Bacteria-induced expression of the pig-derived protegrin-1 transgene specifically in the respiratory tract of mice enhances resistance to airway bacterial infection

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Bacteria-induced expression of the pig-derived protegrin-1 transgene specifically in the respiratory tract of mice enhances resistance to airway bacterial infection

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Abstract

Background: About 70% of all antibiotics produced in the world are used in the farm animal industry. The massive usage of antibiotics during farm animal production has caused rapid development of antibiotic resistance in bacteria, which poses a serious risk to human and livestock health when treating bacterial infections. Protegrin-1 (PG-1) is a potent antimicrobial peptide (AMP), initially identified in pig leukocytes with a broad-spectrum antibacterial and antiviral activity, and a low rate of inducing bacterial resistance.

Results: To develop genetic approaches for reducing the use of antibiotics in farm animal production, we produced transgenic mice carrying a bovine tracheal AMP gene promoter-controlled PG-1 transgene. The PG-1 transgene was specifically expressed in the respiratory tract of transgenic mice upon induction by bacterial infection. These PG-1 transgenic mice exhibited enhanced resistance to nasal bacterial infection as the infected transgenic mice showed a higher survival rate, lower bacterial load and milder histological severity than the infected wild-type control littermates.

Conclusions: The present study provides a promising genetic strategy to prevent airway bacterial infections in animals by bacteria-inducible tissue-specific expression of PG-1 transgene, an approach which may also be helpful for reducing the possibility of inducing bacterial resistance.

Keywords

Antimicrobial peptides, Protegrin-1, Transgenic mice, Respiratory tract

Introduction

About 70% of all antibiotics produced worldwide are used to improve the health and growth performance of farm animals [1]. In some countries, the amount of antibiotics used for farm animals accounts for approximately 80% of the nation's total consumption [2]. This massive usage of antibiotics in food animals contributed significantly to the rapid development of antibiotic resistance, which is increasingly threatening the health of humans and animals [3-5]. Therefore, alternative methods that can reduce or replace the use of antibiotics in farm animal production are needed.

Antimicrobial peptides (AMPs) are a family of small polypeptides that are naturally expressed in many organisms as innate anti-infective agents [6, 7]. AMPs usually display low toxicity to non-target organisms and have little propensity for inducing bacterial drug resistance, unlike traditional antibiotics. Additionally, AMPs also have a broad-spectrum defensive effect on various pathogens, including bacteria, fungi, viruses, and parasites [8-11]. Protegrin-1 (PG-1) is a potent AMP, originally derived from pigs, that has a broad-spectrum antibacterial and antiviral activity [12-19] and a low rate of inducing bacterial resistance [15].

In an attempt to develop new strategies for reducing antibiotic consumption in farm animal production, PG-1 was introduced as a transgene into the genome of mice which resulted in a significantly enhanced ability of these mice to fight against bacterial infections [20]. However, as the PG-1 transgene in these transgenic mice was driven by a constitutive promoter, resulting in the constant and ubiquitous expression of PG-1, development of microbial resistance would be very likely. Therefore, promoters that are inducible only upon microbial infection and are active only in susceptible tissues should be used to control AMP transgene expression in

transgenic animals. Respiratory diseases caused by airway microbial infection are the most devastating infectious diseases in the livestock industry that cause significant economic loss [21-23]. Therefore, using a respiratory tract-specific and bacterium-inducible promoter to drive AMP transgene expression could be an effective approach to improve resistance to infectious respiratory diseases in farm animals while reducing the likelihood of inducing bacterial resistance.

In this study, we generated transgenic mice that carry a PG-1 transgene controlled by the bovine tracheal antimicrobial peptide (TAP) gene promoter to test whether the bacteria-inducible and respiratory tract-specific expression of PG-1 transgene in animals can increase the animals' resistance to airway bacterial infection. PG-1 transgenic mice showed specific expression of PG-1 in their respiratory tract upon induction of bacterial infection. These transgenic mice also exhibited enhanced resistance to nasal challenge of *Actinobacillus pleuropneumoniae* (APP) bacterium, which is the causative pathogen of the highly prevalent porcine pleuropneumonia disease [24].

Materials and methods

Bacterial strain

APP serotype 1 bacteria were purchased from the China Veterinary Culture Collection Center (catalog no: CVCC259, Beijing, China). APP was cultured on tryptic soy agar (TSA) or in tryptic soy broth (TSB) (Difco Laboratories, USA) supplemented with 10 μg/ml of nicotinamide adenine dinucleotide (NAD) and 10% (v/v) filtered bovine serum at 37 °C as described previously [44].

Plasmid construction

A 890-bp DNA fragment containing the bacteria-inducible tracheal epithelial cell-specific bovine TAP promoter[45-47] and the 450-bp pig PG-1-coding sequences (GenBank Accession no: X79868.1) was synthesized by the GENEWIZ Company (Suzhou, China). This fragment was used to replace the PSP-hNGF fragment between the Age I and Asc I sites of the pmPSP-hNGF plasmid,[48] to generate the pTAP-PG-1 plasmid. The DNA sequences of pTAP-PG-1 plasmid were confirmed by sequencing.

Generation of transgenic mice

The pTAP-PG-1 plasmid and the piggyBac transposase expression plasmid pmPB were co-injected into the pronucleus of one-cell-stage mouse embryos (C57BL/6 strain), which were then transferred into the oviducts of Institute of Cancer Research (ICR) strain surrogate mothers. The recipient females were mated with vasectomized stud males of ICR strain the day before embryo transfer. Pregnant recipient mothers were allowed to deliver and raise their pups.

PCR identification of transgenic mice

Genomic DNA was isolated from tail biopsies of founder mice using a tissue DNA extraction kit (Omega, Doraville, GA, USA). The TAP-PG-1 transgene, CMV-EGFP marker gene, and the internal control gene of β-actin gene were amplified by PCR. The PCR amplification products were sequenced to confirm their identities.

Observation of EGFP expression

EGFP expression in the claw tissues of founder transgenic mice was analyzed by fluorescence microscopy. EGFP expression in newborn line 438 transgenic mice was visualized by the Living Organism's fluorescent protein observation system (Model: FBL, BLS company,

Budapest, Hungary).

QPCR analysis of transgene copy number

PG-1 transgene copy number was analyzed as previously reported [49]. A standard set of mixtures of pTAP-PG-1 plasmid DNA with WT mouse genomic DNA representing 1, 10, 100, 1000, 10,000, and 100,000 copies of PG-1 transgene per mouse genome was prepared. The mixture from each standard sample at 2 μl was used as template for qPCR measurement of threshold cycle (Ct) values. A standard curve was established based on the measured Ct values of all the standard samples and their corresponding transgene copy number. The Ct values of all transgenic founder mice's genomic DNA were also measured by qPCR following the same protocol. The Ct value of each transgenic founder mouse genomic DNA was converted into corresponding PG-1 transgene copy number based on the established standard curve.

qPCR analysis of transgene expression

Transgenic mice were injected intraperitoneally with 15 μg of LPS from Salmonella typhimurium (Catalog no. L-7261, Sigma) to induce PG-1 transgene expression. Total RNA was extracted from the collected tissues of injected transgenic mice by E.Z.N.A.^{TM} total RNA kit I (OMEGA, Doraville, GA, USA). cDNA was synthesized by PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China). The Ct values of PG-1 and internal control glyceraldehyde phosphate dehydrogenase were analyzed by qPCR via the Eco real-time PCR system (Illumina, San Diego, CA, USA) and SYBR Premix Ex Taq (Takara, Dalian, China). Relative transgene mRNA level was calculated by the $2^{-\Delta\Delta Ct}$ method. The qPCR products were sequenced to confirm their identities.

