Efficacy of AiiM, an *N*-acylhomoserine lactonase, against *Pseudomonas aeruginosa* in a mouse model of acute pneumonia

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ABSTRACT

Quorum sensing (QS) in *Pseudomonas aeruginosa* (*P. aeruginosa*) regulates the production of many virulence factors and plays an important role in the pathogenesis of *P. aeruginosa* infection. *N*-Acyl homoserine lactones (AHL) are major QS signal molecules. Recently, a novel AHL-lactonase enzyme, AiiM, has been identified. The aim of this study was to evaluate the effect of AiiM on the virulence of *P. aeruginosa* in a mouse model of acute pneumonia.

We developed a *P. aeruginosa* PAO1 strain harbouring an AiiM-expressing plasmid. The production of several virulence factors by the AiiM-expressing strain was examined. Mice were intratracheally infected with an AiiM-expressing PAO1 strain. Lung histopathology, bacterial burden, and bronchoalveolar lavage (BAL) were assessed at 24 h post-infection.

AiiM expression in PAO1 reduced production of AHL-mediated virulence factors and attenuated cytotoxicity against human lung epithelial cells. In a mouse model of acute pneumonia, AiiM expression reduced lung injury and greatly improved the survival rates. The levels of pro-inflammatory cytokines and

myeloperoxidase activity in BAL fluid were significantly lower in mice infected with AiiM-expressing PAO1.

Thus, AiiM can strongly attenuate *P. aeruginosa* virulence in a mammalian model and is a potential candidate for use as a therapeutic agent against *P. aeruginosa* infection.

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative pathogen responsible for opportunistic and health-care associated infections such as pneumonia and urinary tract-, surgical site-, and catheter-related blood stream infections (1). Often, these infections are difficult to treat because of biofilm formation and intrinsic resistance to many antibiotics. In addition, *P. aeruginosa* easily develops resistance to many currently used antibiotics, and therefore, the development of novel treatment strategies is imperative.

Recently, quorum sensing (QS) system has attracted attention as a new therapeutic target (2). *P. aeruginosa* has two well-characterised QS systems (the LasR-LasI and the RhlR-RhlI systems) which mainly utilise two *N*-acyl homoserine lactones (AHL) molecules, namely, *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL) (3). These systems regulate the production of various virulence factors, including pyocyanin, elastase, and rhamnolipid, which play important roles in promoting the infection (4). In addition, 3-oxo-C12-HSL itself has immunomodulatory activity, stimulates cytokine production, and induces neutrophil or macrophage apoptosis (5, 6).

Previous studies showed that AHL-negative mutant *P. aeruginosa* had considerably lower virulence than the wild-type strain (7, 8). Thus, AHL inhibitor could be a therapeutic agent for infections by gram-negative bacteria.

The first AHL-inactivating enzyme, AHL-lactonase AiiA, was isolated from *Bacillus* species (9). Recently, Wang et al. have found a novel AHL-lactonase, termed AiiM, from *Microbacterium testaceum,* which resides on the leaf surface of potato (10, 11). AiiM is a member of the α/β hydrolase fold family and inactivates a broad range of AHL. They have also reported that expression of the *aiiM* gene in the gram-negative plant pathogenic bacterium *Pectobacterium carotovorum* subsp. *carotovorum*, resulted in significantly reduced pathogenicity to the potato slices (11). To date, although many AHL-lactonase enzymes (approximately 20) have been discovered, the efficacy of AHL-lactonase in infectious diseases has been evaluated only in non-mammalian species (9, 10). The aim of the present study was to investigate whether AHL-lactonase AiiM would attenuate *P. aeruginosa* pathogenicity both *in vitro* and in a mammalian model.

MATERIALS AND METHODS

Bacterial strains and growth conditions

P. aeruginosa reference strain PAO1 (12) was grown at 37°C in Luria-Bertani (LB) medium with shaking at 250 rpm. The AHL-reporter strains *Chromobacterium violaceum* (*C. violaceum*) CV026 (13) and *C. violaceum* VIR07 (14) were grown at 30°C in LB medium. When required, antibiotics were added to the medium at the following final concentrations to maintain the plasmid: carbenicillin (50 µg/mL) for *P. aeruginosa* and kanamycin (40 µg/mL) for *C. violaceum*. A final concentration of 0.5% L-arabinose was used as an inducer *in vitro*. Since there was some residual expression of AiiM, *in vivo* experiments were performed in the absence of arabinose induction.

