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Cover Letter

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February 20th, 2020

JoVE
Dr. Vineeta Bajaj

Re: Manuscript Number: JoVE61163-R2

Title: **Cigarette smoke modulates *Pseudomonas* infection in lung epithelial cells**

Dear Dr. Bajaj,

Submitted is a revised manuscript (#: JoVE61163-R2) entitled " **Cigarette smoke modulates *Pseudomonas* infection in lung epithelial cells"** for consideration for publication in the ***JoVE***. This original research manuscript has not been published nor is it under consideration for publication elsewhere.

We thank the editor for the helpful comments that improve the manuscript. We have followed the editor's comments substantially revised the manuscript. We have added new Figures (new Figure 3, 4, 5, 6, and 9) to present data on human primary cells (new Figure 3), cell viability (new Figure 4, 5, 6), and fluorescent image (new Figure 9). We have made other changes as requested and carefully re-written the manuscript (please see the point by point response).

We hope this revised manuscript is adequate and acceptable and look forward to your editorial decision.

With respect and gratitude,

Chunbin Zou, M.D./Ph.D.
Associate Professor of Medicine
Acute Lung Injury Center of Excellence
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TITLE:

Studying Effects of Cigarette Smoke on *Pseudomonas* Infection in Lung Epithelial Cells

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KEYWORDS:

cigarette smoke exposure, cigarette smoke extract, *Pseudomonas,* infection, bacterial load, lung epithelial cell, lung injury

SUMMARY:

Described here is a protocol to study how cigarette smoke extract affects bacterial colonization in lung epithelial cells.

ABSTRACT:

Cigarette smoking is the major etiological cause for lung emphysema and chronic obstructive pulmonary disease (COPD). Cigarette smoking also promotes susceptibility to bacterial infections in the respiratory system. However, the effects of cigarette smoking on bacterial infections in human lung epithelial cells have yet to be thoroughly studied. Described here is a detailed protocol for the preparation of cigarette smoking extracts (CSE), treatment of human lung epithelial cells with CSE, and bacterial infection and infection determination. CSE was prepared with a conventional method. Lung epithelial cells were treated with 4% CSE for 3 h. CSE-treated cells were, then, infected with *Pseudomonas* at a multiplicity of infection (MOI) of 10. Bacterial loads of the cells were determined by three different methods. The results showed that CSE increased *Pseudomonas* load in lung epithelial cells. This protocol, therefore, provides a simple and reproducible approach to study the effect of cigarette smoke on bacterial infections in lung epithelial cells.

INTRODUCTION:

Cigarette smoking affects the public health of millions of people worldwide. Many deleterious diseases, including lung cancer and chronic obstructive pulmonary disease (COPD), are reported to be related to cigarette smoking[1,2]. Cigarette smoking increases susceptibility to acute microbial infections in the respiratory system[3-5]. Furthermore, mounting evidence proves that cigarette

smoking enhances the pathogenesis of many chronic disorders[6-8]. For instance, cigarette smoking may increase viral or bacterial infections that cause COPD exacerbation[9]. Among the bacterial pathogens that etiologically contribute to acute exacerbation of COPD, an opportunistic gram-negative bacillus pathogen, *Pseudomonas aeruginosa*, causes infections that correlate with poor prognoses and higher mortalities[10,11]. COPD exacerbation worsens the disease by accelerating pathological progression. There are no effective therapies against COPD exacerbation except for the antisymptomatic management[12]. COPD exacerbation promotes patient mortality, decreases quality of life, and increases economic burden on society[13].

The respiratory airway is an open system, continuously subjected to various microbial pathogens present externally. Opportunistic bacterial pathogens are usually detected in the upper airways but sometimes are observed in the lower airways[14,15]. In animal models *P. aeruginosa* can be detected in alveolar sacs as soon as 1 h after infection[16]. As a major defense mechanism, immune cells such as macrophages or neutrophils eliminate the bacteria in the airways. Lung epithelial cells, as the first physiological barrier, perform a unique role in the host defense against microbial infections. Lung epithelial cells may regulate microbial invasion, colonization, or replication independent of immune cells[17]. Some molecules found in epithelial cells, including PPARγ, exert antibacterial functions, thereby regulating bacterial colonization and replication in lung epithelial cells[18]. Cigarette smoking may alter the molecules and impair normal defense function in lung epithelial cells[19,20]. Recent studies reported direct exposure of cigarette smoke to lung epithelial cells using robot smoking apparatus[21,22]. Exposure to smoke can be performed in other ways, however, including application of CSE. Preparation of CSE is a reproducible approach with potential applications in other cell types, including vascular endothelial cells that are indirectly exposed to cigarette smoke.

This report describes a protocol to generate cigarette smoke extract to alter bacterial load in lung epithelial cells. CSE increases the bacterial load of *P. aeruginosa*, and it may contribute to the recurrence of bacterial infections usually seen in COPD exacerbation. A conventional method is used for the preparation of CSE. Lung epithelial cells, at their exponential growth stage, are treated with 4% CSE for 3 h. Alternatively, monolayer-cultured lung epithelial cells can be directly exposed to cigarette smoke in an air-liquid interface. CSE-treated cells are then challenged with *Pseudomonas* at a multiplicity of infection (MOI) of 10. The bacteria are propagated at a particular shaking speed to ensure the morphology of their flagella remains intact to retain their full invasive capacity. Gentamycin is employed to kill the bacteria left in the culture medium, thereby reducing the potential contamination during the subsequent determination of the bacterial load. The protocol also uses GFP-labeled *Pseudomonas*, which has been utilized as a powerful tool in studying *Pseudomonas* infection in different models. A representative strain is *P. fluorescens Migula*[23]. The degree of infection or bacterial load after CSE treatment is determined in three ways: the drop plate method with colony counting, quantitative PCR using *Pseudomonas* 16S rRNA-specific primers, or flow cytometry in cells infected with fluorescent *Pseudomonas*. This protocol is a simple and reproducible approach to study the effect of cigarette smoke on bacterial infections in lung epithelial cells.

PROTOCOL:

1. 100% CSE preparation

1.1. Draw 10 mL of serum-free cell culture media (DMEM/F12 for BEAS-2B cells; airway epithelial cell basal medium for HSAEC cells) into a 60 mL syringe.

1.2. Reversely attach an appropriately trimmed 1 mL pipette tip to the nozzle of the syringe as an adapter to hold the cigarette (3R4F).

1.3. Remove the filter of the cigarette. Attach a cigarette to the tip adaptor and combust the cigarette.

1.4. Draw 40 mL of smoke-containing air into 10 mL of serum-free media. Mix the smoke with the medium by vigorously shaking (30 s per draw).

1.6. Repeat step 1.4 about 11x in ~7 min until the cigarette is completely burned out.

1.7. Filter the 10 mL of smoked media with a 0.22 µm filter to exclude any microorganisms and insoluble particles. Transfer to a closed sterile tube. Prepare the 100% CSE no more than 30 min before the subsequent assay.

2. *Pseudomonas* culture

2.1. Inoculate frozen *P. aeruginosa* (strain PAO1) or *P. fluorescens Migula* (strain PAO143) into a Tryptic Soy Broth (TSB) agar plate for overnight culture at 37 °C.

NOTE: To obtain enough bacteria for culturing, spread as much bacteria onto the TSB agar plate as possible.

2.2. Collect a bacterial smear and incubate in 20 mL of TSB with 5% of glycerol as the carbon source.

2.3. Shake the bacterial suspension in a 37 °C incubator at 200 rpm for 1 h until the OD_{600} value = 0.6.

CAUTION: Do not let the shaking speed exceed 200 rpm. Higher shaking speeds may damage the morphology of the bacterial flagella and impact the bacterial invasion into lung epithelia. Likewise, limit the shaking time to 1 h to obtain highly invasive bacteria. Measure the OD_{600} value to estimate the number of bacteria. An OD_{600} = 1 corresponds to ~10^9 colony forming units (CFU)/mL.