ELISA analysis of protein concentration

The PG-1 protein concentration in the tissues was analyzed by enzyme-linked immunosorbent assay (ELISA) kit for pig PG-1 (catalog no: E1705p, EIAab, Wuhan, China) by following the manufacturer's instructions. Serum IL-1β concentrations were also measured by ELISA kits (Boster Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instructions.

APP challenge and post challenge monitoring

Transgenic mice and their WT littermates from line 438 were independently raised in their own cages under the same conditions. PCR analysis was performed to identify transgenic mice and their WT littermates. All the PCR-identified transgenic and WT mice were confirmed through the observation of EGFP expression on their claw tissue. All animals were housed in a pathogen-free unit in a temperature-controlled environment with 12 h light/12 h dark cycle and allowed free access to food and water. Serological testing confirmed that all animals were free of the usual viral and bacterial pathogens before the APP challenge. APP was cultured in TSB supplemented with 10 μg/ml of NAD and 10% (v/v) filtered bovine serum at 37 °C. APP bacterial concentration (CFU/ml) was determined from the optical density value at 600 nm by the standard curve method. Mice were infected by nasal drop of 20 μl of APP. Mice were monitored every 2 h to determine their status of survival or death.

Blood parameter analysis

Blood (200–400 μl) was collected into EDTA-containing tubes from the retro-orbital plexus of surviving mice at 6 hpi of APP. Collected blood samples were stored at 4 °C and analyzed through an automatic hematology analyzer within 1 h.

154 **Bacterial load measurement**

155 Surviving mice were euthanized at 6 hpi. Tissues (approximately 10 mg to 40 mg) were
156 collected immediately after euthanization and placed in pre-weighed sterile 2-ml tubes. The
157 weight of each tissue was measured, and sterile phosphate-buffered saline (PBS) was added to
158 each tissue sample at a ratio of 10 µl of sterile PBS to 1 mg of tissue. All samples were
159 immediately homogenized with a homogenizer. The resulting tissue homogenate was spread
160 on three 100 µl TSA plates. The plates were incubated overnight at 37 °C in an atmosphere of
161 5% CO_2. Bacteria showing the characteristic APP phenotype were counted.

162 **Histological analysis**

163 Tissues were collected from the euthanized mice at 6 hpi. The tissues were gently instilled
164 with 10% buffered formalin, immersed in the same solution for fixation, embedded in paraffin,
165 sectioned, and stained with hematoxylin–eosin. The stained slides were examined under a
166 microscope.

167 **PCR identification of APP**

168 Bacterial colonies recovered from tissues of challenged transgenic and WT mice at 6 hpi
169 were isolated from the TSA plates and grown in TSB supplemented with 10 µg/ml of NAD and
170 10% (v/v) filtered bovine serum at 37 °C for 8 h. One microliter of bacteria-containing medium
171 was used as template for PCR to amplify a gene in the genome of APP. The PCR product was
172 sequenced, and the sequencing result was blasted against APP genomic DNA.

173 **Statistical analysis**

174 Chi-square test was used to determine differences in survival rate between the two groups,
175 whereas student t-test was used to compare differences in other values between the two groups.

Results

Generation of PG-1 transgenic founder mice

A pTAP-PG-1 plasmid harboring a piggyBac transposon that carries a bacteria-inducible tracheal epithelial cell-specific bovine TAP promoter-driven PG-1 gene was constructed (**Figure 1A**). This plasmid also contains a cytomegalovirus (CMV) promoter-controlled fusion selectable marker gene, which was composed of the neomycin (Neo) gene and an enhanced green fluorescence protein (EGFP) gene (**Figure 1A**). The pTAP-PG-1 plasmid was co-injected with the piggyBac transposase expression plasmid pmPB [25] into the pronuclei of mouse zygotes (C57BL/6 strain). Fifty-six pups were born following the transfer of 362 microinjected embryos into the oviducts of 12 surrogate mothers (ICR strain), and 11 of these pups were identified as transgenic founder mice (**Table 1**). All 11 transgenic founder mice carried both the PG-1 and the EGFP gene in their genomes as detected by polymerase chain reaction (PCR) (**Figure 1B**). EGFP expression, albeit at varied levels, was observed in the claw tissues of all 11 transgenic founder mice (**Figure 1C**). No abnormal behavior or phenotype was detected in these 11 transgenic founder mice.

Selection of transgenic mouse lines

Quantitative PCR (qPCR)-based transgene copy number analysis indicated that the copy number of the inserted PG-1 transgene varied from 1 to 24 in the 11 founders (**Figure 2A**). F_1 offspring from the different founder lines expressed PG-1 mRNA at various levels in the trachea and lung tissues that contain tracheal epithelial cells (**Figure 2B**). PG-1 transgene copy number was not positively correlated to its transcription level, as for example transgenic mice

198 from a high transgene copy number line (line 434) and transgenic mice from two low transgene
199 copy number lines (lines 438 and 439) expressed similar PG-1 mRNA levels (**Figures 2A and**
200 **2B**). This finding could be due to the difference in the insertion sites of the randomly integrated
201 transgene among different transgenic mouse lines. Transgenic F_1 progenies from lines 434, 435,
202 436, 443, and 444 carrying multiple copies of PG-1 transgene exhibited larger variations in
203 PG-1 mRNA expression levels than transgenic offspring from lines 438 and 439, which carried
204 only one copy of PG-1 transgene (see the error bars in **Figure 2B**). This phenomenon may have
205 resulted from the segregation of the multiple copies of independently inserted transgenes after
206 their transmission from the same line founder to its transgenic progeny. Therefore, line 438
207 transgenic mice were chosen for subsequent investigation as transgenic mice from this line
208 carried only one copy of PG-1 transgene and yet expressed a relatively high level of PG-1
209 mRNA in their respiratory tract tissues (**Figures 2A and 2B**).

210 **Characterization of transgene expression in transgenic progenies from line 438**

211 Epifluorescence expression of EGFP was observed over the whole body of newborn
212 transgenic progeny produced by mating transgenic founder 438 with wild-type (WT) mice
213 (**Figure 3A**). The PG-1 transcript levels in the trachea and lung tissues of line 438 transgenic
214 mice injected with lipopolysaccharide (LPS) were much higher than those in other tested
215 tissues, including heart, muscle, liver, brain, and skin (**Figure 3B**). This finding suggests that
216 the PG-1 transgene was selectively expressed in respiratory tract tissues by the TAP promoter.
217 Transgenic mice injected with LPS expressed a higher PG-1 mRNA level in the trachea and
218 lung tissues than transgenic mice without LPS treatment (**Figure 3B**). Hence, the TAP
219 promoter-driven PG-1 transgene in transgenic mice was inducible upon bacterial infection. The

PG-1 protein levels in the trachea and lung tissues of line 438 transgenic mice treated with LPS were 4.37 ± 0.23 and 3.01 ± 0.08 μg/g, respectively (**Figure 3C**).

PG-1 transgenic mice showed enhanced resistance to airway bacterial infection

Susceptibility testing demonstrated that nasal inoculation of APP at a dosage of 6.55×10^7 colony forming unit (CFU) per mouse resulted in a 58.3% death rate in WT mice (**Figure 4A**). Therefore, this dosage is close to the median lethal dose and was used as the nasal challenge dosage to compare the resistance to APP infection of transgenic and WT mice. After nasal infection with APP at a dosage of 6.55×10^7 CFU/mouse, typical clinical signs, such as labored breathing, ruffled hair coat, lethargy, and hunched posture, were seen after about 5 hours post infection (hpi), and death was observed at 10 hpi in infected WT mice and 14 hpi in infected transgenic mice. The death rate of APP-challenged PG-1 transgenic mice was substantially lower than that of their infected WT littermates from 14 hpi to 192 hpi, and the overall mortality rates of transgenic and WT groups were 20.83% and 65.22%, respectively, during the monitoring period of 192 hpi (**Figure 4B**).