Construction of a plasmid containing *aiiM* and *P. aeruginosa* mutants

A 1029-bp fragment containing *aiiM* was PCR-amplified with NheI/EcoRI ends from pUC118-aiiM (11) and cloned into pMJT1 (15), which contains arabinose-inducible araBAD promoter to generate pMJT1-aiiM. The constructed plasmid pMJT1-aiiM and pMJT1 (vector control) were transferred to *P. aeruginosa* by chemical transformation (15). The strains, plasmids, and primers used in this

119 study are listed in Tables 1 and 2.

120

121 **Detection of AHLs by thin-layer chromatography (TLC)**

122 CV026 and VIR07 were used to detect short-chain AHLs (C4-HSL and
123 C6-HSL) and a long-chain AHL (3-oxo-C12-HSL), respectively. AHLs were
124 extracted from culture supernatants and separated by TLC according to the
125 methods described previously (13, 14, 16), with slight modifications. *P. aeruginosa*
126 strains were grown overnight in 50 mL of LB medium and cell-free culture
127 supernatants were obtained by centrifugation (1,700 *g* for 45 min at 4°C), followed
128 by filtration through a 0.22-µm filter (Millipore Corp., USA). The supernatants were
129 extracted twice with the same volume of acidified ethyl acetate (0.1% v/v acetic
130 acid) in a separating funnel. The organic layers were dried over anhydrous
131 magnesium sulphate, and evaporated. The residues were resuspended in 100 µL of
132 ethyl acetate.

133 Eight microlitres of the solution was spotted on and separated using
134 reverse-phase TLC plates (RP-18 F245; Merck) with methanol/ water (60:40, v/v)
135 for the short chain AHLs and normal-phase TLC plates (Silica gel 60F254; Merck)

with hexane/acetone (45:55, v/v) for 3-oxo-C12-HSL. Then, TLC plates were dried and overlaid with 120 mL of LB medium containing 0.5% (w/v) agar mixed with 12 mL of overnight culture of either *C. violaceum* CV026 or *C. violaceum* VIR07. After incubation for 24 h at 30°C, AHL-induced violacein production in *C. violaceum* appeared as purple spots. AHL standards were purchased from Cayman Chemical, MI, USA.

Assay for pyocyanin and elastase production

Cell free culture supernatants were obtained by the same method as described above for AHL detection assay. Pyocyanin production was determined using a slightly modified procedure as previously described (17). Four millilitres of the supernatant was mixed with 3 mL of chloroform and centrifuged at 1,700 *g* for 5 min. Two millilitres of the lower organic layer containing pyocyanin was collected, and extracted with 2 mL of 0.2 M HCl. After centrifugation, the absorbance of the pink top layer was measured at 520 nm. Concentrations of pyocyanin were determined by multiplying the absorbance by 17.072 (18).

The elastase activity of the same culture supernatants was measured by the elastin Congo red (ECR) assay (19). Ten microlitres of the supernatant was mixed with 250 µL of ECR buffer (30 mM Tris containing 2.5 mg of ECR, pH 7.5). After 14-h incubation, the absorbance of the supernatant was measured at 495 nm, and the elastase activity was calculated using porcine elastase as a standard.

Assessment of cytotoxicity

The human alveolar epithelial cell line A549 was cultured in RPMI 1640 medium with 10% foetal bovine serum, 100 U of penicillin/mL and 100 µg of streptomycin/mL at 37°C with 5% CO_2. After achieving confluence, the cells were diluted to 2.0×10^5 cells/mL and 100 µL aliquots were dispensed in a 96-well plate. After overnight incubation, the cells were washed and infected with *P. aeruginosa* strains at a concentration of 4.0×10^6 cfu/well or normal saline (negative control) for 1, 3, 6, 9, 12, and 15 h in serum-free RPMI 1640. The cytotoxicity was assessed by the amount of lactate dehydrogenase (LDH) in the culture medium by using Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany).

Mouse model of *P. aeruginosa* acute lung infection

Animal experiments were approved by the Ethics Review Committee for Animal Experimentation and performed on the basis of the Guidelines for Animal Experimentation at Nagasaki University. Pathogen-free 6-week-old male ddY mice (body weight, 30–35 g) were purchased from SLC Japan (Tokyo, Japan). All animals were housed under constant temperature and light conditions (12/12-h light/dark cycle) with free access to sterile food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University.

