN recruitment at the early stage of infection. Our findings indicate that the enhancement of BD-2 may be an efficacious intervention for chronic *P. aeruginosa* lung infection. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords β-defensin-2; bacterial infection; gene transfer; host defense peptides; innate immunity; *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa, a Gram-negative bacillus, is a major opportunistic pathogen that causes disease in patients with impaired host defenses and is often a cause of life-threatening nosocomial infection in critically ill

and immunocompromised patients [1–4]. In addition, persistent infection of the lungs with *P. aeruginosa* complicates many chronic lung diseases, including cystic fibrosis, bronchiectasis, chronic obstructive lung disease, and long-term mechanical ventilation [5,6]. Despite our clinical armamentarium, improvements the morbidity and mortality from *Pseudomonas* pneumonia remain substantial, suggesting that development of new and efficacious antimicrobial intervention is desirable.

Mammalian defensins, which are widespread in nature, act by mechanisms that extend beyond their capacity to serve as gene-encoded antibiotics. For example, defensins alter the properties of the mammalian membrane or interact with its receptors to influence diverse cellular processes, including cytokine release, chemotaxis, antigen presentation, angiogenesis, wound healing and tissue remodeling [7–10]. Based on sequence homology and the connectivity of the six conserved cysteine residues, mammalian defensins are classified into α-, β- and θ-defensins [7].

Human beta-defensin-2 (BD-2), primarily expressed in the epithelial tissues, is an inducible antimicrobial peptide, which can be induced by Gram-negative bacteria (e.g. *P. aeruginosa*) and their products [e.g. lipopolysaccharides (LPS)] as well as proinflammatory mediators such as TNF-α and IL-1β [11–15]. Human BD-2 (hBD-2) is also expressed in mesenchymal cells (e.g. articular chondrocytes, synoviocytes, and osteoblasts) and in glial and meningeal cells [16–22]. Now, more evidence indicates that BD-2 is an important mediator involved in the control of *P. aeruginosa* infection. Singh *et al*. [23] found that inactivated hBD-2 in the respiratory tract was associated with the incidence of *P. aeruginosa* infections in patients with cystic fibrosis. Similarly, Chen *et al*. [24] reported that decreased levels of β-defensins in cystic fibrosis likely contributed to a secondary defect of the local host defense. However, the relative contribution that BD-2 makes to host defenses in the lung remains unclear.

Animal models have been helpful for analysis of the role of BD-2 in pulmonary defenses. Rat BD-2 (rBD-2), a homologue of human BD-2 [25], is expressed in lung. Shu *et al*. [26] showed that overexpression of rBD-2 protected against acute *P. aeruginosa* pneumonia and lung injury by cecal ligation and double puncture in rats. *Pseudomonas aeruginosa* is an opportunistic pathogen; therefore, chronic infections are obtained more readily [6]. Nonetheless, the effects of rBD-2 on chronic *P. aeruginosa* lung infection have not been clearly described.

Increasing evidence indicates that early polymorphonuclear neutrophils (PMN) recruitment is important for combating bacterial infection [27] and PMN recruitment may be impaired in the early stages of *P. aeruginosa* infection in cystic fibrosis patients [28–30]. Therefore, an increase in early PMN recruitment may be beneficial for chronic lung infection. Previous studies have revealed that several cytokines and chemokines are important for neutrophil migration, which include the direct neutrophil chemoattractant kertinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2), and indirect attractants interleukin (IL)-1β and tumor necrosis factor (TNF)-α [14,24]. BD-2 has been shown to modulate the production of proinflammatory cytokines [31]. In addition, Niyonsaba *et al*. [32] reported that BD-2 was a potent chemoattractant for neutrophils *in vitro*.

In the present study, we explored whether the overexpression of rBD-2 has a beneficial effect on bacterial clearance and host survival in the setting of *P. aeruginosa* pneumonia. We also proposed the hypothesis that the enhancement of rBD-2 modulated the production of chemokines/cytokines and increased PMN recruitment, which are probably responsible for bacterial clearance [27,33]. Accordingly, we modelled chronic *P. aeruginosa* lung infection by infecting rats with *P. aeruginosa* strain PAO1 embedded in alginate beads via intratracheal challenge [6,34,35]. A nonviral gene transfer approach was used, in which plasmid DNA was complexed to a linear polycationic polymer, polyethylenimine (PEI), and used for delivering rBD-2 gene to rat lungs.

Materials and methods

Construction of rBD-expressing plasmid, cell culture, and transient transfection

A plasmid encoding rBD-2 or an enhanced green fluorescent protein (GFP) was constructed and referred to as pCMV-rBD-2 and pCMV-GFP, respectively. The control plasmid containing no insert (empty vector) is referred to as pCMV-null. Transient transfection of cultured Chinese hamster ovary (CHO) cells with pCMV-rBD-2, pCMV-null, or pCMV-GFP was performed.

Intratracheal gene delivery to rat lungs

Linear *in vivo*-jetPEI™ (Polyplus-transfection, Illkirch, France) was complexed with 20 μg of plasmid DNA using a charge ratio of 8 (charge ratio is expressed as PEI nitrogen to DNA phosphate). The DNA/PEI complex was prepared and delivered to the rat lungs *in vivo* via an intratracheal catheter as described previously [34,36].

Preparation of bacteria for challenge

PAO1, a fully sequenced strain of *P. aeruginosa* [37], was immobilized in seaweed alginate beads as previously described [38–40]. The suspension of PAO1 was then adjusted to 5×10^8 colony-forming units (CFU) ml^{-1} and the yield was confirmed by colony counts.

Rat model of chronic lung infection and collection of lung

All animal experiments were carried out according to the guidelines set by the Chinese Council on Animal

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Care and approved by the Tongji Medical College Committee on Animal Experimentation. The research protocol was reviewed and approved by the Tongji Medical College Committee. Seventy-two pathogen-free male Sprague-Dawley (SD) rats (aged 8–12 weeks and weighing 180–210 g) were obtained from the animal supply centre at Tongji Medical College (Wuhan, Hubei Province, China). The animals were randomly divided into four groups: the normal group ($n = 18$), the untreated-infection group ($n = 18$), the pCMV-null/PEI-treated group ($n = 18$), and the pCMV-rBD-2/PEI-treated group ($n = 18$). The rat model of chronic *P. aeruginosa* lung infection was established as described by Johansen *et al*. [27]. In brief, each rat received 0.1 ml alginate beads containing 5×10^7 CFU of PAO1. The incision was sutured with silk and healed without any complications. At 3 and 7 days post-infection (p.i.), lung tissue was obtained for the detection of cytokines and myeloperoxidase (MPO) activity, for bacterial CFU counting (left lobes), as well as for histology, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot analysis (right lobes) [30].