At 6 hpi, no APP bacteria were recovered from the liver and spleen of surviving WT and transgenic mice challenged by nasal inoculation, but the number of APP bacteria recovered from the lung and trachea of infected WT mice was higher than that of their transgenic littermates (**Figure 5A**). Severe hemorrhage in the lung was observed in infected WT mice but not in infected transgenic mice or non-infected negative control (NC) mice at 6 hpi (**Figure 5B**). Infected WT and transgenic mice showed normal features in the liver and spleen sections. Infected transgenic mice exhibited mild neutrophilic infiltrate while infected WT mice exhibited severe neutrophilic infiltrate and congestion in the lung sections compared with NC

mice at 6 hpi (**Figure 5B**). Congestion was observed in the tracheal sections of WT mice but not in transgenic mice at 6 hpi (**Figure 5B**). The serum concentration of IL-1β, which is a PG-1-regulated cytokine,[26] was higher in infected transgenic mice than in NC mice and challenged WT mice at 6 hpi (**Figure 5C**).

An analysis of blood, collected at 6 hpi indicated that mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width-coefficient of variation (RDW-CV) were similar between WT and transgenic mice, but these three blood indexes were lower in infected WT and transgenic mice than those in NC mice (**Table 2**). All other blood parameters measured at 6 hpi were similar among the three groups of mice except the neutrophil number (Neu#) in transgenic mice was higher than those in NC and WT mice (**Table 2**).

Genomic DNA of recovered bacteria was subjected to PCR amplification for an APP bacteria-specific gene (APXIVA) followed by sequencing of the PCR products to confirm that the bacteria recovered from the lung, liver, spleen, and trachea tissues of challenged transgenic and WT mice were derived from the inoculated APP. Sequencing results confirmed that the recovered bacteria were derived from the inoculated APP (data not shown).

Discussion

In this study, we produced transgenic mice carrying a PG-1 transgene under the control of a bacteria-inducible and respiratory tract tissue-specific promoter. These PG-1 transgenic mice were healthy and predominately fertile. In comparison with their WT littermates, they exhibited enhanced resistance to APP bacterial infection as evidenced by their higher survival rate, lower

tissue bacterial load, and milder histological severity after APP inoculation. These results were similar to those reported in the transgenic mice that exhibited ubiquitous expression of PG-1 under the control of a CMV promoter [20]. Yet, with the transgenic mice generated in the present study the likelihood of inducing bacterial resistance to antibiotics is further reduced as PG-1 expression is restricted to the respiratory tract only upon induction by bacterial infection. Expressions of many AMPs in their native host animals are induced via microbial infection and are tissue-specific [27-29]. This phenomenon could be the evolution outcome to minimize the chances of inducing microbial resistance. Therefore, bacteria-inducible and tissue-specific promoters should be used to direct the AMP transgene expression in transgenic animals if AMP genes are introduced into the genome of animals to control bacterial infectious diseases.

Some AMPs not only directly target and destroy bacteria, but also indirectly inhibit bacterial infection by regulating the host immune responses via several different approaches, such as enhancing proinflammatory cytokine secretion and recruiting immune cells to the infection sites [30-34]. Specifically, PG-1 has direct bacteria-killing activities [35-40] and also participates in immune modulation by stimulating the rapid and efficient release of mature IL-1β from monocytes [26]. Our findings are in agreement with these reports, as serum IL-1β levels were higher in our transgenic mice than those in WT and NC mice at 6 hpi. In addition, our observation that PG-1 transgenic mice have higher numbers of neutrophils than WT or NC mice at 6 hpi is consistent with previous reports of PG-1 to exert immune modulatory effects via promoting neutrophil migration to the infection sites to reduce bacterial colonization [20].

The expression of the TAP promoter-controlled PG-1 in transgenic mice increased the mice's resistance to nasal APP infection. However, whether its expression can enhance the

ability of transgenic animals against other important airway bacterial infections remains to be determined. Therefore, future studies will have to assess if PG-1 transgenic mice produced in this study are also resistant to other respiratory tract pathogens, such as A. suis [41, 42] and Streptococcus suis [43], which also cause serious contagious diseases in farm animals. In addition, the PG-1 transgenic mice created in the present study provide a valuable animal model for investigating the *in vivo* function and mechanism of action of PG-1.

Conclusions

Expression of PG-1 under the control of the TAP promoter enhanced the resistance of transgenic mice to airway bacterial infection. The bacteria-inducible and respiratory tract-specific expression of PG-1 transgene is a promising genetic strategy to control airway bacterial infections in animals. This strategy may also be helpful for reducing the chance of inducing bacterial resistance.

Abbreviations

AMP: antimicrobial peptide

APP: *Actinobacillus pleuropneumoniae*

CFU: colony forming unit

CMV promoter: cytomegalovirus promoter

EGFP: enhanced green fluorescence protein

hpi: hours post infection

LPS: lipopolysaccharide

NC: negative control

PG-1: Protegrin-1

TAP promoter: bovine tracheal antimicrobial peptide gene promoter

WT: wild-type

Declarations

Ethics approval and consent to participate

This study was conducted in Guangdong Province of China in strict accordance with ‘‘Guidelines with Respect to Caring for Laboratory Animals’’ issued by the Ministry of Science and Technology of China. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of South China Agricultural University. All efforts were made to minimize animal suffering.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Fang Zeng, Zicong Li and Zhenfang Wu designed the experiments. Fang Zeng, Chengcheng Zhao, Xiao Wu, Rui Dong, Guoling Li, Qingchun Zhu, Enqin Zheng and Dewu Liu performed the experiments. Fang Zeng, Jinzeng Yang, Stefan Moisyadi, Johann Urschitz, Zicong Li and Zhenfang Wu wrote the manuscript.

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Tables:

Table 1. Summary of the production of PG-1 transgenic mice by pronuclear microinjection

No. of injected embryos	No. of transferred injected embryos	No. of surrogate mothers	No. of born mice	No. of transgenic mice
400	362	12	56	11

Table 2. Comparison of routine blood parameters among non-infected NC, APP-infected WT, and APP-infected transgenic live mice at 6 hpi.

	NC (n=6)	WT (n=4)	TG (n=4)
WBC# (10^9/L)	3.77±0.83	3.52±0.64	3.51±0.81
Bas# (10^9/L)	0.022±0.01	0.01±0.000	0.018±0.015
Neu# (10^9/L)	0.89 ± 0.43^{A}	0.57 ± 0.19^{A}	1.13 ± 0.26^{B}
Eos# (10^9/L)	0.008±0.009	0.005±0.006	0.015±0.006
Lymph# (10^9/L)	2.84±0.81	2.77±0.41	1.95±0.67
Bas% (%)	0.62±0.26	0.58±0.25	0.65±0.27
Neu% (%)	23.87±12.46	28.17±9.70	36.85±8.36
Eos% (%)	0.23±0.19	0.30±0.25	0.50±0.16
Lymph% (%)	75.23±12.52	70.95±9.68	61.95±8.58
RBC (10^{12}/L)	8.45±1.54	9.17±1.30	9.56±0.24
HGB (g/L)	128.67±15.01	142.75±17.89	147.75±6.18
MCV (fL)	49.57 ± 0.27^{A}	48.45 ± 0.95^{B}	47.98 ± 0.46^{B}
MCH (pg)	15.20±0.34	15.65±0.42	15.43±0.34