Overnight cultures of *P. aeruginosa* strains were grown, adjusted to an OD_{600} of 0.1, and 30-μL aliquots were transferred to 3 ml of fresh LB medium. Following 6 h (late log phase) of incubation, bacteria were harvested by centrifugation (13,000 *g* for 1 min at 4°C) and resuspended in sterile normal saline at a final concentration of 2×10^8 cfu/mL based on optical density. Inoculated bacterial counts were confirmed by spreading the appropriately diluted suspension on LB plates. Mice were infected with an intratracheal instillation of 0.05 mL of bacterial suspension (1×10^7 cfu/mouse) under anaesthesia with pentobarbital sodium. As a non-infected control, intratracheal instillation was performed with normal saline in the same manner. For

186 survival studies, 10–12 mice in each group were observed at least once daily until
187 the seventh day post infection. Another group of infected mice was sacrificed by
188 cervical dislocation at 24 h after the infection, and the lungs, spleen, blood, and
189 bronchoalveolar lavage (BAL) fluid were collected for further analysis.
190
191 **Bacteriological and histopathological examination**
192 Blood was collected by right ventricular puncture using heparin-coated
193 syringes. Lungs and spleens were dissected out under aseptic conditions. For
194 bacteriological examination, the organs were suspended in normal saline (1 mL for
195 the lung sample and 0.5 mL for the spleen sample) and homogenised using a
196 homogeniser (AS One Co., Osaka, Japan). Each specimen (blood, lung, and
197 spleen) was serially diluted and plated on LB agar, followed by incubation at 37°C
198 for 24 h. For histopathological examination, lung specimens were fixed in 10%
199 buffered formalin, and the paraffin-embedded sections were stained with
200 haematoxylin-eosin.
201
202 **Analysis of BAL fluid**

After the pulmonary vasculature was flushed with 5 mL of normal saline via the right ventricle, an 18-gauge plastic intravenous catheter was inserted into the trachea and the lungs were lavaged three times with 1 mL of normal saline. Total and differential cell counts were determined manually.

Hemoglobin concentrations in BAL fluid were analyzed by using previously described methods with some modification (20, 21). A 200-μL aliquot of the BAL fluid was mixed with 800 μL of distilled water to lyse the red blood cells. Then the sample was mixed with an equal volume of Drabkin's reagent (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 15 min. Hemoglobin content was quantified by measuring the absorbance at 540 nm using purified human hemoglobin (Sigma-Aldrich, St. Louis, MO) as the standard.

Cell-free BAL supernatants were obtained by centrifugation (15,000 g for 5 min at 4°C) of BAL fluids. Albumin concentration and LDH activity in the supernatant fluid were determined by using mouse albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX) and Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The concentrations of myeloperoxidase (MPO), tumour necrosis factor (TNF)-α,

interleukin (IL)-1β, IL-6, and macrophage inflammatory protein (MIP)-2 in cell-free BAL supernatants were quantified using ELISA kits for MPO glycoprotein (Hycult Biotech, Uden, The Netherlands) and mouse cytokine and chemokine ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Statistical analyses

The data are expressed as mean ± standard error of the mean (SEM). Differences between groups were examined using the unpaired Student's *t* test or the Mann-Whitney's *U* test, as appropriate depending on the normality of data distribution. Survival analysis was performed using the log rank test, and the survival rate was calculated by the Kaplan-Meier method. A significant difference was defined as a *P* value less than or equal to 0.05.

RESULTS

Expression of AiiM in *P. aeruginosa* reduced AHL accumulation

As seen in Fig. 1, short- and long-chain AHL (C4-HSL, C6-HSL, and 3-oxo-C12-HSL) were detected as purple spots on the TLC plates from the extracts

of the wild-type strain PAO1 (PAO1) and the mutant carrying the empty vector (PAO1/pMJT1). In contrast, none of these AHLs were detected from the mutant carrying the plasmid with *aiiM* gene (PAO1/pMJT1-aiiM). The growth curve of PAO1/pMJT1-aiiM and PAO1/pMJT1 showed no difference (data not shown). Therefore, the results indicated that AiiM expressed in PAO1/pMJT1-aiiM degraded AHL.

AiiM expression reduced virulence factor production in *P. aeruginosa*

Since the production of pyocyanin and elastase, the important virulence factors of *P. aeruginosa*, are controlled by QS system, we examined whether AiiM expression could inhibit the production of these virulence factors. As shown in Fig. 2, the levels of pyocyanin and elastase decreased to a large extent in *P. aeruginosa* PAO1/pMJT1-aiiM compared to PAO1/pMJT1.