Macroscopic pathology of the lungs

Macroscopic lung pathology was expressed as the lung index of macroscopic pathology (LIMP), which was calculated by dividing the area of the lung showing pathological changes by the total area of the whole lung [39,41]. In addition, the gross pathological changes in the lungs were also assigned four different scores according to the severity of the inflammation as previously described [38,40,42]: I, normal lungs; II, swollen lungs, hyperemia, and small atelectasis (<10 mm^2); III, pleural adhesions and atelectasis (<40 mm^2); and IV, abscesses, large atelectasis, and hemorrhages. The evaluation was conducted in a double-blind way to avoid bias.

Histopathology of the lungs

Lung histological examination was performed with six lungs from each group of rats at each time point as described previously [38,40]. The lower section of the left lung lobe was fixed in formalin, embedded in paraffin wax, cut at 5 μm in thickness and stained with hematoxylin and eosin. The lung pathology was assigned microscopically one of four scores according to the severity of the inflammation: I, normal histology; II, mild focal inflammation; III, moderate to severe focal inflammation with areas of normal lung tissue; and IV, severe inflammation to necrosis or severe inflammation throughout the lung.

Lung bacteriology

Lung samples from each group of rats ($n = 6$ per group/time) at each time point were prepared for quantitative bacteriological examination as described by Johansen *et al*. [35]. In brief, each lung was mixed with 5 ml of cold sterile phopshate-buffered saline and the mixture was homogenized in a blender. Serial ten-fold dilutions of the homogenate were plated on *Pseudomonas* isolation agar (Difco, Franklin Lakes, NJ, USA) in triplicate to determine the number of bacterial CFU after 24 h incubation at 37 °C. Results are reported as $\log_{10}$ number of CFU per lung (± SEM).

Lung MPO assay

The MPO assay was used to determine the infiltration of PMN into the lungs of the rats as described previously [30,43]. Briefly, samples in duplicate (75 μl) were mixed with equal volumes of the substrate (3 mM 3,3′,5,5′-tetramethyl-benzidine dihydrochloride, 120 μM resorcinol, and 2.2 mM H_2O_2) for 2 min. The reaction was stopped by adding 150 μl of 2M H_2SO_4. The optical density was measured at 450 nm.

Real-time RT-PCR

Real-time quantitative RT-PCR was performed using the ABI 7900HT Fast real-time PCR System (Applied Biosystems, Foster City, CA, USA). Total RNA was isolated from individual lungs for analysis using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and quantified by spectrophotometric determination (260 nm). One microgram of total RNA was reverse transcribed to cDNA using RT-PCR kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. The cDNA was analysed immediately or stored at −20 °C. Next, cDNA was amplified using SYBR Green Master mix (Takara) according to the manufacturer's instructions. Briefly, the 20-μl reaction system contained 10 μl of SYBR Green PCR Master mix, 0.5 μM primers, 2 μl of cDNA (diluted 1 : 5), and diethyl pyrocarbonate water. Sequences of primer sets for real-time PCR are shown in Table 1. Relative mRNA levels were calculated using the relative standard curve method that compares the amount of target normalized to an endogenous reference, β-actin. Briefly, the mean ± SD values of replicate samples were calculated. Results are expressed as the relative amount of mRNA between experimental test samples and normal control samples (all normalized to β-actin). In preliminary experiments, the products were analysed by gel electrophoresis, and a single product was obtained with each primer set. In addition, dissociation curves yielded single peaks.

Western blot analysis

The rBD-2 protein in the lung or cell-free supernatants was evaluated by western blotting analysis using a primary Rabbit polyclonal antihuman BD antibody (Alpha

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Table 1. Nucleotide sequence of the specific primers used in PCR amplification

Gene	Primer sequence (5′- to 3′)	
β-actin	GGAGATTACTGCCCTGGCTCCTA	F
	GACTCATCGTACTCCTGCTTGCTG	R
rBD-2	TCACATGCCTGACCAAAGGA	F
	AACCGCCAGTGCATGGAC	R
MCP1	TCACGCTTCTGGGCCTGTTG	F
	CAGCCGACTCATTGGGATCATC	R
MIP2	GGCAAGGCTAACTGACCTGGAAAG	F
	CACATCAGGTACGATCCAGGCTTC	R
KC	TGGATGCGTTTCATCGATGGTCGT	F
	GCACAGTGGTTGACACCTAATGGT	R
TNF-α	AACTCGAGTGACAAGCCCGTAG	F
	GTACCACCAGTTGGTTGTCTTTGA	R
IL-Iβ	GCTGTGGCAGCTACCTATGTCTTG	F
	AGGTCGTCATCATCCCACGAG	R

Diagnostic, San Antonio, TX, USA) as described previously [27].

Enzyme-linked immunoasorbent assay (ELISA) determination of cytokine/chemokines concentrations

The concentrations of cytokines/chemokines (IL-1β, TNF -α, KC and MIP-2) in supernatants of the lung homogenate (from the detection of lung bacteriology) from each group of rats at 3 days and 7 days p.i., were determined by commercially available sandwich-type ELISAs, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). For quantification, reference curves, obtained using increasing concentration of recombinant rat cytokines, were performed in parallel. Protein concentrations in lung homogenates were determined using the Bradford dye binding procedure. Cytokine/chemokine concentration was calculated as pg mg^{-1} of total protein for each sample.

Statistical analysis

Survival curves (Kaplan–Meier plots) were compared by log-rank test. The pathologic score was compared by the nonparametric Kruskall–Wallis test. One-way analysis of variance with the Newman–Keuls test was used to determine the significance of viable bacterial counts, MPO, real-time RT-PCR, and protein assays. $p < 0.05$ was considered statistically significant.