MCHC (g/L)	306.50±7.04^{A}	322.50±2.38^{B}	321.75±4.99^{B}
RDW-CV (%)	13.15±0.50^{A}	12.25±0.60^{B}	11.83±0.10^{B}
RDW-SD (fL)	25.85±0.87	23.58±0.99	22.75±0.13
HCT (%)	41.92±7.64	44.35±5.61	45.88±1.50
PLT (10^9/L)	703.17±212.09	620.00±214.11	747.75±194.51
MPV (fL)	6.38±0.89	5.68±0.36	5.83±0.43
PDW (%)	15.10±0.14	14.97±0.13	15.05±0.58
PCT (%)	0.43±0.09	0.35±0.11	0.43±0.10

WBC#, white blood cell number; Bas#, basophil number; Neu#, neutrophil number; Eos#, eosinophil number; Lymph#, lymphocyte number; Bas%, percentage of basophils; Neu%, percentage of neutrophils; Eos%, percentage of eosinophils; Lymph%, percentage of lymphocytes; RBC, red blood cell number; HGB, hemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW-CV, red cell distribution width-coefficient of variation; RDW-SD, red cell distribution width-standard deviation; HCT, hematocrit; PLT, platelet number; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit. Values in the same row labelled with different superscript are significantly different at $P < 0.05$.

Figures:

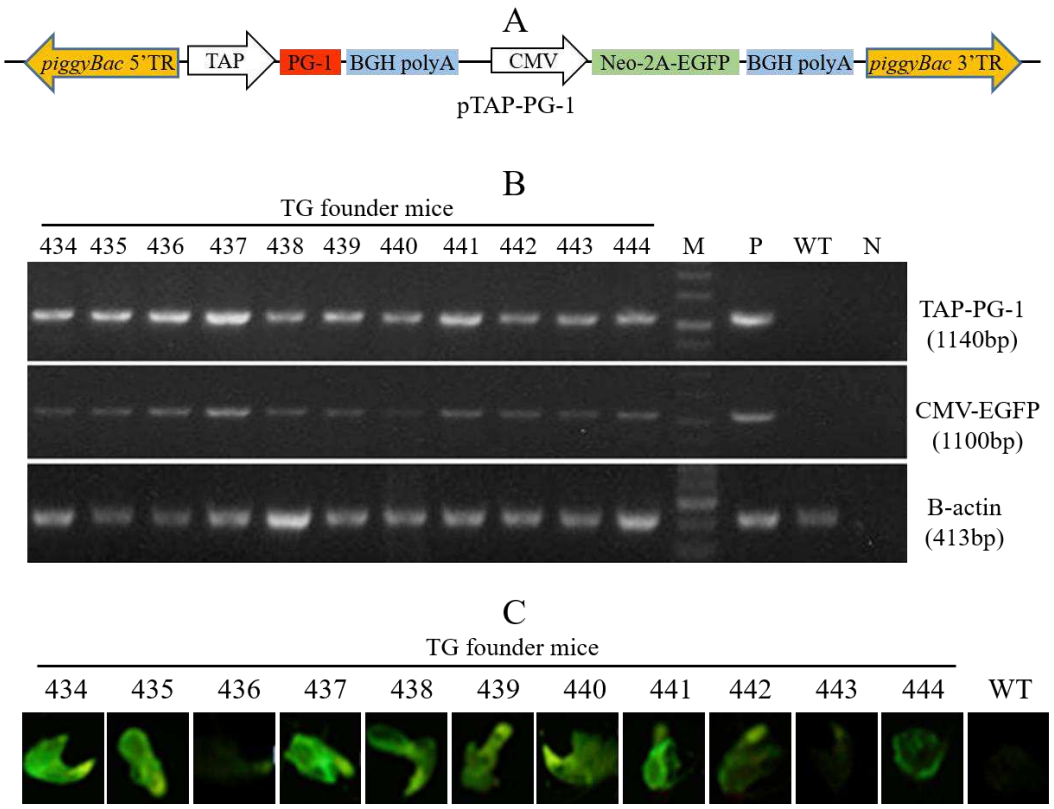
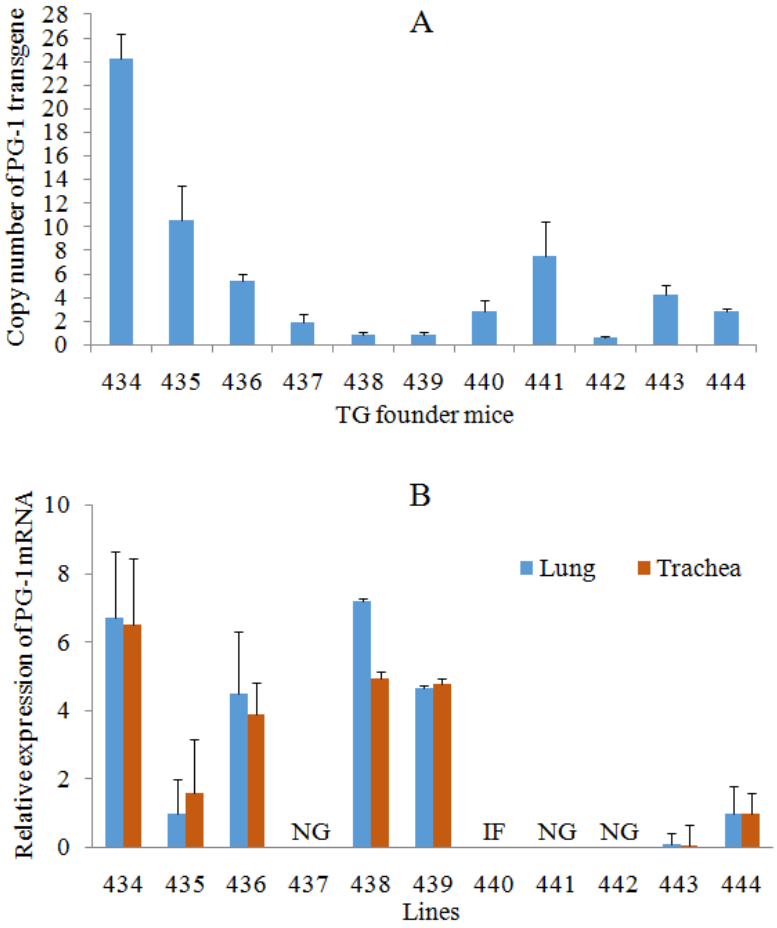


Figure 1. Generation of PG-1 transgenic mice. **A**. Map of the constructed pTAP-PG-1 plasmid. The piggyBac 5′ and 3′ TRs, the piggyBac transposon 5′ and 3′ terminal repeat elements; TAP, the bovine trachea antimicrobial peptide gene promoter; PG-1, the pig-derived antimicrobial peptide protegrin-1 gene; BGH polyA, bovine growth hormone signal; CMV, cytomegalovirus promoter; Neo, the neomycin gene; 2A, the 2A peptide linker; EGFP, the enhanced green fluorescence protein gene. **B**. PCR identification of PG-1 transgenic founder mice. M, marker; P, positive control using pTAP-PG-1 plasmid as template for PCR; WT, wild-type mice; N, negative control using water as template for PCR. β-Actin was amplified as internal control. **C.** EGFP expression in the claw tissues of PG-1 transgenic founder mice. TG, transgenic mice. WT, wild-type mice.



496

497 **Figure 2**. Selection of transgenic mouse lines. **A**. Analysis of PG-1 transgene copy number in
498 transgenic founder mice. Data shown are the means ± SEMs from triplicate experiments. **B**.
499 Comparison of PG-1 transgene expression level in the respiratory tract tissues among different
500 transgenic mouse lines. Four to six transgenic mice were analyzed for each line, and data shown
501 are the means ± SEMs. NG, no germline transmission of transgene; IF, infertile.