AiiM expression attenuated cytotoxicity against human lung epithelial cells A549

253 To test the influence of AiiM expression on the infectivity of *P. aeruginosa*, we
254 evaluated the cytotoxicity of each strain against A549 cells by measuring the
255 release of LDH following infection. *P. aeruginosa* PAO1/pMJT1-aiiM
256 showed approximately half the level of cytotoxicity compared with PAO1/pMJT1 at
257 9–15 h post infection (Fig. 3).
258
259 **AiiM expression improved survival in murine models of acute lung infection**
260 **and reduced systemic dissemination of bacteria**
261 Our preliminary results encouraged us to investigate the *in vivo* efficacy of AiiM
262 expression in *P. aeruginosa.* Mortality was significantly lower in the mice infected
263 with PAO1/pMJT1-aiiM compared to those infected with PAO1/pMJT1 (Fig. 4a).
264 We further investigated whether AiiM could reduce local infection and systemic
265 dissemination. The bacterial count in the lungs did not significantly differ between
266 the groups. However, the bacterial counts in the blood and spleen of mice infected
267 with PAO1/pMJT1-aiiM was significantly lower than in those of the mice infected
268 with PAO1/pMJT1 (Fig. 4b), indicating that AiiM expression contributed to reduction
269 of systemic dissemination, but did not alter the local bacterial burden.

AiiM expression decreased the lung injury induced by *P. aeruginosa* infection

Next, we examined whether AiiM expression could reduce lung injury and inflammation. In spite of the identical bacterial burden in both groups, PAO1/pMJT1-aiiM-infected mice showed mild and localised inflammation, while *P. aeruginosa* PAO1/pMJT1-infected mice exhibited a large area of diffuse inflammation accompanied by haemorrhage into the alveolar spaces (Fig. 5a and b). In addition, the reduced levels of haemoglobin, albumin, and LDH in the cell-free BAL fluids of PAO1/pMJT1-aiiM infected mice (Fig. 5c-e) were also observed.

AiiM expression altered the host inflammatory response to *P. aeruginosa* lung infection

The total cell counts and neutrophil percentage in the BAL fluid of both the infected groups increased compared to the uninfected control, with no significant differences between the two groups (Fig. 6a and b); however, MPO activity, a marker of neutrophil activation, in the BAL fluid of the mice infected with *P.*

286 *aeruginosa* PAO1/pMJT1-aiiM was significantly lower than that of the
287 PAO1/pMJT1-infected mice (Fig. 6c).

288 The levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the BAL
289 fluid were also significantly lower in the PAO1/pMJT1-aiiM-infected mice than in the
290 PAO1/pMJT1-infected mice (Fig. 7a-c). MIP-2 level tended to be lower in the
291 PAO1/pMJT1-aiiM-infected group than in the PAO1/pMJT1-infected group,
292 although the decrease was not statistically significant (Fig. 7d). Therefore, these
293 results suggest that AiiM expression could reduce lung injury and inflammation and
294 systemic dissemination by reducing bacterial virulence, but it does not reduce
295 bacterial count.

296

297 **DISCUSSION**

298 Although several studies have reported the therapeutic potential of AHL
299 lactonases against gram-negative pathogens in non-mammalian models (22, 23,
300 24), it has never been considered as therapeutic agent for mammalian infections.
301 This is the first study demonstrating that AiiM could serve as a potent inhibitor of *P.*

302 *aeruginosa* QS and attenuate bacterial virulence in the mouse model of *P.*
303 *aeruginosa* lung infection.

304 AHL-mediated virulence factors such as pyocyanin and elastase and AHL itself
305 have been reported to cause cytotoxic effects and tissue damage, and play an
306 important role in the pathogenesis of *P. aeruginosa* (6, 25, 26). Expression of AiiM
307 in *P. aeruginosa* PAO1 strongly suppressed AHL accumulation, including two major
308 ones (C4-HSL and 3-oxo-C12-HSL) and the production of the QS-dependent
309 virulence factors. Furthermore, we demonstrated that AiiM-expressing PAO1
310 mutant exhibit a significantly decreased virulence, both *in vitro* and *in vivo*. A
311 previous report that described less virulence in AHL-negative mutant *P. aeruginosa*
312 lung infection in neonatal mice supports our results (7).

313 It is unclear whether systemic bacterial dissemination is directly involved with
314 the mortality in our model, although development of bacteraemia has been reported
315 to be related to the high mortality rate in acute *Pseudomonas* infection (27). The
316 decreased lung injury in the mice infected with AiiM-expressing mutant may
317 contribute less to mortality and also minimise airway epithelial permeability, thereby
318 resulting in reduced systemic dissemination (28).