3. Human lung epithelial cell culture and CSE treatment

3.1. Culture human BEAS-2B cells in HITES medium (500 mL of DMEM/F12, 2.5 mg insulin, 2.5 mg transferrin, 2.5 mg sodium selenite, 2.5 mg transferrin, 10 µM hydrocortisone, 10 µM β-estradiol, 10 mM HEPES, and 2 mM L-glutamine) supplemented with 10% fetal bovine serum (FBS) as previously described[24].

3.2. Culture human primary small airway epithelial cells (HSAEC) in the airway epithelial cell culture medium (500 mL of Airway Cell Basal Medium, 500 µg/mL HSA, 0.6 µM linoleic acid, 0.6 µg/mL lecithin, 6 mM L-glutamine, 0.4% extract P, 1.0 µM epinephrine, 5 µg/mL transferrin, 10 nM T3, 1 µg/mL hydrocortisone, rh EGF 5 ng/mL, and 5 µg/mL rh insulin). Incubate the cells at 37 °C in 5% CO_2.

3.3. Dissociate the cells with 1 mL of 0.25% trypsin for 5 min until the cells completely detach from the bottom of the plate.

3.4. Add 10 mL of complete HITES medium to neutralize trypsin and collect the cells in a 15 mL tube. Centrifuge at 4 °C at 300 x *g* for 5 min.

CAUTION: Carefully monitor the time for trypsin digestion by microscopy, because overdigestion may cause cell death.

3.5. Discard the supernatant and resuspend the cells in 2 mL of HITES medium with 10% FBS.

3.6. Pipette 10 µL of the above cell suspension onto the plate and insert it into an automated cell counter to obtain the concentration in cells/mL.

3.7. Plate BEAS-2B cells at a concentration of 3×10^5 cells/mL into 6 well plates in a total volume of 2 mL in HITES medium supplemented with 10% of FBS for overnight culture.

3.8. Treat the cells at approximately 80% confluency, or 5×10^5 cells/mL, with 4% CSE for 3 h. Before CSE treatment, change the medium with HITES medium with 1% of FBS.

4. Bacterial infection

4.1. Add *P. aeruginosa* or *P. fluorescens Migula* (~1×10^7 CFU/mL) to each well of the CSE-treated cells and incubate for 1 h at 37 °C in 5% CO_2.

4.2. Aspirate the supernatants and replace with 2 mL of fresh HITES medium to treat with 4% CSE and 100 µg/mL gentamicin.

NOTE: Gentamicin is used because it is unable to penetrate human lung epithelial cellular membranes. Thus, it can kill all the bacteria in the medium but not those that invaded the lung epithelial cells.

4.3. After 1 h of CSE/gentamicin treatment at 37 °C in 5% CO_2, aspirate the supernatants and wash the cells 3x with PBS for the subsequent bacterial concentration determination.

NOTE: To confirm the internalized bacteria, cells infected with GFP-labeled *P. fluorescens Migula* were observed under fluorescent microscopy.

5. Determination of bacterial concentration using the drop plate method

5.1. To determine bacterial load in infected cells with the drop plate method, wash the gentamycin-treated cells 2x with 2 mL of cold PBS.

5.2. Add 1 mL of cell lysis buffer (0.5% triton X-100 in PBS) to each well.

5.3. Dilute the cell lysates containing the internalized bacteria in a gradient (1:10, 1:100, 1:1,000, and 1:10,000) for the following inoculation to the TSB agar plate.

5.4. After 16 h of incubation, obtain the results of CFU by counting the bacterial colonies.

6. RT-qPCR detection of bacterial 16S rRNA

6.1. Treat the *Pseudomonas*-infected lung epithelial cells (~1 x 10^6 cells/mL) with gentamycin as described above. Aspirate the medium and wash the cells 2x with 2 mL of cold PBS.

6.2. Add 0.35 mL of the guanidium thiocyanate lysis buffer per well of a 6 well plate. Collect the cells with a cell scraper. Pipette the lysate into a microcentrifuge tube and mix gently with the pipette.

6.3 Add the same volume (0.35 mL) of freshly prepared 70% ethanol into the lysate and mix well. Transfer all samples to a spin column placed in a 2 mL collection tube. Centrifuge at 10,000 x *g* for 30 s at 20–25 °C. Then, discard the buffer in the collection tube.

6.4. Wash the column with 0.7 mL of wash buffer 1. Centrifuge the column at 10,000 x *g* for 30 s. Wash the column 2x with 0.5 mL of buffer to wash the membrane-bound RNA. Repeat the centrifugation at 10,000 x *g* for 2 min.

6.5. Place the column into a new 1.5 mL collection tube. Add 30–50 µL RNase-free water. Centrifuge at 10,000 x *g* for 1 min. Collect the flow-through and measure the RNA concentration.

6.6. Perform a reverse transcription reaction according to the manufacturer's protocol. Mix 1 µg of total RNA with 10 µL of reaction buffer, 1 µL of reverse transcriptase, and RNase-free water for a 20 µL reaction. Conduct the reverse transcription reaction at 37 °C for 1 h and then 95 °C for 5 min.

6.7. Mix together the cDNA templates (1 µL of each reverse transcription reaction above), 5 µL of the Master Mix containing SYBR dye, 1 µL of each 200 nM specific primers, and water in a 20 µL mixture for the following PCR analysis, according to the manufacturer's recommendations.

NOTE: The following are the primers targeting the 16S rRNA of *P. aeruginosa*: forward 5' -CAAAACTACTGAGCTAGAGTACG-3'; reverse 5'-TAAGATCTCAAG GATCCCAACGGC-3'. *GAPDH* was used as a loading control with the following primers: forward: 5'-GGCATGGACTGGTCATGA-3'; reverse: 5'-TTCACCATGGAGAAGGC-3'.

6.8. Use the comparative CT method to determine the expression.

7. Detection of fluorescent *Pseudomonas* with flow cytometry

7.1 Treat the above fluorescent *Pseudomonas*-infected lung epithelial cells (~1 x 10^6 cells/mL) with gentamycin as previously described. Aspirate the medium and wash the cells 2x with 2 mL of cold PBS.

7.2. Analyze the samples with a flow cytometer at a wavelength of 509 nm for the detection of GFP. Terminate each read at 100,000 counts. Analyze the acquired data with related software.

REPRESENTATIVE RESULTS:

A diagram is used to illustrate the protocol in **Figure 1**. Lung epithelial BEAS-2B cells were treated with CSE and challenged with *Pseudomonas*. *Pseudomonas* in the culture medium were killed by the added gentamycin and the cells were subjected to the drop plate assay, RT-qPCR detection of *Pseudomonas* ribosome 16S RNA, and flow cytometry. Compared with control, CSE treatment substantially increased bacterial infection in drop plate methods (**Figure 2**). Correspondingly, CSE affected bacterial load in HSAEC (**Figure 3**). Cell viability did not change considerably after 3 h of 4% CSE treatment, in 1 h of *Pseudomonas* infection, or in 1 h of gentamycin treatment (**Figure 4**, **Figure 5**, **Figure 6**). The 16S rRNA-target RT-qPCR method (**Figure 7**) and flow cytometry (**Figure 8**) demonstrated similar results. Results from fluorescent microscopy showed that GFP-labeled bacteria colocalized with BEAS-2B cells in a *P. fluorescens Migula* infection experiment (**Figure 9**). These results suggest that cigarette smoking increased *Pseudomonas* load in BEAS-2B cells.

FIGURE LEGENDS:

Figure 1: Schematic presentation of the protocol to study cigarette smoke effects on *Pseudomonas* infection in lung epithelial cells. Lung epithelial cells grown in cell culture inserts or conventional culture plates or lung organoids were exposed to cigarette smoke for 16 min via smoking robot or treated with prepared 4% CSE for 3 h. These cells were then infected with *P. aeruginosa* for 1 h (MOI = 10). Gentamycin was used to eliminate live *Pseudomonas* in the culture medium. The above cells were subject to the drop plate method, qRT-PCR, or flow cytometry approaches to determine the bacterial load.