Results

rBD-2 elaboration after *P. aeruginosa* agarose bead infection in rats

To determine whether rBD-2 was present in the lungs before and after infection with *P. aeruginosa*, mRNA expression levels in normal, uninfected and infected lungs were tested by real-time RT-PCR. Representative data are provided in Figure 1. The mRNA level for rBD-2 was constitutively expressed in normal uninfected lungs; however, it was up-regulated after infection with *P. aeruginosa*.

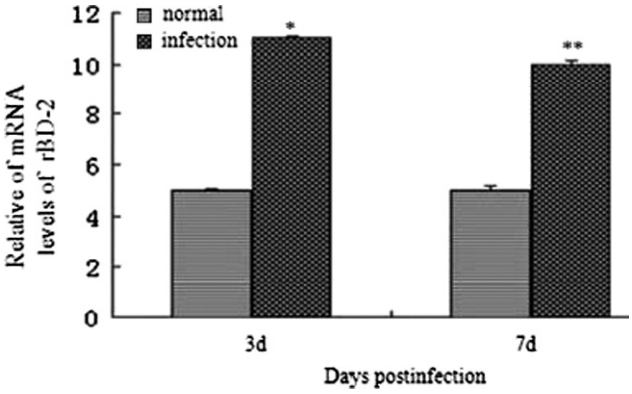

Figure 1. rBD-2 expression in the lungs of rats. mRNA expression levels of rBD-2 were significantly increased in the infected lungs at 3 and 7 days p.i. in comparison to normal uninfected lungs (*$p < 0.001$, **$p < 0.05$ versus normal, uninfected lungs). Data are the mean ± SEM and represent two individual experiments each with six animals per group per time

Transgene expression of rBD-2 *in vitro* and *in vivo*

Expression of rBD-2 in vitro

We used CHO cells to verify rBD-2 expression from the plasmid pCMV-rBD-2. Concentrated supernatant was analysed by means of western blotting. rBD-2 was detectable in the supernatant harvested from cells transduced with plasmid pCMV-rBD-2, whereas no rBD-2 was detected in the supernatant from untransfected cells or cells transfected with pCMV-null (Figure 2A). On the basis of data obtained by counting GFP positive cells under a fluorescence microscope [34,36], the transfection efficiency was as high as approximately 80%.

Detection of rBD-2 in vivo

The highest and significant levels of transfection were obtained at days 1 and 3, and was reduced at day 7 after an intratracheal injection with pCMV-GFP/PEI complexes [34]. rBD-2 gene was delivered via the airway to lung using the PEI as a gene carrier 48 h before *P. aeruginosa* infection. In addition, one group received the empty control vector pCMV-null; this group acted as a control group for vector delivery. We detected rBD-2 mRNA and protein expression levels in rat lung tissue by real-time RT-PCR and western blotting, respectively. Rat lungs from the pCMV-rBD-2/PEI treatment group exhibited a higher level of rBD-2 mRNA and protein expression at 3 days p.i. compared to untreated lungs ($p < 0.05$) or those treated with

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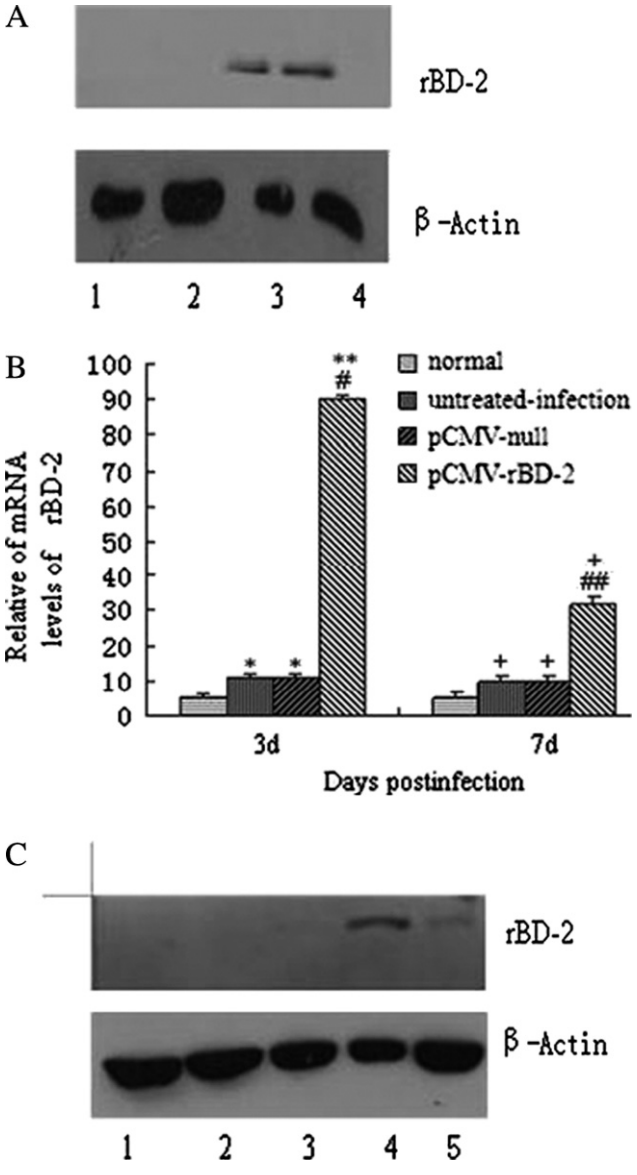

Figure 2. rBD-2 protein and mRNA expression *in vivo* and vitro. (A) Forty-eight hours after infection with pCMV-rBD-2 or pCMV-null, the supernatant of CHO cells was collected and concentrated ten- and 20-fold using a superfilter column. The expression of rBD-2 in the supernatant was analysed by western blotting. Lane 1, ten-fold concentrated supernatant from CHO infected with pCMV-null; lane 2, 20-fold concentrated supernatant from CHO infected with pCMV-null; lane 3, ten-fold concentrated supernatant from CHO infected with pCMV-rBD-2; lane 4, 20-fold concentrated supernatant from CHO infected with pCMV-rBD-2. (B) mRNA expression levels of rBD-2 were significantly increased in lung treated with pCMV-rBD-2/PEI complex at 3 and 7 days p.i. Results are the mean ± SEM. *$p < 0.05$, +$p < 0.05$, **$p < 0.01$ versus normal lungs; #$p < 0.05$, ##$p < 0.05$ versus all other infection groups including the untreated group and those treated with the empty control vector (pCMV-null). (C) Western blotting analysis shows that rBD-2 protein was clearly expressed in lungs treated with pCMV-rBD-2/PEI complex. Lane 1, normal; lane 2, pCMV-null at 3 days p.i.; lane 3, pCMV-null at 7 days p.i.; lane 4, pCMV-rBD-2 at 3 days p.i.; lane 5, pCMV-rBD-2 at 7 days p.i.

the empty vector pCMV-null/PEI ($p < 0.05$; Figures 2B and 2C).