Despite pro-inflammatory cytokines, chemokine and MPO activities in BAL fluid were lower in mice infected with the AiiM-carrying mutant than in the control mutant. Neutrophil counts in BAL fluid were identical in both groups. While the 3-oxo-C12-HSL attract neutrophils either directly or through the induction of chemokines (IL-8, MIP-2) (29, 30), the pro-apoptotic effect of 3-oxo-C12-HSL on neutrophils is known (6). AHL-mediated virulence factors, namely, pyocyanin and rhamnolipid, also cause apoptotic or necrotic cell death of neutrophils in BAL fluid (31, 32). Thus, part of migrated neutrophils may be killed by these factors, with no change in the BAL neutrophil counts.

AHL-lactonase enzymes are highly specific for AHL and are expected to have beneficial effects in patients with *P. aeruginosa* infection. Thus, our results indicate that a novel strategy using AHL may be useful for treating intractable *Pseudomonas* infections, though there are still some issues for clinical use. We consider several possibilities of AiiM for clinical applications in patients with infectious diseases. First, the aiiM gene can be used to improve probiotics to enhance their positive effects through genetic modification. There are, however, some additional issues with mutant probiotics, such as human safety or possibly adverse effects on the

environment, and thus it is not yet ethically practical. Second, the use of the purified AiiM protein will be the most convenient and practical method. Local administration of the purified AiiM protein may reduce the severity of lung infections due to *P. aeruginosa* and may have an efficacy in preventing infection, though it is not technically available at the present point. Therefore, further studies will be required to evaluate the efficacy and safety of purified AiiM protein. In conclusion, this study demonstrates that AiiM can disturb the QS systems of *P. aeruginosa* and attenuate bacterial virulence in a mouse model of acute lung infection. Although the mechanism of host immune response to QS-related factors is not fully understood, we have found that quenching QS signals by AiiM may result in the reduction of excessive inflammation and help in inciting an immune response against *P. aeruginosa* infection. Thus, our results are expected to supply attractive candidates to develop novel prophylactic or therapeutic strategies using AHL lactonases against *Pseudomonas* infections.

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REFERENCES

1. **Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK.** 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infection Control and Hospital Epidemiology **29:**996–1011.
2. **Sintim HO, Smith JAI, Wang J, Nakayama S, Yan L.** 2010. Paradigm shift in discovering next-generation anti-infective agents: targeting quorum sensing,

c-di-GMP signaling and biofilm formation in bacteria with small molecules. Future Medicinal Chemistry **2:**1005–1035.

3. **Pesci EC, Pearson JP, Seed PC, Iglewski BH.** 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. Journal of Bacteriology **179:**3127–3132.

4. **Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH.** 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. Journal of Bacteriology **185:**2080–2095.

5. **Smith RS, Harris SG, Phipps R, Iglewski B.** 2002. The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-(3-oxododecanoyl)homoserine lactone contributes to virulence and induces inflammation in vivo. Journal of Bacteriology **184:**1132–1139.

6. **Tateda K, Ishii Y, Horikawa M, Matsumoto T, Miyairi S, Pechere JC, Standiford TJ, Ishiguro M, Yamaguchi K.** 2003. The *Pseudomonas aeruginosa* autoinducer *N*-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils. Infection and Immunity **71:**

5785–5793.

7. **Pearson JP, Feldman M, Iglewski BH, Prince A.** 2000. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. Infection and Immunity **68:**4331–4334.

8. **Imamura Y, Yanagihara K, Tomono K, Ohno H, Higashiyama Y, Miyazaki Y, Hirakata Y, Mizuta Y, Kadota J, Tsukamoto K, Kohno S.** 2005. Role of *Pseudomonas aeruginosa* quorum-sensing systems in a mouse model of chronic respiratory infection. Journal of Medical Microbiology **54:**515–518.

9. **Dong YH, Xu JL, Li XZ, Zhang LH.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proceedings of the National Academy of Sciences of the United States of America **97:**3526–3531.

10. **Morohoshi T, Someya N, Ikeda T.** 2009. Novel *N*-acylhomoserine lactone-degrading bacteria isolated from the leaf surface of *Solanum tuberosum* and their quorum-quenching properties. Bioscience, Biotechnology, and Biochemistry **73:**2124–2127.

11. **Wang W-Z, Morohoshi T, Ikenoya M, Someya N, Ikeda T.** 2010. AiiM, a novel

class of *N*-acylhomoserine lactonase from the leaf-associated bacterium *Microbacterium testaceum*. Applied and Environmental Microbiology **76:**2524–2530.

12. **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson M V.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.

13. **McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology **143:**3703–3711.

14. **Morohoshi T, Kato M, Fukamachi K, Kato N, Ikeda T.** 2008. *N*-Acylhomoserine lactone regulates violacein production in *Chromobacterium*

violaceum type strain ATCC 12472. FEMS Microbiology Letters **279:**124–130.