Figure 2: Drop plate method to determine bacterial load in lung epithelial BEAS-2B cells. BEAS-2B cells were treated with 4% CSE for 3 h. Cells were then subjected to *P. aeruginosa* (strain

PAO1) infection for 1 h followed by gentamycin treatment for another 1 h. Cells were lysed and the cell lysates were diluted to inoculate TSB plates for 16 h. Colonies were counted; the CFU numbers are illustrated in the plot. Graph shows mean ± SD, and "*" denotes $P < 0.05$. Results are representative of n = 3 experiments. Two-way unpaired Student *t*-test was used for smoke-treated and untreated groups. $P < 0.05$ indicates statistical significance.

Figure 3: Drop plate method to determine bacterial load in HSAEC cells. Human primary small airway epithelial cells were treated with 4% CSE for 3 h. Cells were then subjected to *P. aeruginosa* (strain PAO1) infection for 1 h followed by gentamycin treatment for another 1 h. The cells were lysed and the cell lysates were diluted to inoculate on TSB plates for 16 h. Colonies were counted; the CFU numbers are illustrated in the plot. Graph shows mean ± SD, and "*" denotes $P < 0.05$. Results are representative of n = 3 experiments. Two-way unpaired Student *t*-test was used for smoke-treated and untreated groups. $P < 0.05$ indicates statistical significance.

Figure 4: Determination of cell viability in CSE-treated lung epithelial BEAS-2B cells. Lung epithelial BEAS-2B cells were treated with 4% CSE for 3 h. Cells were stained with trypan blue and cell viability was measured with cell counter. Graph shows mean ± SD. Results are representative of n = 3 experiments.

Figure 5: Determination of cell viability in *Pseudomonas*-infected lung epithelial BEAS-2B cells. Lung epithelial BEAS-2B cells were infected with *P. aeruginosa* (MOI = 10) for 1 h. Cells were stained with trypan blue and cell viability was measured with a cell counter. Graph shows mean ± SD. Results are representative of n = 3 experiments.

Figure 6: Determination of cell viability in gentamycin-treated lung epithelial BEAS-2B cells. Lung epithelial BEAS-2B cells were treated with 100 µg/mL gentamycin for 1 h. Cells were stained with trypan blue and cell viability was measured with a cell counter. Graph shows mean ± SD. Results are representative of n = 3 experiments.

Figure 7: qRT-PCR to determine bacterial load in lung epithelial cells. Treated cells in **Figure 2** were subjected to total RNA extraction. An equivalent amount of RNA from each sample was reverse transcribed into cDNA, and the amount of 16S RNA of *P. aeruginosa* was determined with quantitative PCR using specific primer pairs. Results from qPCR are plotted in the graph. Graph shows mean ± SD, and "*" denotes $P < 0.05$. Results are representative of n = 3 experiments. Two-way unpaired Student *t*-test was used for smoke-treated and untreated groups. $P < 0.05$ indicates statistical significance.

Figure 8: Flow cytometry to determine bacterial load in lung epithelial cells. BEAS-2B cells were treated with CSE and infected with *P. fluorescens Migula* (strain PAO143). Infected cells were treated with gentamycin and digested with trypsin to make a cell suspension. Cell suspensions were passed through flow cytometer and fluorescent *Pseudomonas*-positive cells were determined at a wavelength of 509 nm. Results from flow cytometry are plotted in the graph. Graph shows mean ± SD, and "*" denotes $P < 0.05$. Results are representative of n = 3

experiments. Two-way unpaired Student *t*-test was used for smoke treated and untreated groups. $P < 0.05$ indicates statistical significance.

Figure 9: Observation of bacterial infection with fluorescent microscopy. BEAS-2B cells were infected with *P. fluorescens Migula* (strain PAO143) for 1 h. The cells were treated with gentamycin for another 1 h and washed 2x with cold PBS. The GFP-labeled bacteria were observed under a fluorescent microscope at a wavelength of 480 nm and BEAS-2B cells were visualized with a phase image. The images were merged, and the representative result is shown. Scale bar = 10 μm.

DISCUSSION:

Bacterial invasion into lung epithelial cells is a crucial step in the pathogenesis of bacterial infections. The process of bacterial invasion into the cells can be broken down into the following three steps: First, the bacteria contact and adhere to the surface of the epithelial cell using their flagella. Second, the bacteria either undergo internalization or penetrate the cellular membrane. Finally, the bacteria replicate and colonize the cells if they successfully escape cellular defense mechanisms[25,26]. Approaches for the observation of bacterial infections in lung microphages have long been developed but with limited knowledge of lung epithelial cells[27,28]. This study determined bacterial load in the lung epithelial cells via three approaches: a drop plate assay, RT-qPCR for *Pseudomonas* ribosome 16S RNA, and flow cytometry. All three approaches work well with similar sensitivities. Choosing the approaches to determine bacterial load in lung epithelial cells depends on the availability of equipment and time. In these experiments, keeping the bacterial flagella undamaged and intact is crucial for successful lung epithelial cell infection[29]. A shorter shaking time with limited speed may help to further promote bacterial invading capacity[30].

A major obstacle in determination of the bacterial concentration in lung epithelial cells is the bacterial contamination from the culture medium. In cell infection experiments, the bacteria are added into the culture medium for a specific time period (i.e., 1 h). Within that time, part of that bacterial load successfully invades the cytoplasmic compartment, but residues may attach to the outer membrane of the cells. An antibiotic, gentamycin, is used to kill the bacteria in the culture medium and the residues that attached to the outer membrane of the cells[31]. Gentamycin is considered not permeable to the cellular membrane and thus will not affect the bacteria already within the cells. Treatment with gentamycin makes it ideal for this system to exclude the potential contamination from outside the infected lung epithelial cells.

To study the effects of cigarette smoke on bacterial infection in lung epithelial cells, lung cells must be exposed to cigarette smoke prior to bacterial infection in cellular models. A conventional approach is preparing fresh CSE to treat cells. Generation of CSE is a cost-effective method for studies. CSE is easy to handle, the process of making CSE is simple, and CSE intratracheal injection is effective in the generation of emphysema in animal models in a short time period[32]. Approaches of direct cigarette exposure have also been developed for both in vitro cellular models and in vivo rodent models. Six months of daily cigarette smoke exposure to mice is widely

used to generate emphysema[33]. Direct cigarette smoke exposure requires complicated equipment that combines cigarette combusting and cell culture systems. Cigarette combusting produces cigarette smoke, but also generates a sum amount of heat that may affect the culture chamber's temperature and humidity. Fortunately, recent techniques make direct cigarette smoke exposure easier[21]. An International Organization for Standardization (ISO) protocol has been implemented for direct cigarette smoke exposure experiments[22]. Exposure to the cigarette smoke can be performed once or multiple times. In addition, along with the progress of cell culture techniques, lung primary epithelial cells could also be grown on transparent inserts to obtain a confluent lung epithelial cell monolayer[34]. Lung epithelial cell monolayers structurally mimic the physiological conditions in lung tissues. Cells can then be exposed to apical gaseous smoke or air at the air-liquid interface[35]. Furthermore, culture of lung organoids has emerged in current lung studies[36,37]. It will be interesting to know how cigarette smoke affects bacterial infection in lung organoids. The approach described may mimic human infection in tissues instead of cultured cells and may make possible further insights in lower respiratory bacterial infection.

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DISCOSURES:

The authors have nothing to disclose.

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37. Miller, A. J. et al. Generation of lung organoids from human pluripotent stem cells in vitro. *Nature Protocols.* **14** (2), 518-540 (2019).