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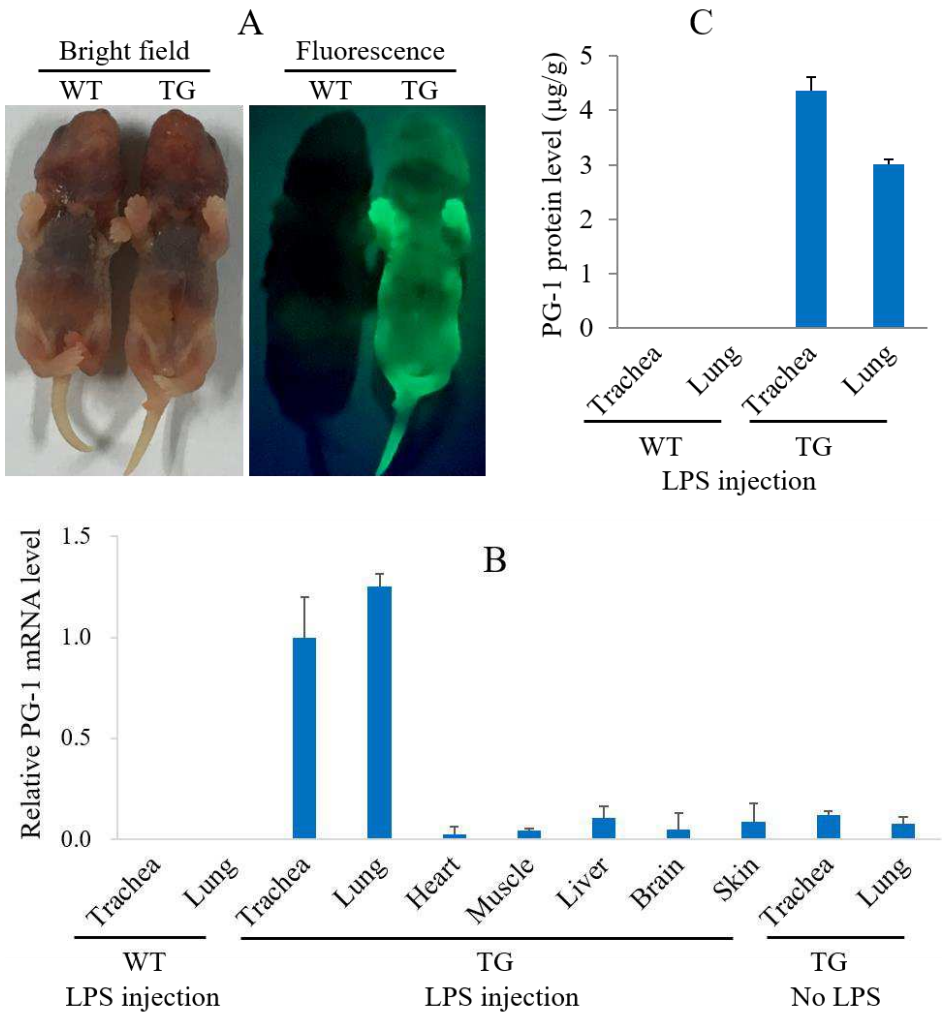

503

504 **Figure 3**. Characterization of transgene expression in transgenic mice from line 438. **A**.
505 Expression of EGFP marker gene in transgenic offspring from line 438. **B**. Relative expression
506 levels of PG-1 mRNA in the trachea, lung, heart, muscle, liver, brain, and skin tissues of line
507 438 transgenic mice with or without LPS treatment. **C**. PG-1 protein levels in the trachea and
508 lung tissues of line 438 transgenic mice injected with LPS. Four transgenic mice were analyzed
509 in B and C, and data shown are means ± SEMs.

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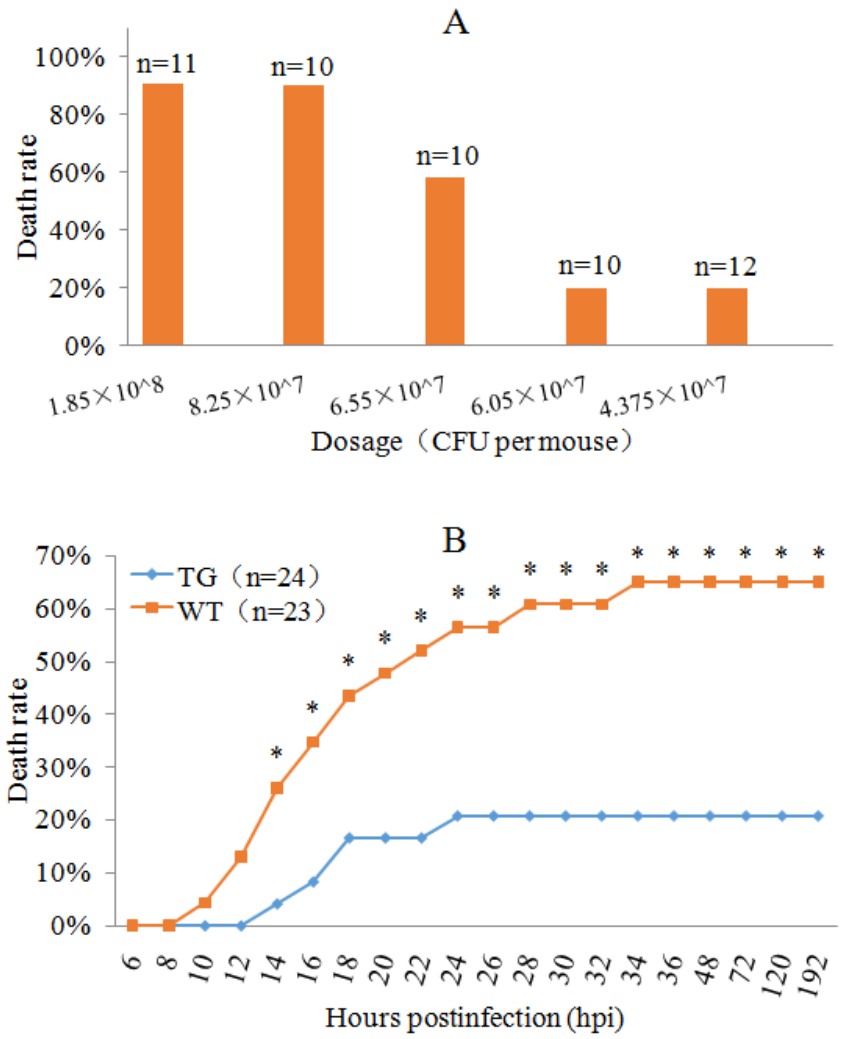

511

512 **Figure 4**. Analysis of resistance of PG-1 transgenic mice to nasal APP infection. **A**. Evaluation
513 of susceptibility of WT mice to APP infection by nasal inoculation of the different APP dosages.
514 Infected mice were monitored for 8 days (192 h) to calculate death rate. **B**. Comparison of
515 death rate between line 438 transgenic mice and their WT littermates after nasal inoculation of
516 APP at 6.555×10^7 CFU per mouse. * mean difference between two groups reaches level of
517 significance at $P < 0.05$.