Effect of rBD-2 expression on survival rate

rBD-2 improved 3-day survival rate

The animal survival rate at 3 days p.i. in the treatment with pCMV-rBD-2/PEI complex group was 83% compared to 66% in the untreated-infection group and the pCMV-null/PEI-treated group ($n = 18$ per group; Figure 3A). By log-rank test analysis, it was significantly different ($p < 0.05$). The rats died between 1 and 3 days p.i., and no mortality was observed subsequently. Autopsies showed that more than 50% of the lungs of each dead rat became acutely consolidated and the animals became significantly dehydrated (data not shown). These findings indicated that those animals might have died from respiratory failure and infectious shock.

Effect of rBD-2 expression on morphologic properties

Macroscopic pathology

Lung consolidation with hemorrhage (at 3 days p.i.) and atelectasis (at 7 days p.i.) could be seen in all infection groups (Figures 3B to 3G). The macroscopic lung pathology in the untreated-infection group (Figures 3B and 3E) and the pCMV-null/PEI-treated group (Figures 3C and 3F) was dominated by a large area of lung consolidation with hemorrhage, or abscess, whereas, in the pCMV-rBD-2/PEI-treated group (Figures 3D and 3G), it was dominated by lung atelectasis without indications of abscess.

LIMP

Significantly lower LIMP scores were found in the pCMV-rBD-2/PEI-treated group at 3 and 7 days p.i. compared to all other infection groups ($p < 0.05$ and $p < 0.01$, respectively; Figure 3H). In the pCMV-rBD-2/PEI-treated group, the difference in the LIMP between 3 and 7 days p.i. ($p < 0.05$) was significant. By contrast, the difference in the LIMP between 3 and 7 days p.i. in the untreated-infection and the pCMV-null/PEI-treated groups was not significant ($p > 0.05$). Lower LIMP scores indicate less lung pathology.

Macroscopic lung scoring

The lung pathology scoring in the pCMV-rBD-2/PEI-treated group showed mainly milder lung pathology (scores in the range 2–3) at 3 and 7 days p.i. By contrast, the untreated infection and the pCMV-null/PEI-treated groups were predominated by more severe lung pathology (scores in the range 3–4). The differences were statistically significant (Table 2).

Histopathology of the lungs

The untreated-infection and the pCMV-null/PEI-treated groups showed significant infiltration of inflammatory

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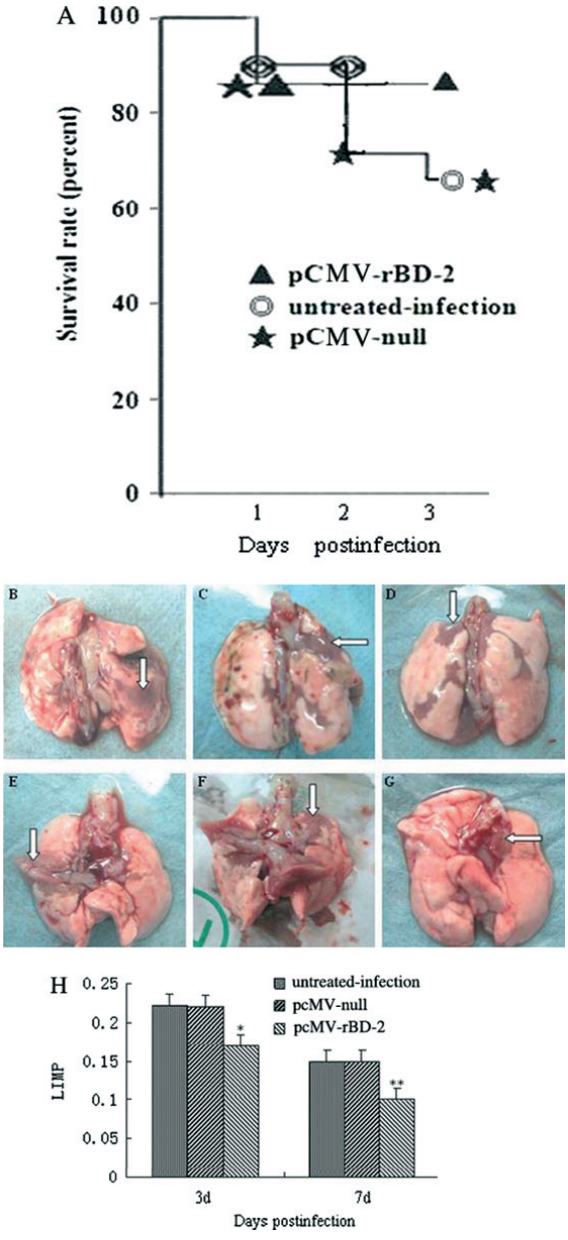

Figure 3. (A) Animal survival. Animal survival rate at 3 days p.i. was improved by treatment with pCMV-rBD-2/PEI complexes ($p < 0.05$). (B–G) Comparison of macroscopic lung pathology. (B) untreated-infection lung at 3 days p.i. (C) pCMV-null/PEI-treated lung at 3 days p.i. (D) pCMV-rBD-2/PEI-treated lung at 3 days p.i. (E) untreated- infection lung at 7 days p.i. (F) pCMV-null/PEI-treated lung at 7 days p.i. (G) pCMV-rBD-2/PEI-treated lung at 7 days p.i. At 3 days p.i. (B, D), lung consolidation with hemorrhage could be seen in all infection groups. However, at 7 days p.i., a large area of lung consolidation with hemorrhage, or abscess was still seen in the untreated infected lung and pCMV-null/PEI=treated lung (E, F), whereas, in the pCMV-rBD-2/PEI-treated group (G), only a small amount of atelectasis was seen. (H) Comparison of the LIMP of the pCMV-rBD-2/PEI-treated group and all other infection groups on different days p.i. A sharply reduced LIMP value with respect to time is observed in the pCMV-rBD-2/PEI-treated group compared to the untreated-infection and the pCMV-null/PEI-treated groups. The results are represented as the mean ± SEM. Significant differences between groups are indicated by asterisks: *$p < 0.01$; **$p < 0.05$