Figure 1

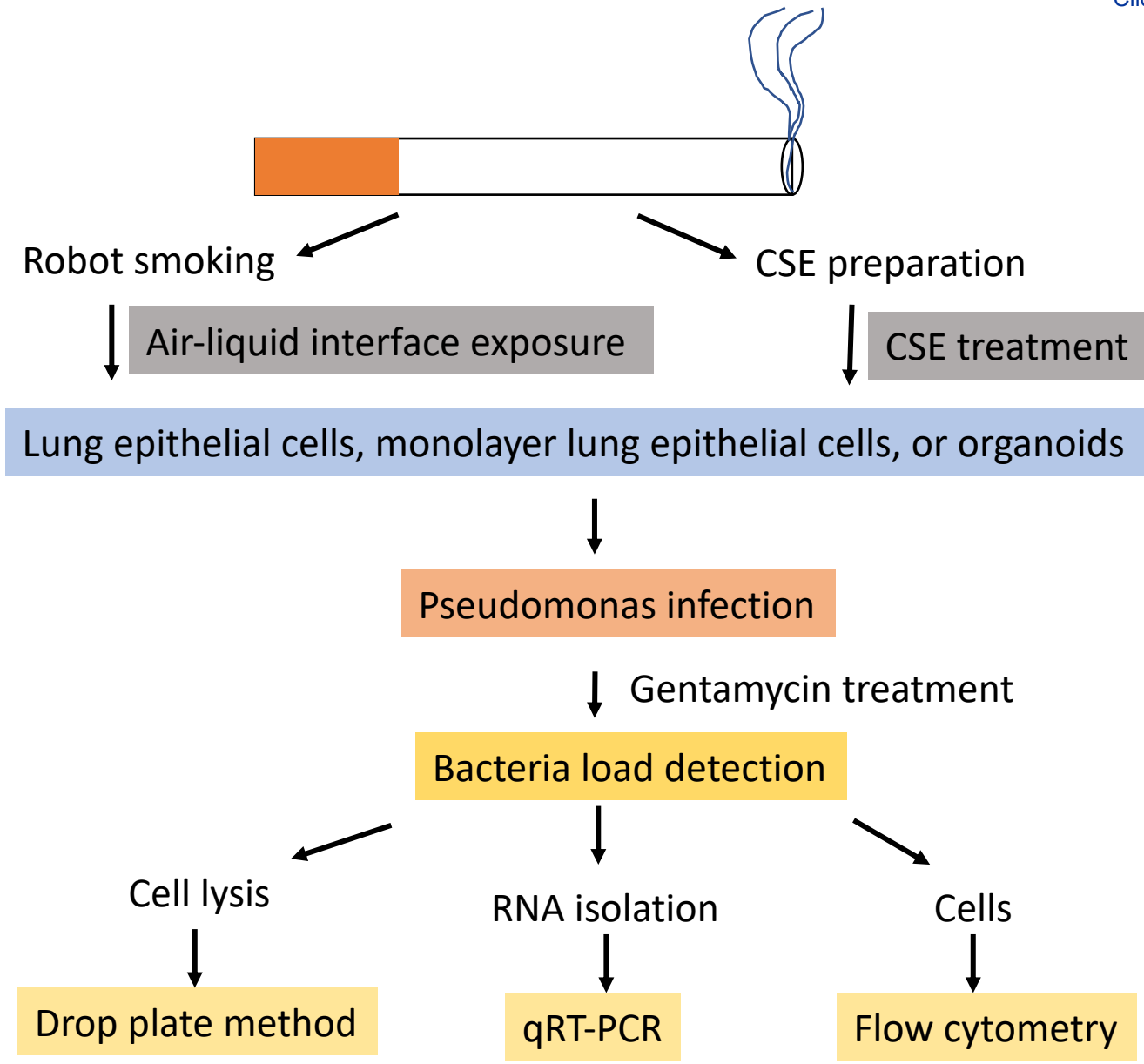


Figure 2

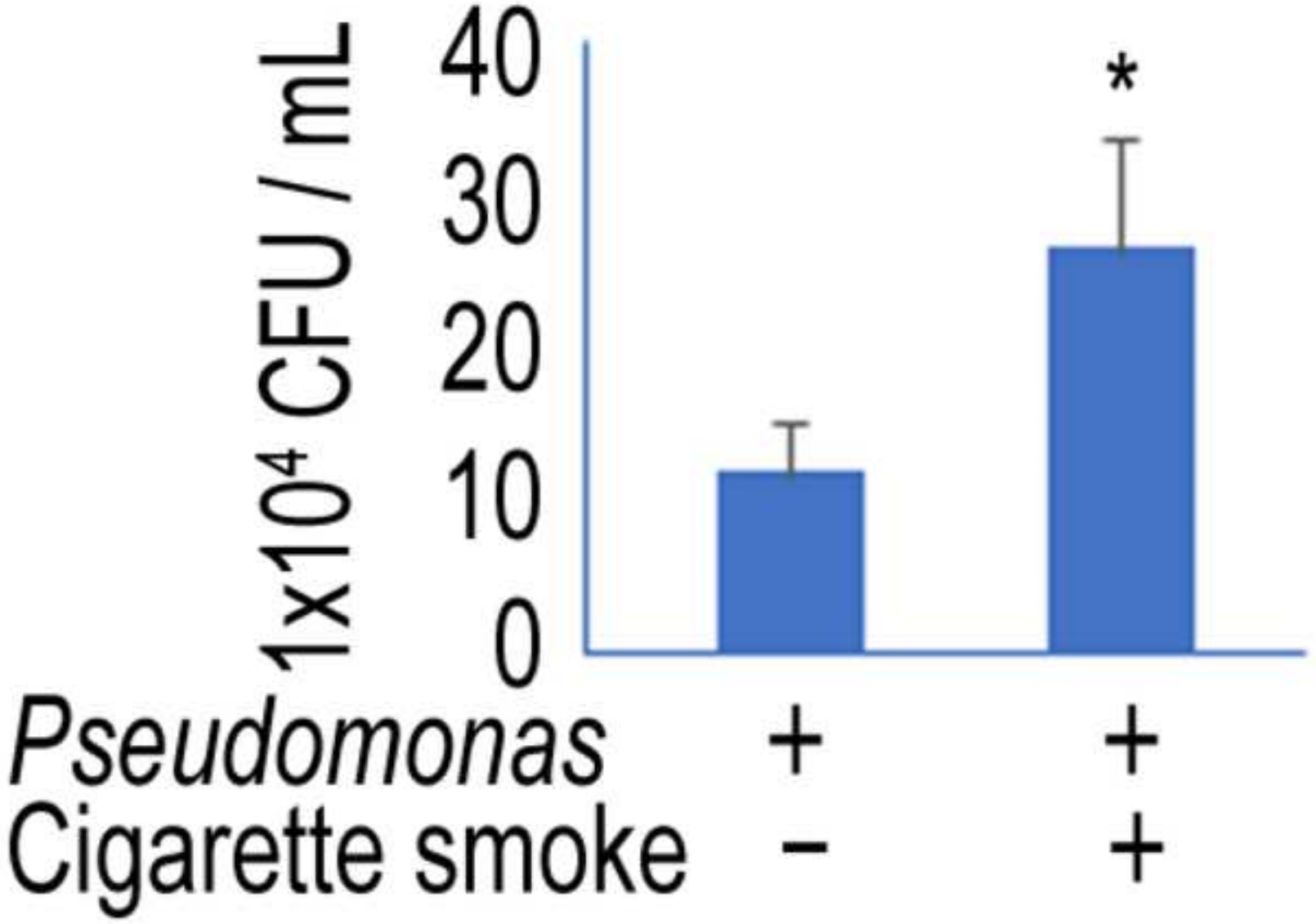


Figure 3