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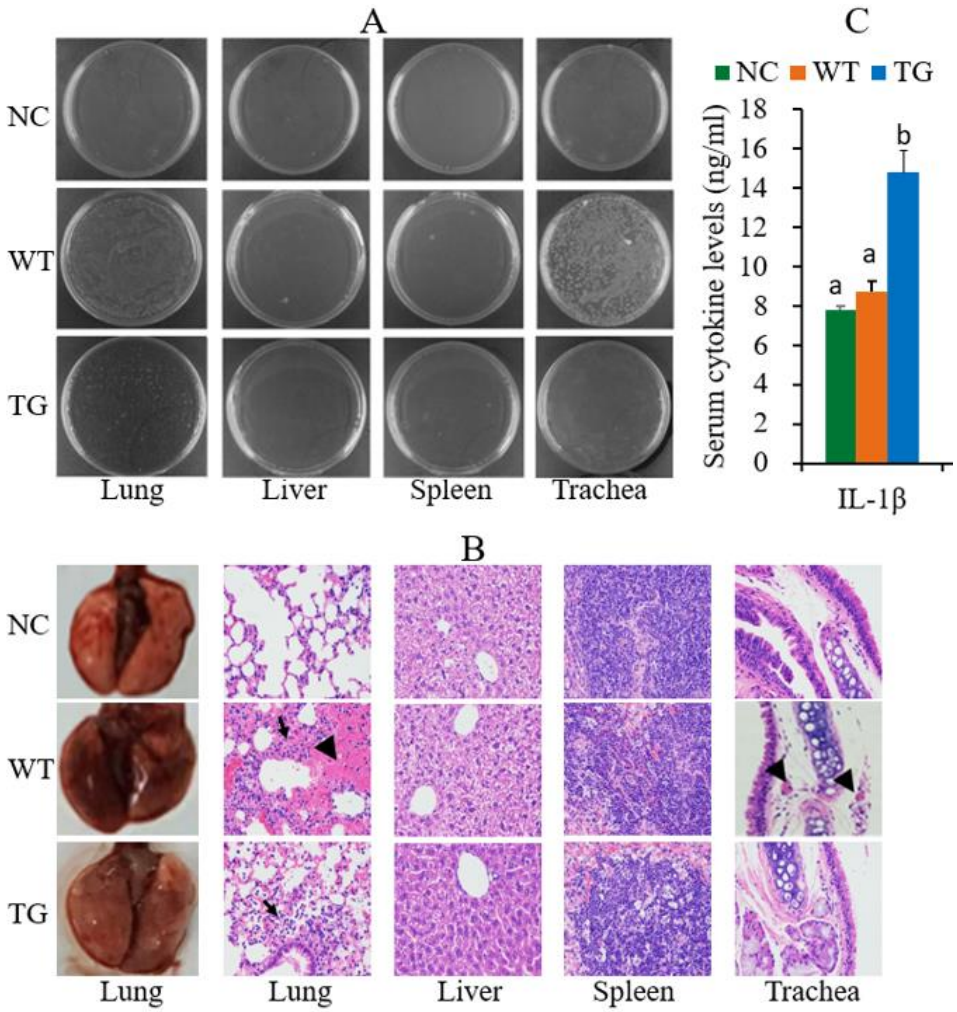

519

520 **Figure 5**. Comparison of APP bacterial load, histological features, and serum cytokine
521 concentrations among surviving transgenic (n = 4) and WT (n = 4) mice at 6 hpi and non-
522 infected NC mice (n = 3). **A**. Representative culture plates of APP colonies recovered from
523 homogenized tissues of surviving transgenic and WT mice at 6 hpi and non-challenged NC
524 mice. **B**. Histological analysis of surviving transgenic and WT mice at 6 hpi and non-
525 challenged NC mice. Representative sections are shown. Lung and trachea sections show that
526 WT mice have severe focal congestion (indicated by arrow head), which was not found in the
527 lungs of NC mice and challenged transgenic mice. Lung sections indicate that the alveolar
528 spaces are clear in NC mice but have mild and severe neutrophilic infiltrates (indicated by

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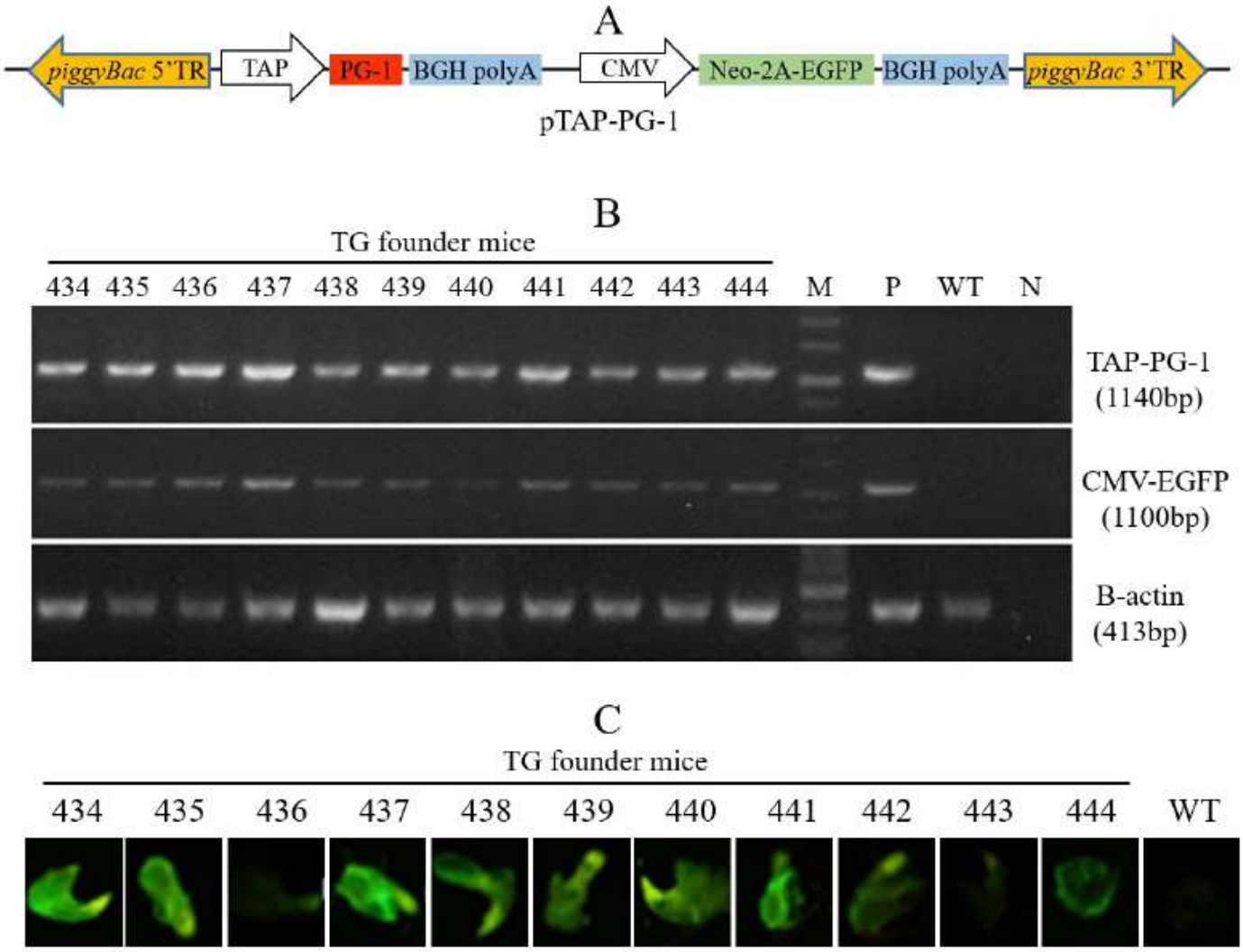
Figures