15. **Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK.** 2007. The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. The Journal of Clinical Investigation **117:**877–888.

16. **Ravn L, Christensen AB, Molin S, Givskov M, Gram L.** 2001. Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics. Journal of Microbiological Methods **44:**239–251.

17. **Li C, Wally H, Miller SJ, Lu C-D.** 2009. The multifaceted proteins MvaT and MvaU, members of the H-NS family, control arginine metabolism, pyocyanin synthesis, and prophage activation in *Pseudomonas aeruginosa* PAO1. Journal of Bacteriology **191:**6211–6218.

18. **Kurachi M.** 1958. Studies of the biosynthesis of pyocyanine. (II) Isolation and determination of pyocyanine. Bull Inst Chem Res Kyoto Univ **36:**174–87.

19. **Rust L, Messing CR, Iglewski BH.** 1994. Elastase assays. Methods in Enzymology **235:**554–562.

20. **Battistini B, Steil AA, Jancar S, Sirois P.** 1998. Roles of endothelins and their receptors in immune complex-induced/polymorphonuclear-mediated lung injury (reversed passive arthus reaction) in CD-1 mice. Pulmonary Pharmacology & Therapeutics **11:**165–172.

21. **Wesselkamper SC, McDowell SA, Medvedovic M, Dalton TP, Deshmukh HS, Sartor MA, Case LM, Henning LN, Borchers MT, Tomlinson CR, Prows DR, Leikauf GD.** 2006. The role of metallothionein in the pathogenesis of acute lung injury. American journal of Respiratory Cell and Molecular Biology **34:**73–82.

22. **Asad S, Opal SM.** 2008. Bench-to-bedside review: Quorum sensing and the role of cell-to-cell communication during invasive bacterial infection. Critical Care **12:**236.

23. **Wang L-H, Weng L-X, Dong Y-H, Zhang L-H.** 2004. Specificity and enzyme kinetics of the quorum-quenching *N*-Acyl homoserine lactone lactonase (AHL-lactonase). The Journal of Biological Chemistry **279:**13645–13651.

24. **Huang W, Lin Y, Yi S, Liu P, Shen J, Shao Z, Liu Z.** 2012. QsdH, a novel AHL lactonase in the RND-type inner membrane of marine *Pseudoalteromonas byunsanensis* strain 1A01261. PloS One **7:**e46587.

25. **Lee K-M, Yoon MY, Park Y, Lee J-H, Yoon SS.** 2011. Anaerobiosis-induced loss of cytotoxicity is due to inactivation of quorum sensing in *Pseudomonas aeruginosa*. Infection and Immunity **79:**2792–2800.

26. **Sadikot RT, Blackwell TS, Christman JW, Prince AS.** 2005. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. American Journal of Respiratory and Critical Care Medicine **171:**1209–1223.

27. **Sawa T, Ohara M, Kurahashi K, Twining SS, Frank DW, Doroques DB, Long T, Gropper MA, Wiener-Kronish JP.** 1998. In vitro cellular toxicity predicts *Pseudomonas aeruginosa* virulence in lung infections. Infection and Immunity **66:**3242–3249.

28. **Azghani AO.** 1996. *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A. American Journal of Respiratory Cell and Molecular Biology **15:**132–140.

29. **Smith RS, Fedyk ER, Springer TA, Mukaida N, Iglewski BH, Phipps RP.** 2001. IL-8 production in human lung fibroblasts and epithelial cells activated by the *Pseudomonas* autoinducer *N*-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2. Journal of

Immunology **167:**366–374.

30. **Wagner C, Zimmermann S, Brenner-Weiss G, Hug F, Prior B, Obst U, Hänsch GM.** 2007. The quorum-sensing molecule *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL) enhances the host defence by activating human polymorphonuclear neutrophils (PMN). Analytical and Bioanalytical Chemistry **387:**481–487.

31. **Allen L, Dockrell DH, Pattery T, Lee DG, Cornelis P, Hellewell PG, Whyte MKB.** 2005. Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. Journal of Immunology **174:**3643–3649.

32. **Jensen PØ, Bjarnsholt T, Phipps R, Rasmussen TB, Calum H, Christoffersen L, Moser C, Williams P, Pressler T, Givskov M, Høiby N.** 2007. Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. Microbiology **153:**1329–1338.