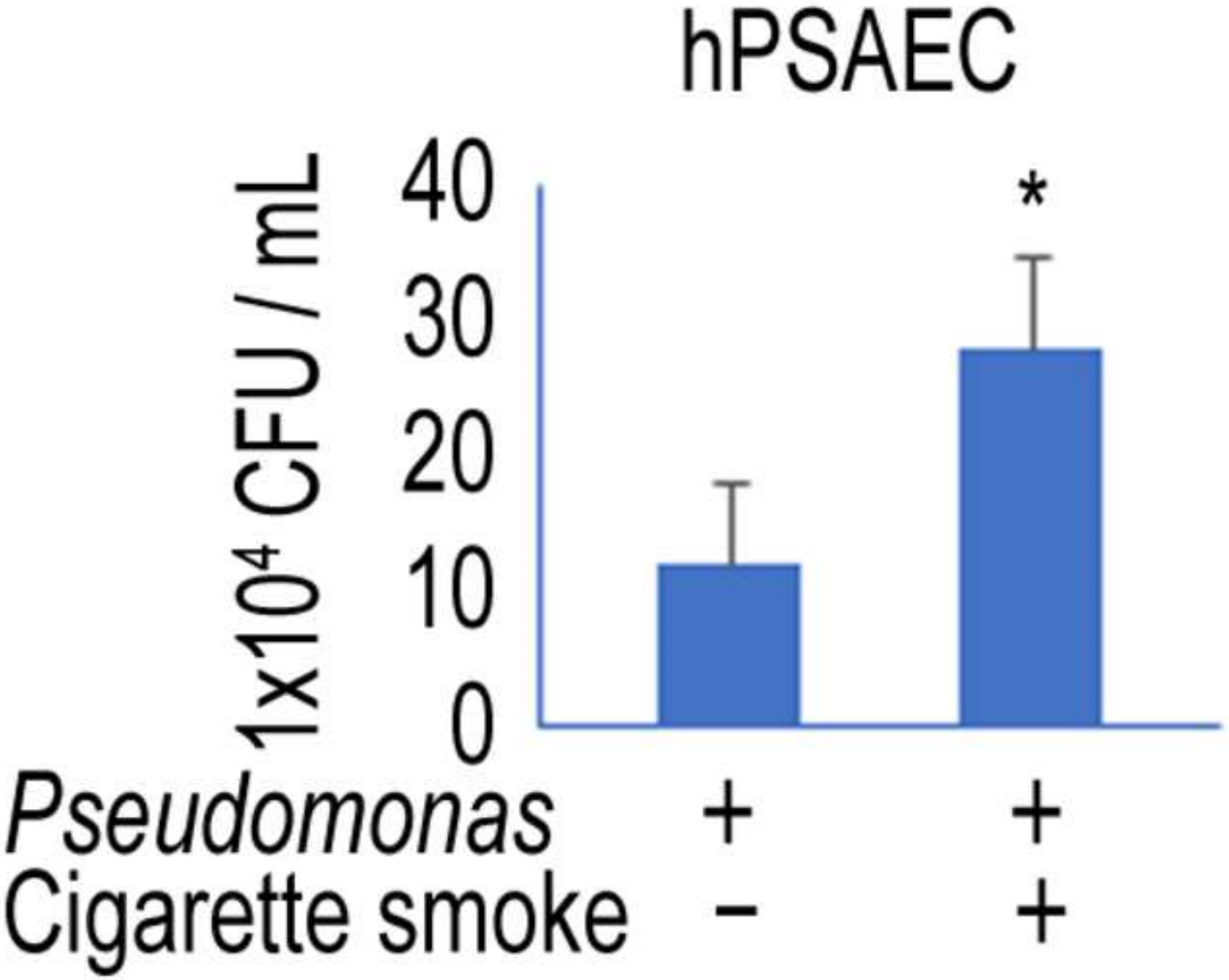


Figure 4

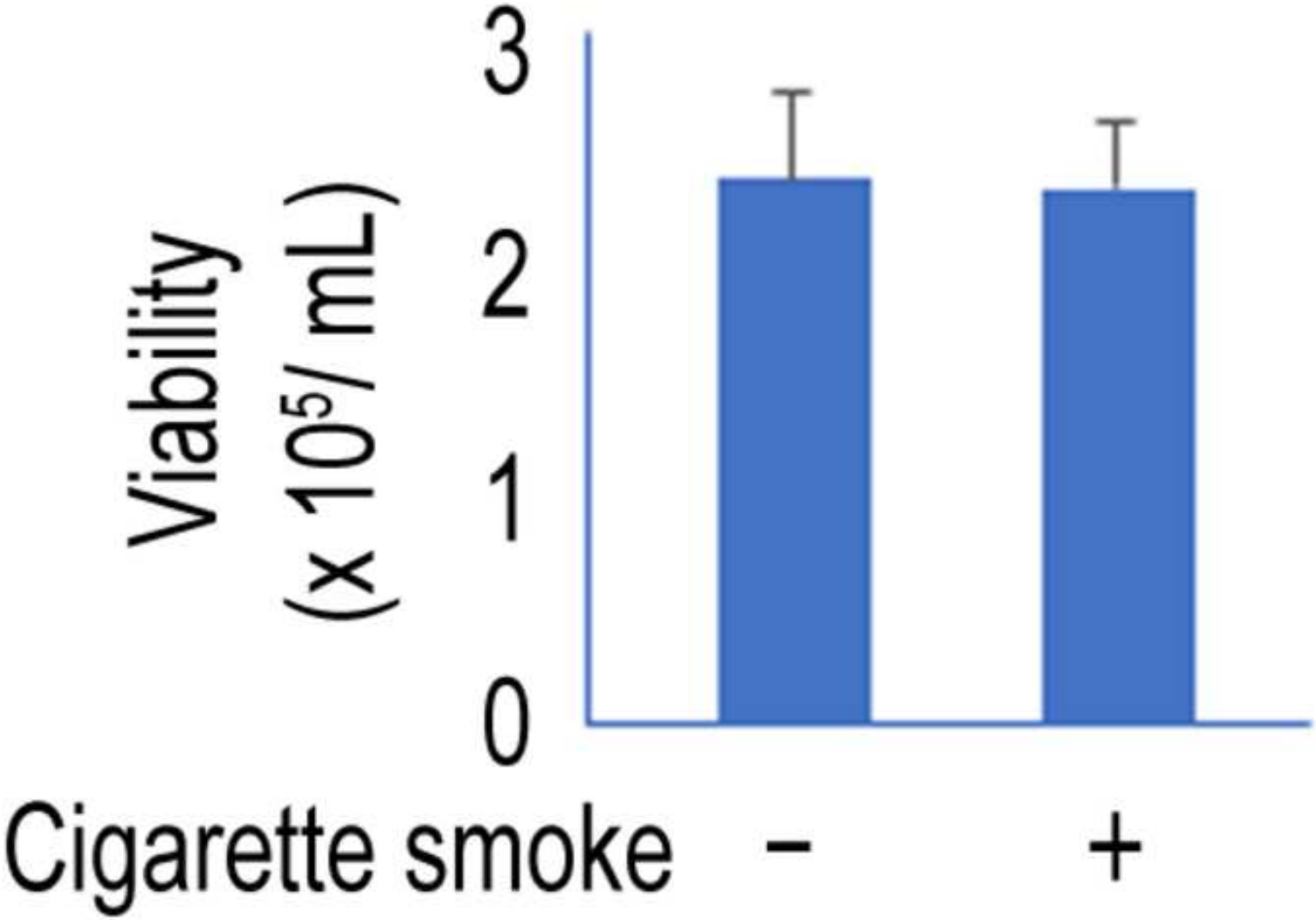


Figure 5