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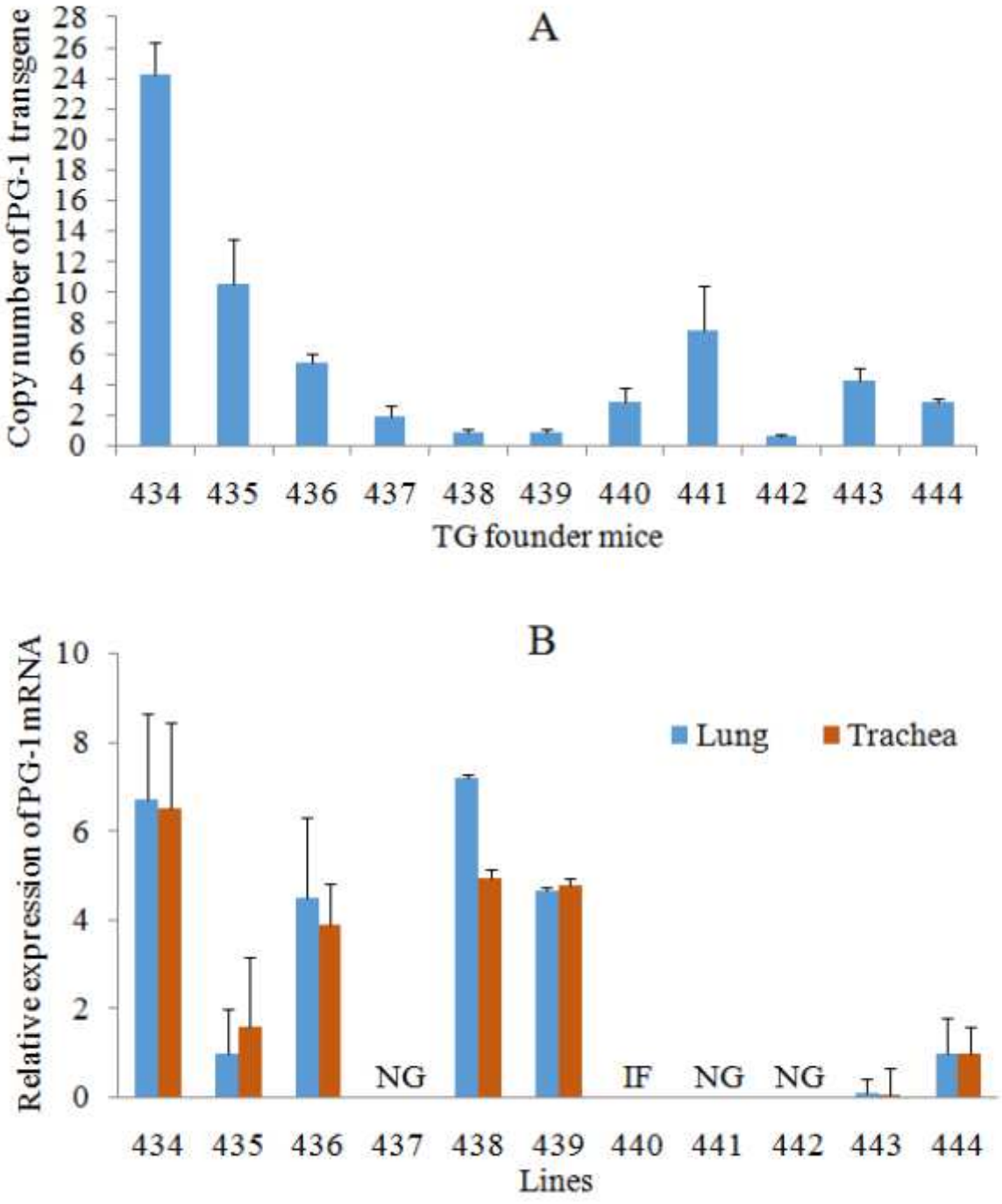

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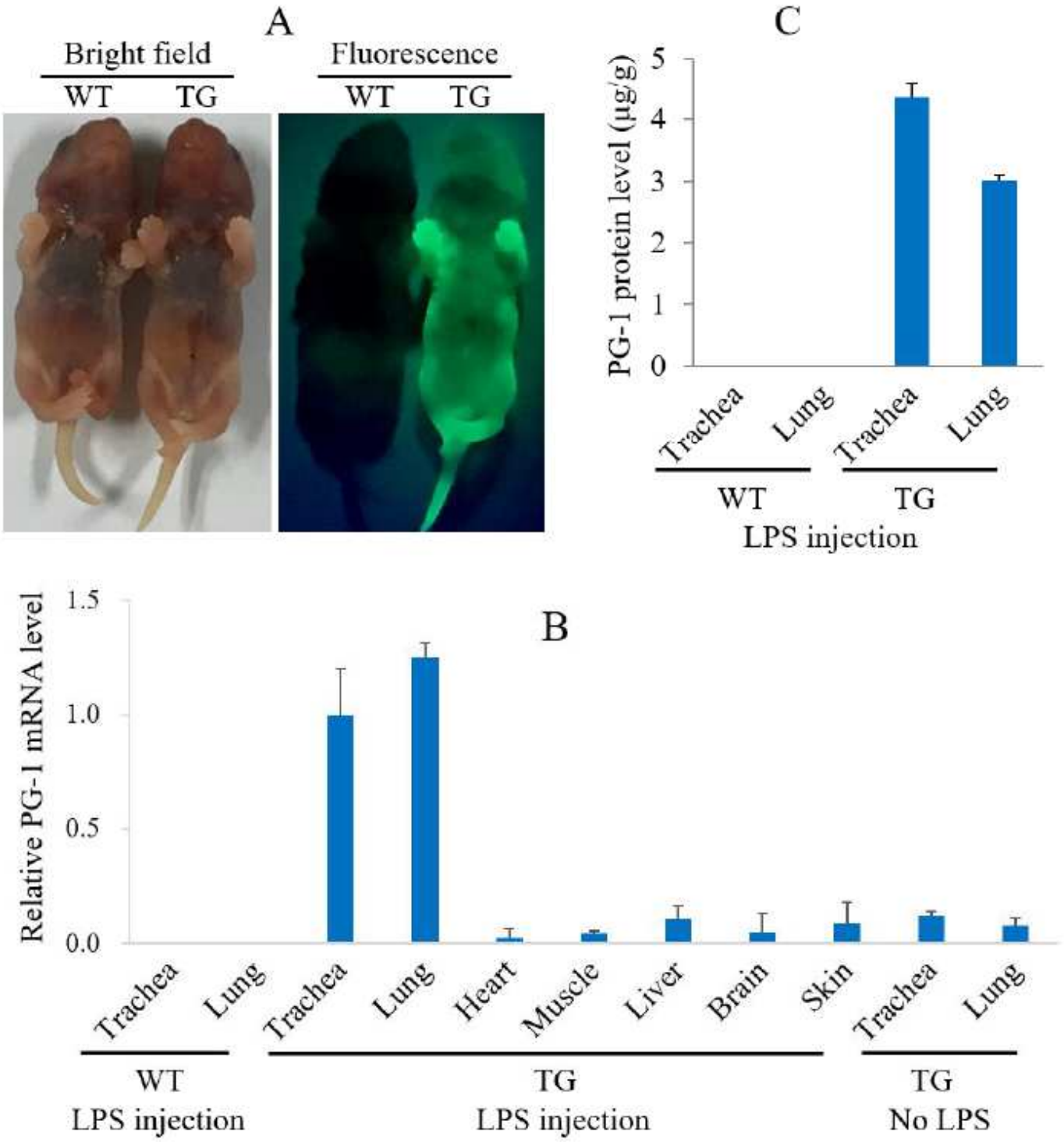