FIGURE LEGENDS

FIGURE 1. TLC analysis of AHL produced by *P. aeruginosa* strains

Lane 1: AHL standards (C4-HSL, C6-HSL, and 3-oxo-C12-HSL). Lane 2: PAO1. Lane 3: PAO1/pMJT1. Lane 4: PAO1/pMJT1-aiiM. Spots were visualised with the AHL-reporter strains *C. violaceum* CV026 for short-chain AHL (a) or *C. violaceum* VIR07 for long-chain AHL (b). None of the three AHLs were detected from PAO1/pMJT1-aiiM.

FIGURE 2. The amount of pyocyanin (a) and elastase (b) activity in the culture supernatant of PAO1/pMJT1 or PAO1/pMJT1-aiiM

Each bar represents the mean of mean of triplicates of the sample and the error bars indicate the standard error of the mean. PAO1/pMJT1-aiiM produced very low levels of both pyocyanin and elastase.

FIGURE 3. Cytotoxicity of A549 cells after infection with *P. aeruginosa* PAO1/pMJT1 (closed circle), PAO1/pMJT1-aiiM (open circle), or uninfected control (cross)

Cytotoxicity was determined by measuring the LDH release. Every data point represents the mean of mean of triplicates of the sample and the error bars indicate the standard error of the mean. *P. aeruginosa* PAO1/pMJT1-aiiM showed reduced cytotoxicity. *, $P < 0.05$ compared with PAO1/pMJT1.

FIGURE 4. Analysis of survival data and bacterial loads

a) Kaplan–Meier survival curve of the mice infected with *P. aeruginosa* PAO1/pMJT1 (closed circle) or PAO1/pMJT1-aiiM (open circle). Survival was followed for 7 days. n = 10–12 mice per group. *P. aeruginosa* PAO1/pMJT1-aiiM

exhibited significantly lower mortality than wild-type control strain. *, $P < 0.01$ compared with PAO1/pMJT1. b) Bacterial loads in the lungs, spleen, and blood at 24 h after infection with *P. aeruginosa* PAO1/pMJT1 (closed circle) or PAO1/pMJT1-aiiM (open circle). Each horizontal line represents the mean of the bacterial counts. n = 8 mice per group. *P. aeruginosa* PAO1/pMJT1-aiiM had a decreased ability to disseminate in the infected mouse.

FIGURE 5. Lung tissue damage caused by *P. aeruginosa* infection

Histological examination of the lungs from the mice infected with *P. aeruginosa* PAO1/pMJT1 (a) and PAO1/pMJT1-aiiM (b). Lung tissue sections were obtained at 24 h post infection, and stained with haematoxylin and eosin. Representative images were shown at an original magnification of 25×. Scale bar = 200 μm. The amount of haemoglobin (c), albumin (d), and LDH (e) in cell-free BAL fluids at 24 h after infection with *P. aeruginosa* PAO1/pMJT1, PAO1/pMJT1-aiiM or uninfected control. Each bar represents average values and error bars show standard error of the mean. n = 8 mice per group. Mice infected with *P. aeruginosa*

PAO1/pMJT1-aiiM developed only small lung lesions and had significantly decreased levels of haemoglobin, albumin, and LDH in BAL fluids.

FIGURE 6. BAL fluid analysis

BAL fluids were harvested 24 h after infection with *P. aeruginosa* PAO1/pMJT1, PAO1/pMJT1-aiiM, or uninfected control. Total cell counts (a) and differential cellular analysis (b) MPO levels (c) in cell-free BAL fluids. Each bar represents average values and the error bars show the standard error of the mean. n = 8 mice per group. Both *P. aeruginosa* PAO1/pMJT1 and PAO1/pMJT1-aiiM induced a similar increase in the total BAL cell and neutrophil numbers in infected mice. The levels of MPO in cell-free BAL fluids were significantly reduced in the mice infected with *P. aeruginosa* PAO1/pMJT1-aiiM. Neu = neutrophils; Mac = macrophages; Lym = lymphocytes.

FIGURE 7. Proinflammatory cytokine and chemokine levels in cell-free BAL fluids at 24 h post infection

Each bar represents average values and the error bars show the standard error of the mean. n = 8 mice per group. Mice infected with *P. aeruginosa* PAO1/pMJT1-aiiM produced lower levels of TNF-α, IL-1β, IL-6, and MIP-2 in cell-free BAL than in mice infected with PAO1/pMJT1.