Table 2. Macroscopic lung scores at different time points after intratracheal challenge and pCMV-rBD/PEI-treatment

	Number of rats in each group[a]							
	Day 3 ($n1 = 7, n2 = n3 = 6$)				Day 7 ($n1 = 8, n2 = n3 = 6$)			
Groups	I[b]	II	III	IV	I	II	III	IV
Untreated-infection	0	0	0	6	0	0	3	3
pCMV-null	0	0	1	5	0	0	3	3
pCMV-rBD-2*	0	1	3	3	0	3	4	1

[a]$n1$, $n2$ and $n3$ are the animal numbers in the pCMV-rBD-2/PEI-treated, pCMV-null/PEI-treated, and untreated-infection groups. [b]See Materials and Methods. *$p < 0.05$ versus all other infection groups.

Table 3. Histopathological lung scores at different time points after intratracheal challenge and pCMV-rBD/PEI -treatment

	Number of rats in each group[a]							
	Day 3 ($n1 = 7, n2 = n3 = 6$)				Day 7 ($n1 = 8, n2 = n3 = 6$)			
Groups	I[b]	II	III	IV	I	II	III	IV
Untreated-infection	0	0	2	4	0	0	3	3
pCMV-null	0	0	2	4	0	0	4	2
pCMV-rBD-2*	0	1	3	3	0	4	3	1

[a]$n1$, $n2$ and $n3$ are the animal numbers in the pCMV-rBD-2/PEI-treated, pCMV-null/PEI-treated, and untreated-infection groups. [b]See Materials and Methods. *$p < 0.05$ versus all other infection groups at 7 days p.i.

cells in the bronchia and adjacent parenchyma with tissue damage, edema, hemorrhage and consolidation at 3 and 7 days p.i. (Figures 4A to 4B, 4D to 4E). By contrast, the area with pathologic changes in the pCMV-rBD-2/PEI-treated group was smaller than in the other infection groups (Figures 4C and 4F). The histopathological score in pCMV-rBD-2/PEI-treated group was lower ($p < 0.05$) compared to the other infection groups at 7 days p.i. (Table 3).

Effect of rBD-2 expression on plate count, MPO

Therefore, we next assessed further the effect of pCMV-rBD-2 treatment on the bacterial component of disease pathogenesis. The pulmonary bacterial counts in the pCMV-rBD-2/PEI-treated group were significantly lower than those in the untreated- infetion and the pCMV-null/PEI-treated groups at 3 and 7 days p.i. ($p < 0.05$ and $p < 0.01$, respectively) (Figure 5A). In the pCMV-rBD-2/PEI-treated group, the lung bacteriology decreased progressively with time [i.e. the number of bacteria in the lungs at 3 days p.i. (4.92 CFU $\log_{10}$ scale) was higher than that at 7 days p.i. (3.3 CFU $\log_{10}$ scale, last time point examined in these studies)]. By contrast, there was no significant difference between the number of bacteria in the lungs at 3 and 7 days p.i. in the untreated-infection group (6.28 CFU $\log_{10}$ scale, 6.12 CFU $\log_{10}$ scale, respectively, $p > 0.05$) and the pCMV-null/PEI-treated group (6.26 CFU $\log_{10}$ scale, 6.04 CFU $\log_{10}$ scale,

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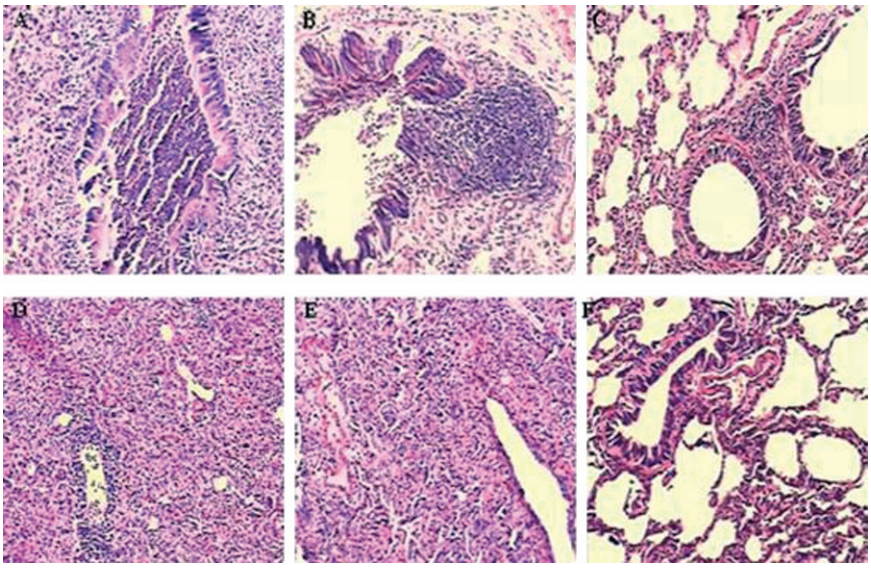