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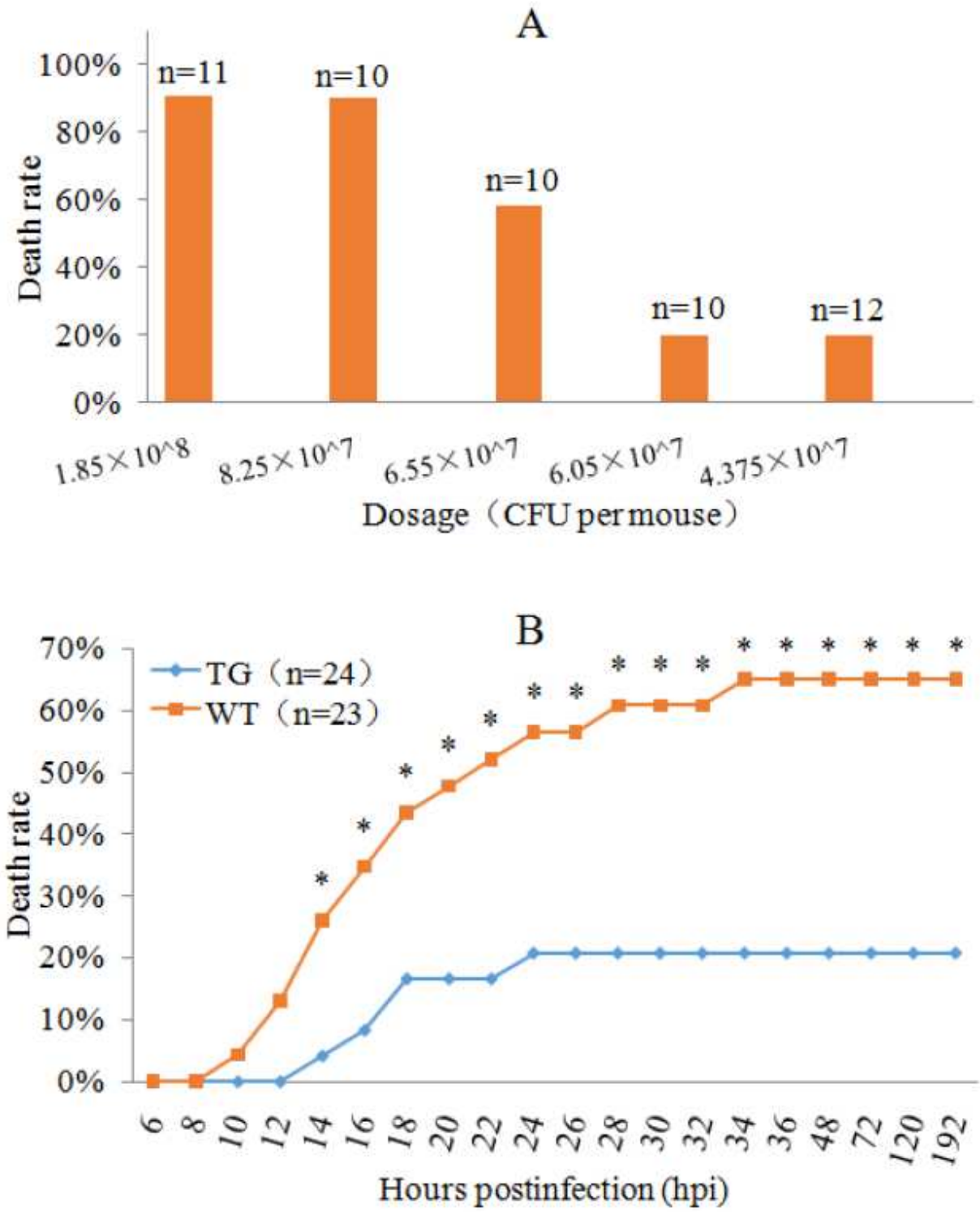

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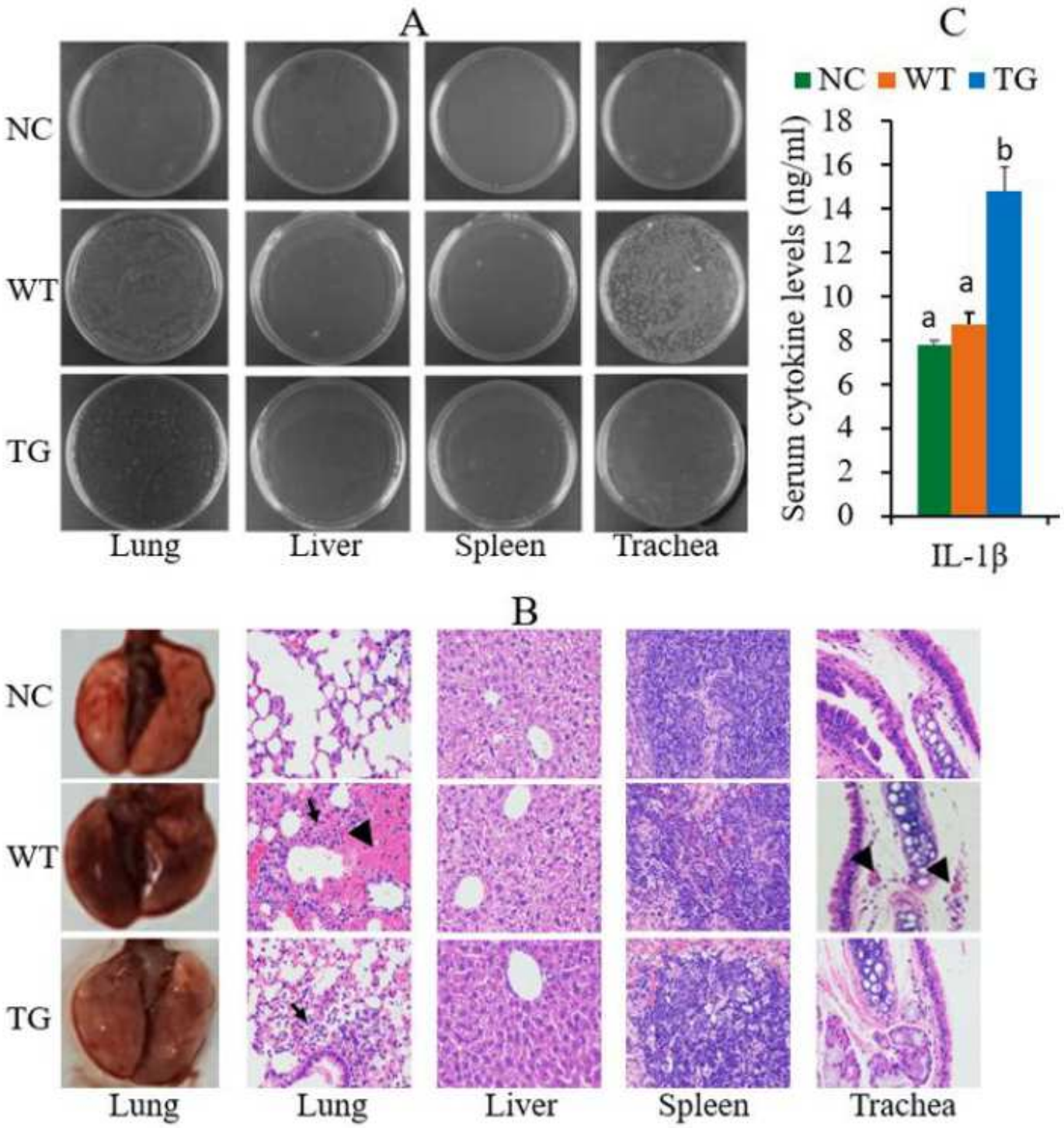

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