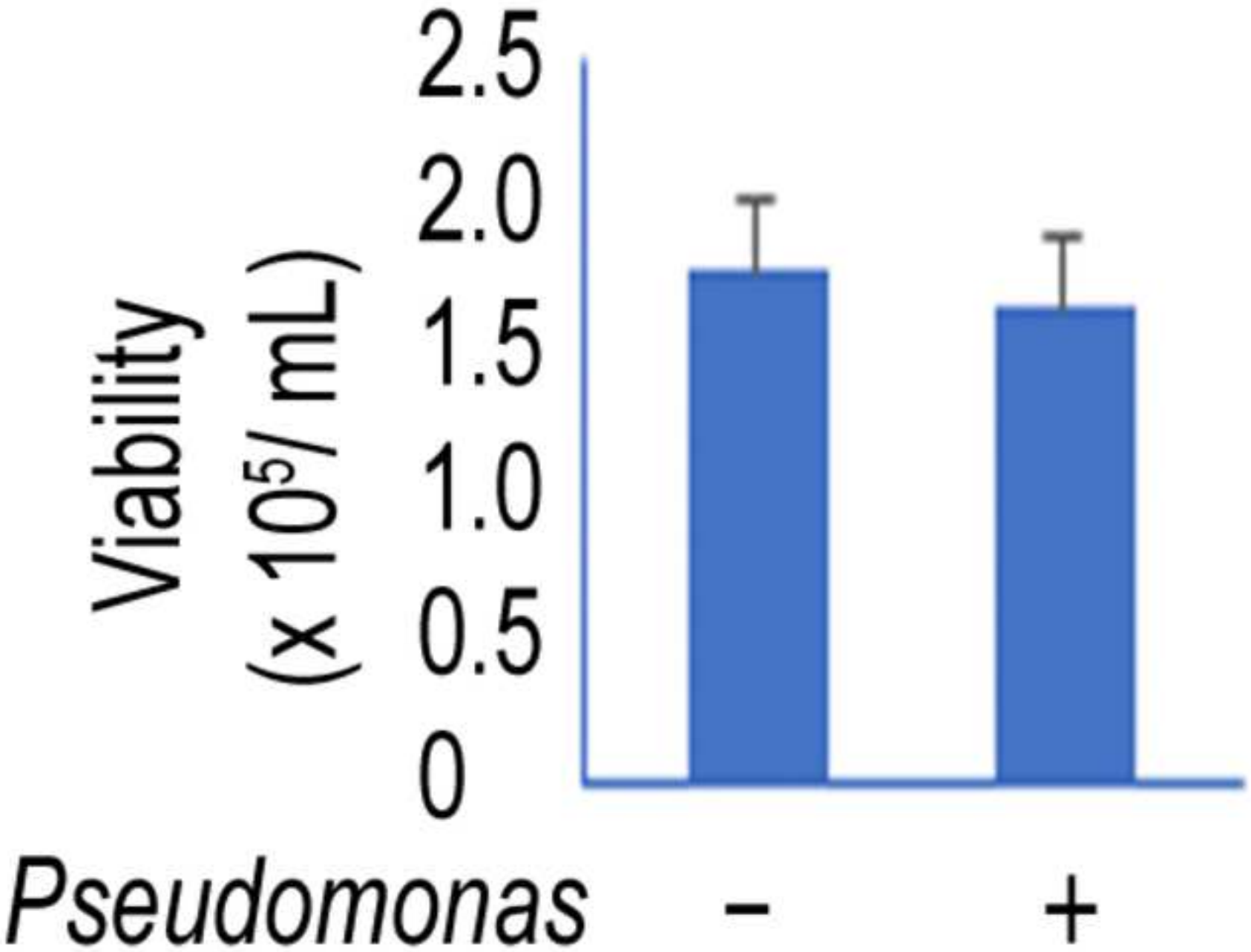


Figure 6

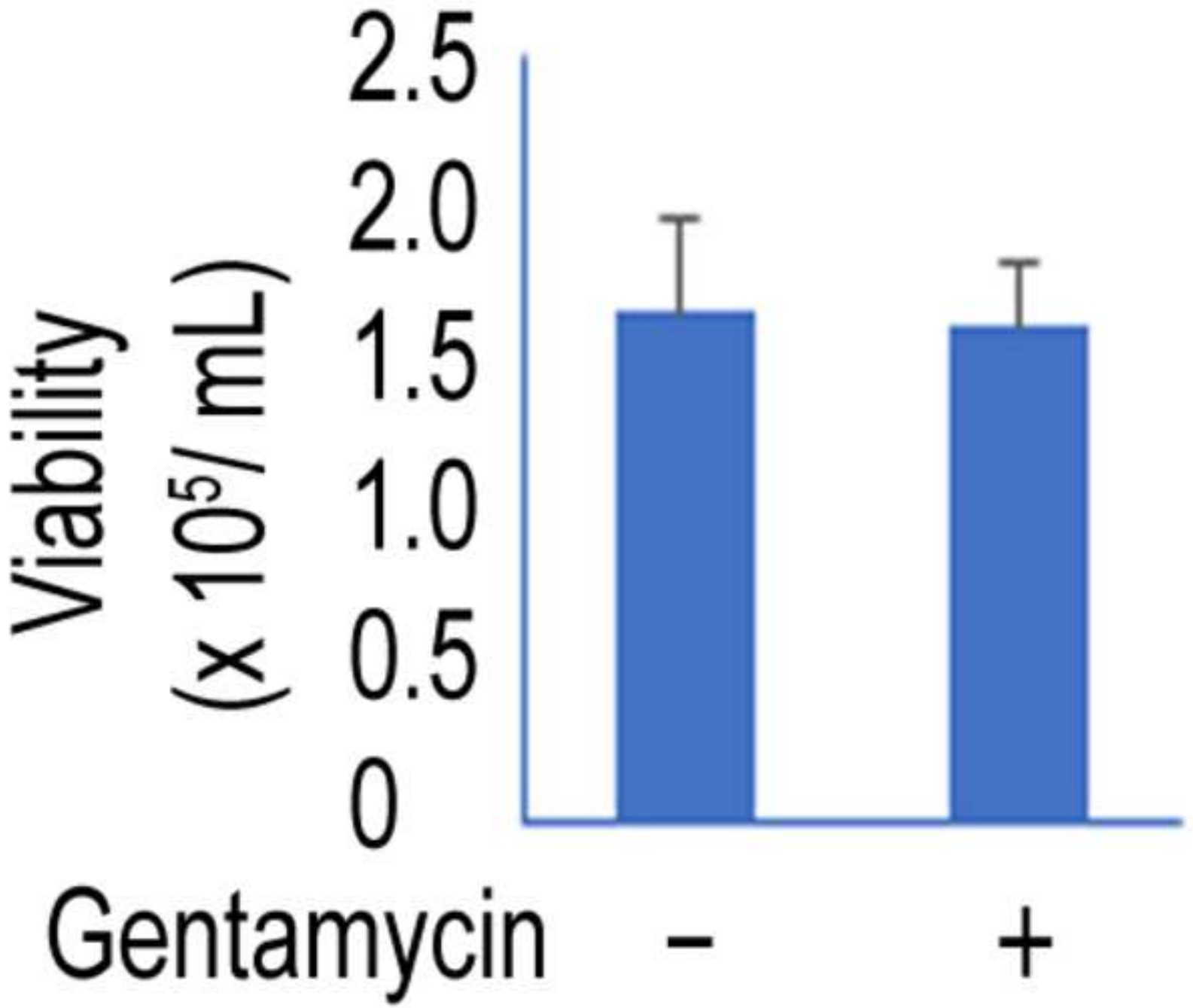


Figure 7

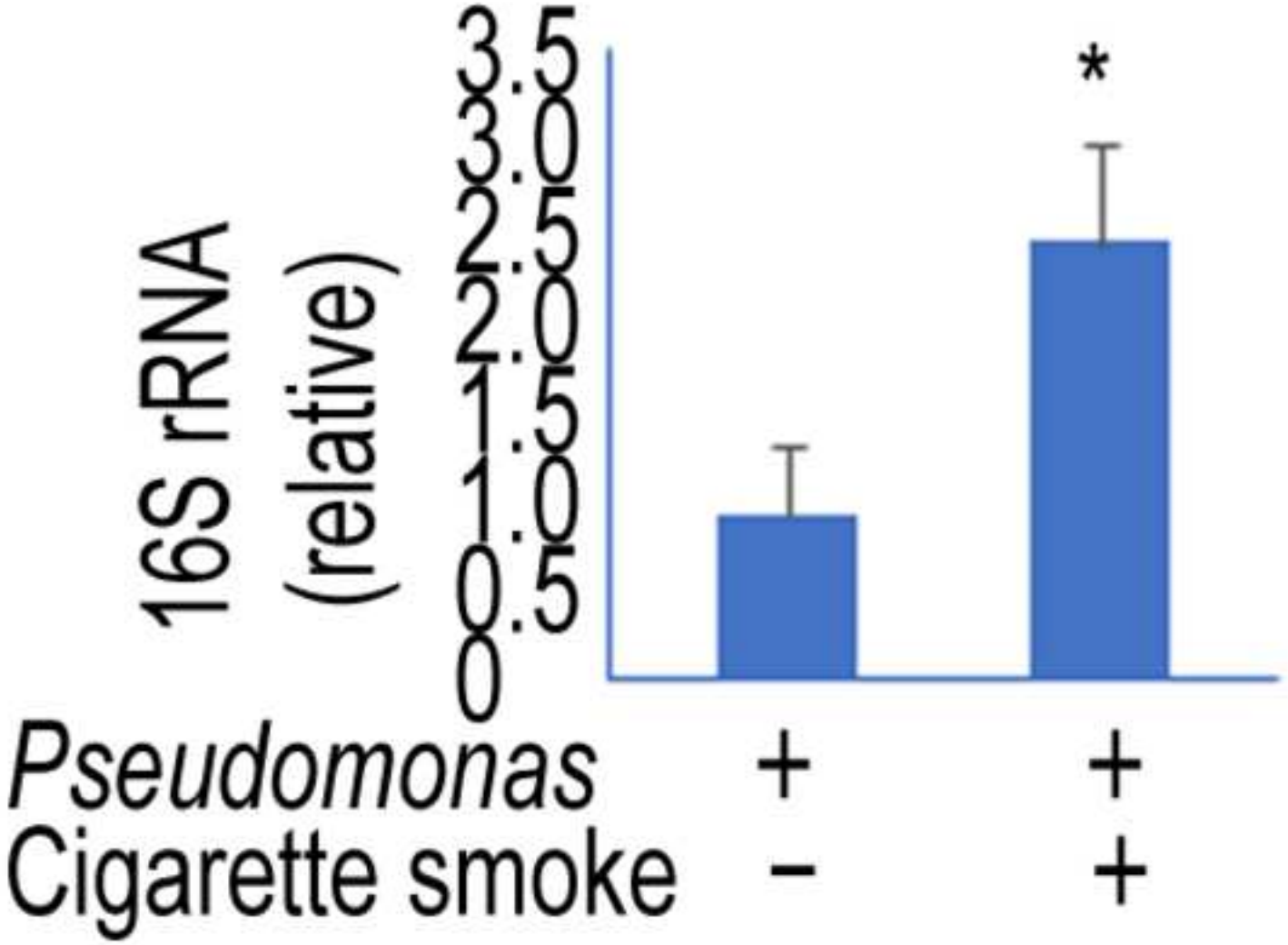