Figure 4. Hematoxylin and eosin staining. (A) a untreated infection lung at 3 days p.i. (B) pCMV-null/PEI-treated lung at 3 days p.i. (C) pCMV-rBD-2/PEI-treated lung at 3 days p.i. (D) untreated-infection lung at 7 days p.i. (E) pCMV-null/PEI-treated lung at 7 days p.i. (F) pCMV-rBD-2/PEI-treated lung at 7 days p.i. The untreated-infection and the pCMV-null/PEI-treated groups showed a significant infiltration of inflammatory cells in the bronchia and adjacent paranchyma with tissue damage, edema, hemorrhage and consolidation at days 3 and 7 p.i., whereas the pCMV-rBD-2/PEI-treated group showed significantly milder lung pathology (original magnification, ×100)

respectively, $p > 0.05$). These results suggested that the overexpression of rBD-2 in the lungs resulted in bacterial clearing away more quickly compared to the other infection groups. We also measured lung MPO activity as an indicator of PMN influx to the lung. The results obtained are shown in Figure 5B. In the pCMV-rBD-2/PEI-treated group, peak MPO activity was observed at 3 days p.i., whereas the activity of MPO continued to increase up to 7 days p.i. in the untreated-infection group and the pCMV-null/PEI-treated group. In the pCMV-rBD-2/PEI-treated group, MPO activity was significantly increased at 3 days p.i. and decreased at 7 days p.i. compared to the untreated-infection lungs ($p < 0.05$) and the pCMV-null/PEI-treated lungs ($p < 0.05$). The data obtained suggest that overexpression of rBD-2 may increase PMN recruitment at the early stage of *P. aeruginosa* lung infection.

Effect of rBD-2 expression on proinflammatory cytokines and chemokines

We next investigated mechanism(s) for the observed beneficial effect provided by genetic up-regulation of rBD-2 in lung against *P. aeruginosa* pneumonia. To determine whether the overexpression of rBD-2 modulated the production of proinflammatory cytokines and chemokines, mRNA expression levels of cytokines/chemokines (IL-1β, TNF-α, KC and MIP-2) were analysed by real-time RT-PCR in all groups (Figure 6). After pCMV-rBD-2/PEI treatment, the mRNA expression levels of IL-1β (Figure 6A) and KC (Figure 6C) were significantly up-regulated at 3 days p.i. and down-regulated at 7 days p.i.; whereas TNF-α (Figure 6B), MIP-2 (Figure 6D) and MCP-1 (Figure 6E) expression levels were similar at 3 days p.i., followed by a significant decrease at 7 days p.i. compared to the untreated-infection group and the pCMV-null/PEI-treated group. Additionally, protein expression levels of IL-1β (Figure 6F), TNF-α (Figure 6G), KC (Figure 6H) and MIP-2 (Figure 6I) were examined by ELISA. Protein expression levels in all groups were assessed at 3 and 7 days p.i. After pCMV-rBD-2/PEI treatment, IL-1β, TNF-α, KC and MIP-2 protein expressions were significantly increased at 3 days p.i. (all $p < 0.01$), but decreased at 7 days p.i. ($p < 0.05$) compared to untreated-infection and the pCMV-null/PEI-treated groups. In the pCMV-rBD-2/PEI-treated group, the expression levels of IL-1β, TNF-α, MIP-2 and KC, decreased progressively with time. By contrast, there were no significant differences of KC and MIP-2 protein expression levels in the lungs at 3 and 7 days p.i. ($p > 0.05$) and IL-1β and TNF-α protein expression levels at 7 days p.i. were significantly higher compared to those at 3 days p.i. in the untreated-infection and the pCMV-null/PEI-treated groups ($p < 0.05$).

Discussion

BD-2, comprising epithelial antibacterial peptides with six conserved cysteine residues, not only has the capability of direct lysis of microbes (especially Gram negative bacteria) [44], but also triggers and tunes immunomodulatory processes such as chemotaxis, cytokine production and dendritic cell maturation [7,8,45,46]. The present study demonstrated that enhancement of rBD-2 improved animal survival after bacterial infection, and significantly

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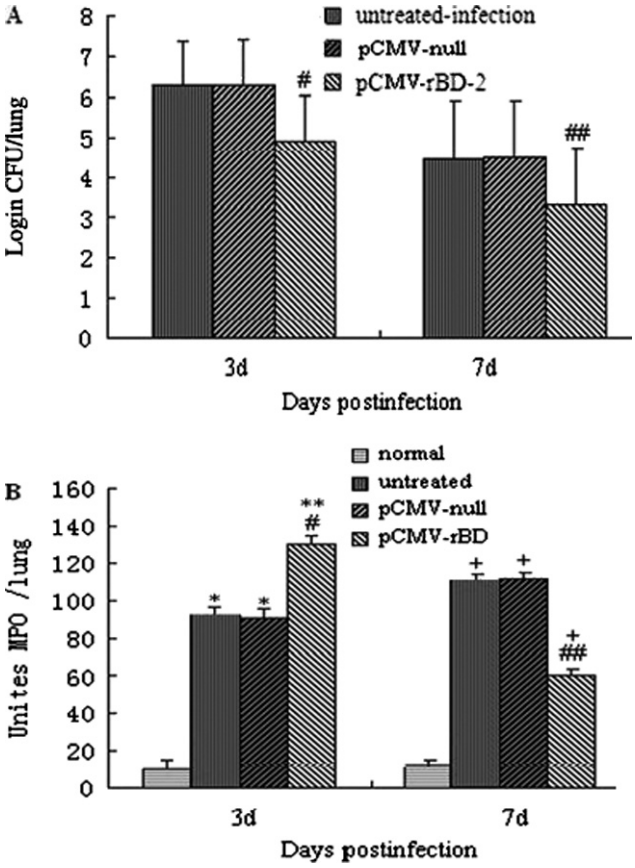

Figure 5. Effect of rBD-2 overexpression on plate count and MPO. (A) The bacterial number decreases more quickly in the pCMV-rBD-2/PEI-treated group than the untreated-infection and the pCMV-null/PEI-treated groups. (B) In the pCMV-rBD-2/PEI-treated group, the MPO activity was up-regulated at 3 days p.i., followed by a significant down-regulation at 7 days p.i. Results are the mean ± SEM ($n = 6$, $n = 7$ or $n = 8$ in groups). $^{*}p < 0.05$, $+p < 0.05$, $^{**}p < 0.01$ versus normal lungs; $\#p < 0.05$, $\#\#p < 0.05$ versus all other infection groups, including the untreated group and those treated with the empty control vector (pCMV-null)

reduced the lung bacterial load. Furthermore, the overexpression of rBD-2 enhanced the production of several cytokines/chemokines (IL-1β, TNF-α, KC and MIP-2) and increased PMN recruitment at the early stage of *P. aeruginosa* infection, which are probably responsible for bacterial clearance.

In the present study, we chose pharmacologic-grade gene carrier linear PEI (L-PEI) to deliver rBD-2 to rats lungs. This is a nonvirus-based system and therefore the inherent drawbacks of viral vectors including inflammation, immunogenic responses and possible insertional mutagenesis could be eliminated [47]. Moreover, a number of studies have demonstrated effective delivery of DNA with L-PEI *in vivo* [34,36,48]. Furthermore, in preliminary experiments, no signs of acute toxicity (inflammation, cellular infiltration, etc.) were detected by direct histopathological analysis after DNA delivery with L-PEI in rats, suggesting that L-PEI did not induce an obvious inflammatory response, which is consistent with previous studies [47]. We used real-time RT-PCR and WB to confirm expression of rBD-2 after intratracheal gene delivery. rBD-2 mRNA and protein expression levels in rat lung tissue of the pCMV-rBD-2/PEI treatment group demonstrated significant increases at 3 and 7 days p.i. compared to the pCMV-null/PEI-treated group. The findings obtained in the present study are similar to previous studies indicating that the highest and significant levels of transfection were obtained at days 1 and 3, and were reduced at day 7, after an intratracheal injection with pCMV-GFP/PEI complexes [34].

Pseudomonas aeruginosa is an opportunistic pathogen, and therefore chronic infections are obtained more readily [6]. In the present study, chronic *P. aeruginosa* lung infection was obtained using *P. aeruginosa* cells impregnated in alginate beads (Figure 4), which closely resembles the lung pathology seen in cyctic fibrosis patients with chronic *P. aeruginosa* lung infection [35,38,41]. BD-1 is often constitutively expressed [49], whereas BD-2 expression is inducible by Gram-negative bacteria (e.g. *P. aeruginosa*), their products (e.g. LPS) and proinflammatory mediators such as TNF-α and IL-1β [11–14,50]. Similarly, the results obtained in the present study demonstrated that the mRNA expression level of rBD-2 was up-regulated in *P. aeruginosa* infection lungs. The up-regulation of the rBD-2 after *P. aeruginosa* infection indicated that rBD-2 might play an important role in lung infection. Moreover, several recent investigations have implicated BD-2 in host defense against lung pathogens, including *P. aeruginosa* [23,26]. Our studies conducted *in vivo* demonstrated that genetic up-regulation of rBD-2 promoted host resistance against chronic *P. aeruginosa* lung infection. Data supporting this tenet included the animal survival rate, morphologic properties, and bacterial plate counts. The pCMV-rBD-2/PEI-treated group displayed increased survival rate (at 3 days p.i.) and dramatically decreased the bacterial load in the lungs (at 3 and 7 days p.i.). They also showed significantly milder lung pathology and quicker recovery compared to the untreated-infection and the pCMV-null/PEI-treated groups.

Increasing evidence suggests that the immunomodulatory functions of BD-2 influence the outcome of infection [15,46,51]. The findings obtained in the present study showed that the overexpression of rBD-2 in the lung enhanced recruitment of PMN to the lung at the early stage of *P. aeruginosa*. The lung neutrophil number assessed by an established MPO assay provided verification that pCMV-rBD-2/PEI-treated group had a significantly greater number of PMN in lung tissue at the early stage (at 3 days p.i.) but decreased at the later stage of infection (at 7 days p.i.) compared to the untreated-infection group and the pCMV-null/PEI-treated group. PMNs, comprising one of the earliest immune cells recruited to the site of infection, play a key role in the early control of bacterial infection. However, at a later stage, their presence contributes to tissue injury [27,52]. In addition, previous studies indicated PMN recruitment may be impaired in the early stages of *P. aeruginosa* infection in cystic fibrosis patients, whereas the infiltration of

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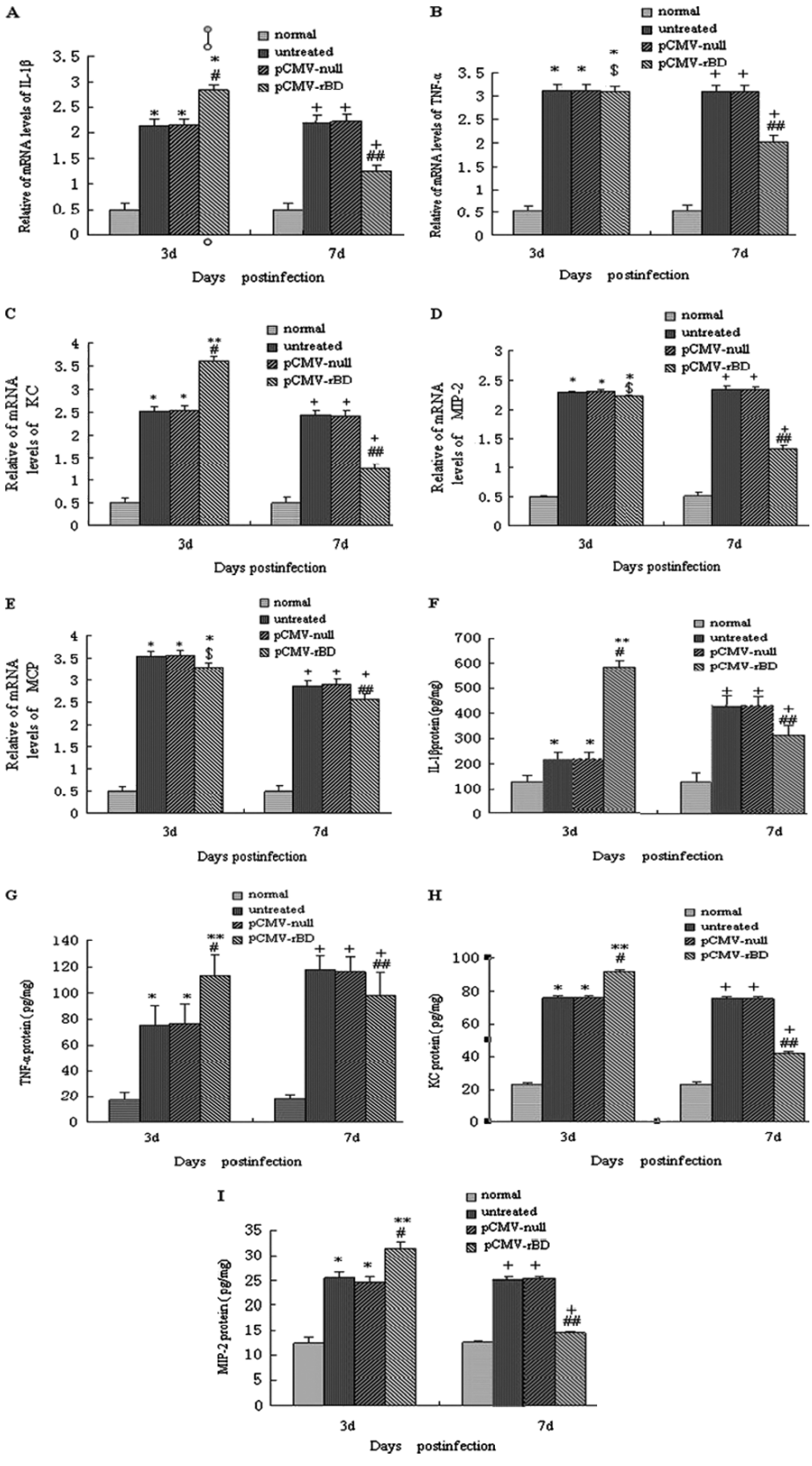