Figure 8

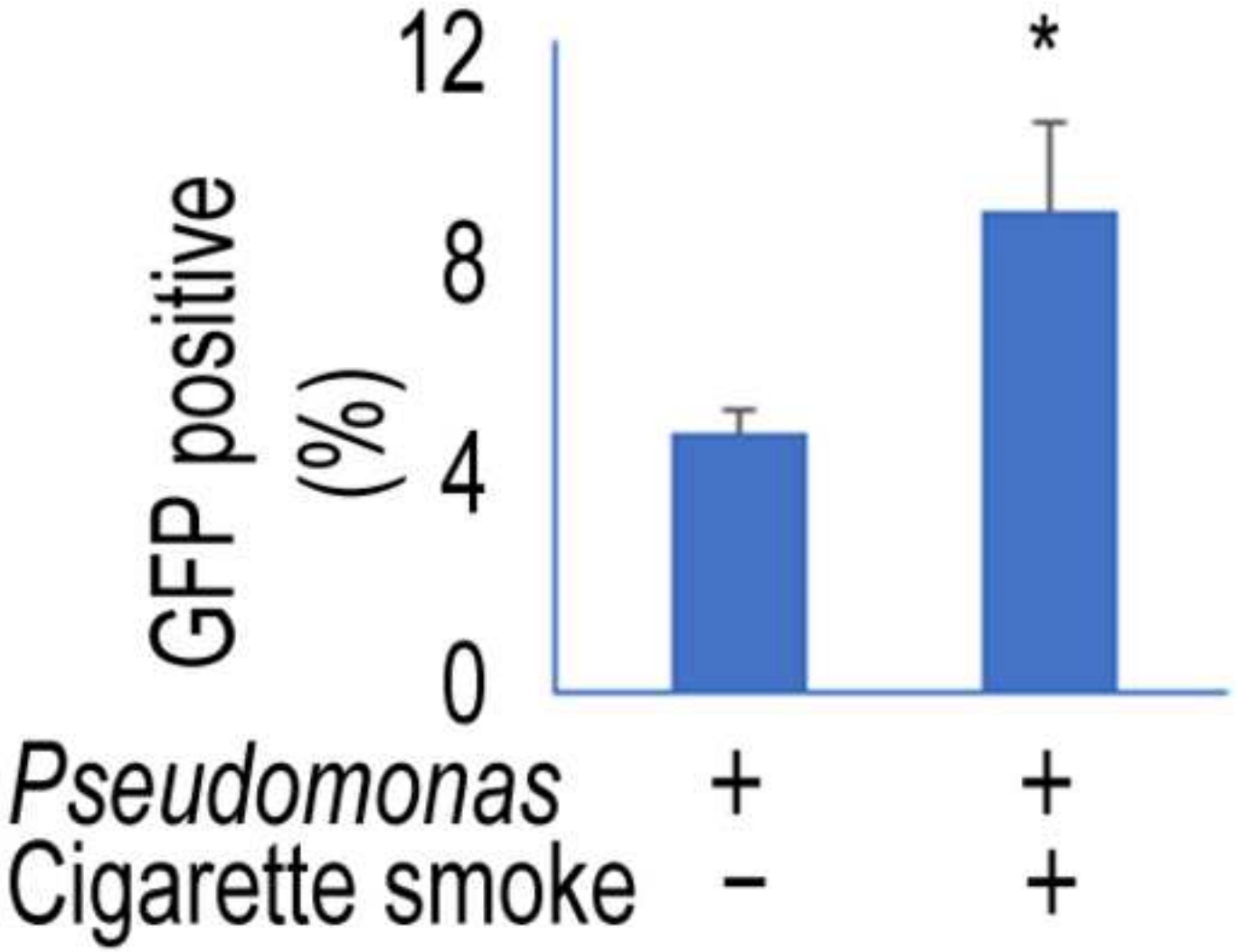
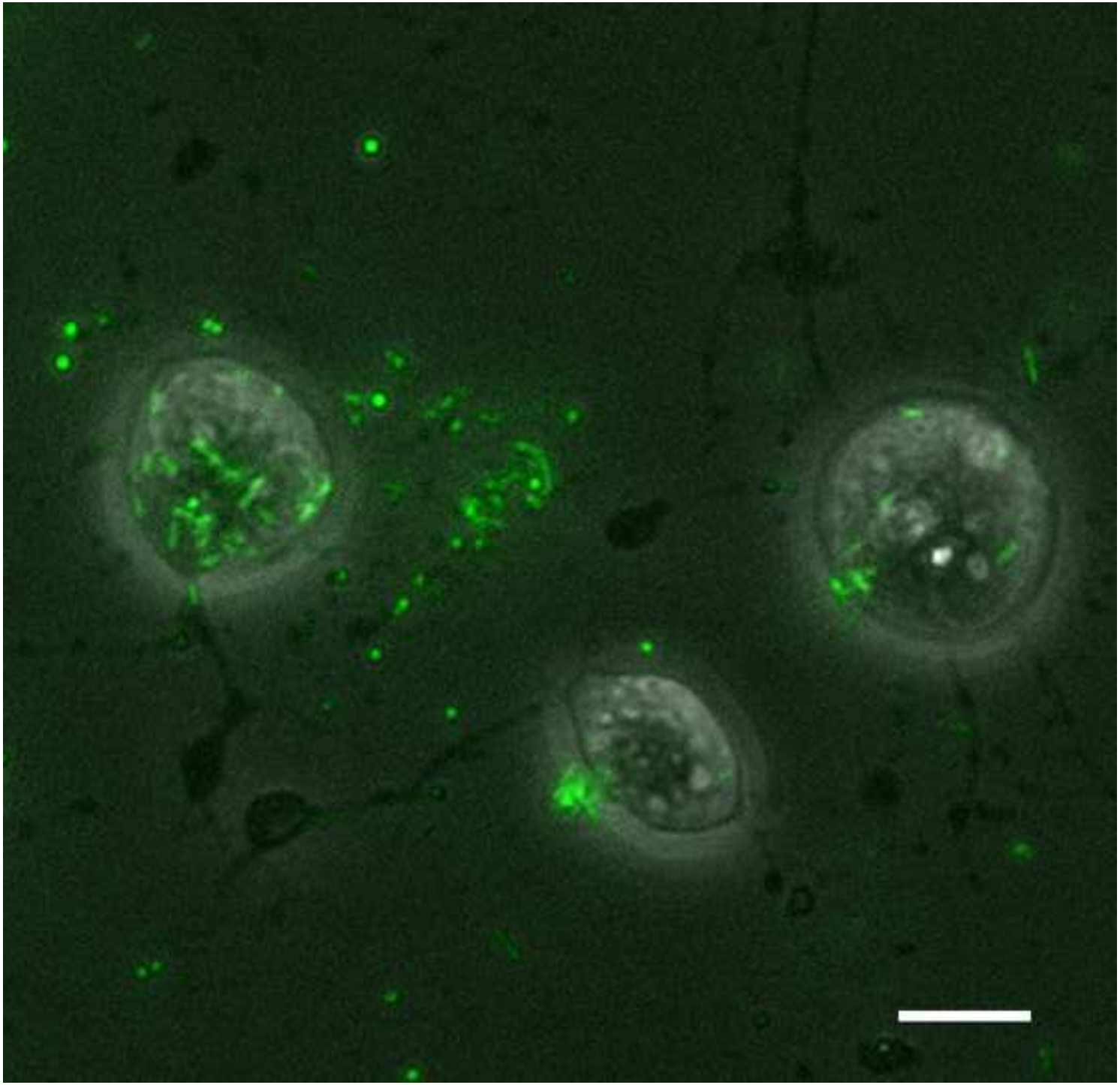