Figure 6. Effect of transgene rBD-2 on proinflammatory cytokines and chemokines. In the pCMV-rBD-2/PEI-treated group, the mRNA expression level of IL-1β (A) and KC (C) was up-regulated at 3 days p.i., followed by a significant down-regulation at 7 days p.i.; TNF-α (B), MIP-2 (D) and MCP-1 (E) were similar at 3 days p.i., followed by a significant decrease at 7 days p.i. compared to untreated-infection and the pCMV-null/PEI-treated groups. The protein levels determined by ELISA indicated a up-regulation of IL-1β (F), TNF-α (G), KC (H) and MIP-2 (I) at 3 days p.i., followed by a significant down-regulation at 7 days p.i. in the pCMV-rBD-2/PEI-treated rats versus untreated infection group. Results are the mean ± SEM ($n = 6$, $n = 7$ or $n = 8$ in groups). *$p < 0.05$, +$p < 0.05$, **$p < 0.01$ versus normal lungs; #$p < 0.05$, ##$p < 0.05$, \$$p > 0.05$ versus all other infection groups, including the untreated-infection group and those treated with the empty control vector (pCMV-null)

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numerous PMN in the lung tissues at the later stages of infection has been shown to be connected to a poor prognosis [28]. Hence, the early increase in PMN recruitment may benefit the patients with chronic lung infection.

Recent studies have shown that BD-2 induces immature DC maturation to trigger Th1 responses *in vivo* as well as proinflammatory cytokine production [31]. It has also been demonstrated that BD-2 and LPS share the same receptor, Toll-like receptor (TLR) 4, and activate the transcription factor nuclear factor-κB through a TLR cascade, leading to proinflammatory cytokine expression [25,31,53]. Wu *et al*. [54] reported that mouse BD-2, a homologue of hBD-2 and rBD-2, modulated the production of proinflammatory cytokines in the *P. aeruginosa* keratitis. Consistent with these findings, the present study also revealed that, in the lung, the enhancemene of rBD-2 can regulate proinflammatory cytokine and chemokines, contributing to host control of *P. aeruginosa* lung infection. With overexpression of rBD-2 in the lung, the protein expression levels of IL-1β, TNF-α, KC and MIP-2 were up-regulated at the early stage of infection (at 3 days p.i.). The infiltration of PMN into the lung is controlled largely by the local production of proinflammatory cytokines (IL-1β, TNF-α) and chemokines (KC, MIP-2) [27,55]. Tsai *et al*. [56] reported that ELR1 CXC chemokines are critical mediators of neutrophil-mediated host defense in *Pseudomonas* pneumonia. In the rat, MIP-2 and KC are functional homologues of the human ELR1 CXC chemokines, IL-8 and GRO-a/b [57]. Moreover, Niyonsaba *et al*. [32] reported that hBD-2 is a potent and specific chemoattractant for TNF-α-treated neutrophils mainly using CCR6 *in vitro*. We deduce that the overexpression of rBD-2 may increase the release of these cytokines/chemokines (IL-1β, TNF-α, KC and MIP-2) as well as serving as chemokines for the directed chemotaxis of PMN, which explains in part the capacity of rBD-2 gene transfer to improve survival and promote bacterial clearance. However, a contribution of direct antibacterial effects of rBD-2 on *P. aeruginosa* is also possible. BD-2 exerts its antimicrobial activity by disrupting membrane integrity and function, ultimately leading to the lysis of microorganisms [45,58].

For proinflammatory cytokines/chemokines (e.g. IL-1β, TNF-α and KC), overexpression of rBD-2 led to a shift in mRNA and protein expression: an up-regulation at an earlier period (3 days p.i.), followed by a down-regulation at 7 days p.i. We hypothesize that, at an early time period, the overexpression of rBD-2 induced the expression of cytokines/chemokines (IL-1β, TNF-α, KC and MIP-2), and enhanced recruitment of PMN to the lung, which improved bacterial clearance in the lungs. By contrast, at the later time period, the expression of rBD-2 decreased, and the bacterial load dramatically decreased; thus, the expression of proinflammatory cytokines and chemokines decreased.

In summary, the present study provides evidence that the overexpression of rBD-2 ameliorates chronic *P. aeruginosa* lung infection in rats. Treatment with pCMV-rBD-2/PEI complexes resulted in a faster and stronger immune response against the bacterial infection in the early phase as judged from enhanced recruitment of PMN to the lung and higher lung IL-1β, TNF-α, KC and MIP-2 production compared to no treatment and treatment with pCMV-null/PEI complex. Subsequently, quicker bacterial clearance from the lungs, milder lung pathology, quicker recovery and increased survival rate (at 3 days p.i.) were also detected in pCMV-rBD-2/PEI treatment group compared to the untreated-infection and the pCMV-null/PEI-treated groups. Given that chronic *P. aeruginosa* lung infection complicates many chronic lung diseases, most notoriously cystic fibrosis, our findings have important clinical implications.

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