Figure 9

Click here to access/download;Figure;61163Fig9.tif



Name of Material/Equipment	Company	Catalog Number	Comments/Description
50mL syringe	BD Biosciences		
airway epithelial cell basal medium	ATCC	PCS-300-030	
Bacteria shaker	ThermoFisher Scientific		
bronchial epithelial cell growth kit	ATCC	PCS-300-040	
Cell Counter	Bio-Rad		
CFX96 Real-Time PCR System	Bio-Rad		
High-Capacity RNA-to-DNA KIT	ThermoFisher Scientific	4387406	
HITES medium	ATCC	ATCC 30-2004	
human BEAS-2B cells	ATCC	ATCC CRL-9609	
human primary small airway epithelial cells	ATCC	ATCC PCS-300-030	
LSRII flow cytometer	BD Biosciences		
Nikkon confocal microscope	Nikkon		
OD reader	USA Scientific		
PCR primers	ITD		
Pseudomonas aeruginosa	ATCC	ATCC 47085	PAO1-LAC
Pseudomonas fluorescens Migula	ATCC	ATCC 27853	*P.aeruginosa GFP*
Research-grade cigarettes (3R4F)	University of Kentucky	TP-7-VA	
RNeasy Mini Kit	Qiagen	74106	
Transprent PET Transwell Insert	Corning Costar		
Tryptic Soy Broth	BD Biosciences		

POINT BY POINT RESPONSE

1. Is it ok to place this in Immunology and infection classification rather than in Medicine?

Answer: Yes. It's ok to place this in Immunology and infection classification.

2. The results shown are with P. fluorescens, please change this here and throughout.

Answer: P. fluorescens Migula was used in flowcytometry and fluorescent microscopic experiments. P. aeruginosa (strain PAO1) was used in all other experiments throughout the manuscript.

3. *P. aeruginosa* can be detected in alveolar sacs as early as 1 h after the infection in animal models. Citations?

Answer: Citation has been added.

4. (It has been reported that lung epithelial cells may regulate microbial invasion, colonization, or replication independent of immune cells.) Citations?

Answer: Citation has been added.

5. Since figure 1 schematics show robot smoking please discuss this part in the introduction, please include the significance of using CSE method instead. Please include citations for the claims.

Answer: We have discussed this robot smoking method in Introduction.

6. Please ensure that each section has a representative result.

Answer: We have presented representative result of each important section.

7. How is this done?

Answer: We suck the air that containing cigarette smoke from 10ml to 50ml scale to get a 40ml volume of smoke.

8. (P. fluorescens Migula (strainPAO143).) Somewhere in the introduction please also include the significance of using this strain for some of the sections to bring out clarity. Please also include citations.

Answer: This strain P. fluorescens Migula (strainPAO143) *is used in flowcytometry experiments. P. aeruginosa PAO1 was used in all other experiments. We have added a sentence and included a citation in Introduction Paragraph 2 Line 76.*

7. Do you use single colonies in this case?

Answer: We don't use the single colonies. We collected pseudomonas in the plate with a policeman to get more bacteria for culturing in tube.

8. Where was this cell line used? If presenting as a primary cell culture model, please include representative results for this as well. If not, please discuss in the discussion as a limitation and how this could be helpful.

Answer: The primary human airway epithelial cell has been used to treated with cigarette smoke. We have added the data of HSAEC in the representative results as new Figure 3.

9. Please include some results to show that cells are indeed viable after the treatment with CSE.

Answer: Results of cell viability under CSE treatment have been presented as new Figure 4.

10. Please include results for this section… what happens to the cells after 1 h of bacteria/CSE treatment? Do they die?

Answer: We have included viability results in the representative results as new Figure 5. Essentially, we did not observe substantial cell death in this experiment.

11. (NOTE: To confirm the internalized bacteria, cells infected with *P. fluorescens Migula* were observed under confocal microscopy.) Please include a Result figure. Also, this can be moved to the representative result section.

Answer: A representative result of P. fluorescens Migula under fluorescent microscopy has been added as new Figure 9.

12. What is used as a control? How do you ensure that the increase is not because of the lung cell number?

Answer: We used equivalent amount of total RNA in reverse transcription and equivalent amount of cDNA for quantitative PCR to avoid potential errors from experimental design.

13. Results can be reprinted from previous publications if reprint permission is obtained and the figure is cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Answer: Thanks for your kind suggestions. We didn't use previous published data.

14. Details on how to perform Robot smoking procedure is not present in the protocol and no results are presented.

Answer: We have added the Robot smoking exposure procedure in protocol in protocol 3.9-3.11. We introduce this method in the protocol but without representative result.

15. Which strain?

Answer: It is P. fluorescens Migula (strainPAO143).

16. Citation for this claim?

Answer: Citations have been added at Line 351-353.

17. Please discuss why this should be done? Why is this a better approach? Please use citations.

Answer: Primary lung epithelial cell monolayer culture and robot smoking in air-liquid interface mimic the physiological cigarette smoke exposure and it's a cellular model other than conventional cell culture to understand cellular responses to cigarette smoke challenge. Besides, citation has been added.

18. Citations.

Answer: Citation has been added.

19. Please bring out clarity on this.

Answer: We have rewritten this sentence as "This approach may mimic human infection in a physiological way and through this approach we may gain insights in lower respiratory bacterial infection".