571 **TABLES**

572 **Table 1.** Bacterial strains and plasmids used

Strain or plasmid	genotype	Source
P. aeruginosa		
PAO1	Wild-type prototroph	12
PAO1/pMJT1	PAO1 with pMJT1	15
PAO1/pMJT1-aiiM	PAO1 with pMJT1-aiiM	This study
C. violaceum		
CV026	ATCC 31532 derivative; cviI::Tn5xylE; KmR SmR	11
VIR07	ATCC 12472 derivative; cviI::KmR; AmpR	11
Plasmid		
pUC118-aiiM	pUC118 (cloning vector, Takara Bio) containing homoserine lactonase gene	11

	aiiM; AmpR	
pMJT1	Broad-host-range expression vector containing the araBAD promoter and the araC gene; AmpR, CarbR	15
pMJT1-aiiM	pMJT1 with arabinose-inducible *aiiM*; AmpR, CarbR	This study

573 *P. aeruginosa*: *Pseudomonas aeruginosa*; *C. violaceum*: *Chromobacterium*
574 *violaceum*.

575

576 **Table 2.** Amplification primers

Primer name	sequence
AiiMrbsNheI_F	TACgctagcGATATGCTCGGGCAAAGCCCG
AiiMendEcoRI_R	CTCgaattcTCGACGACGACATCCAGCTCCACG

577

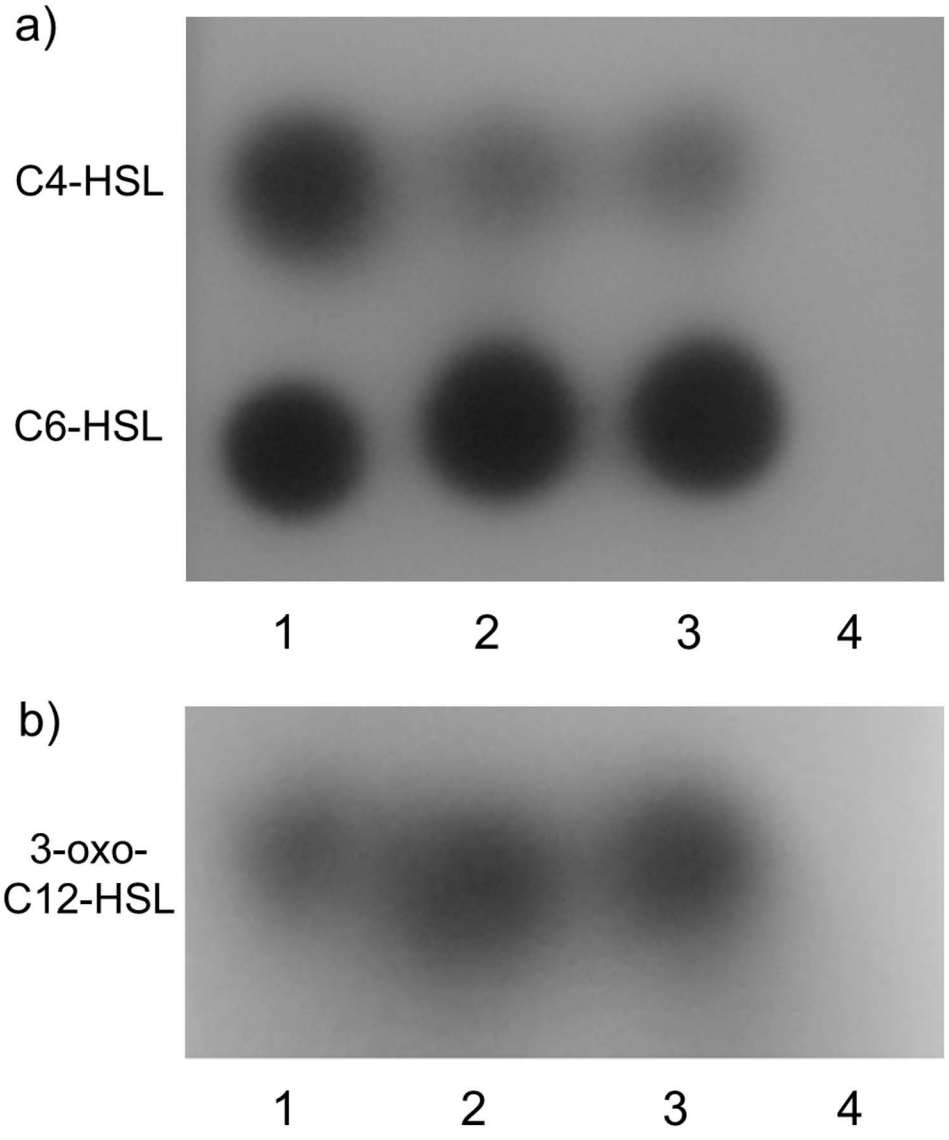

FIGURE 1.

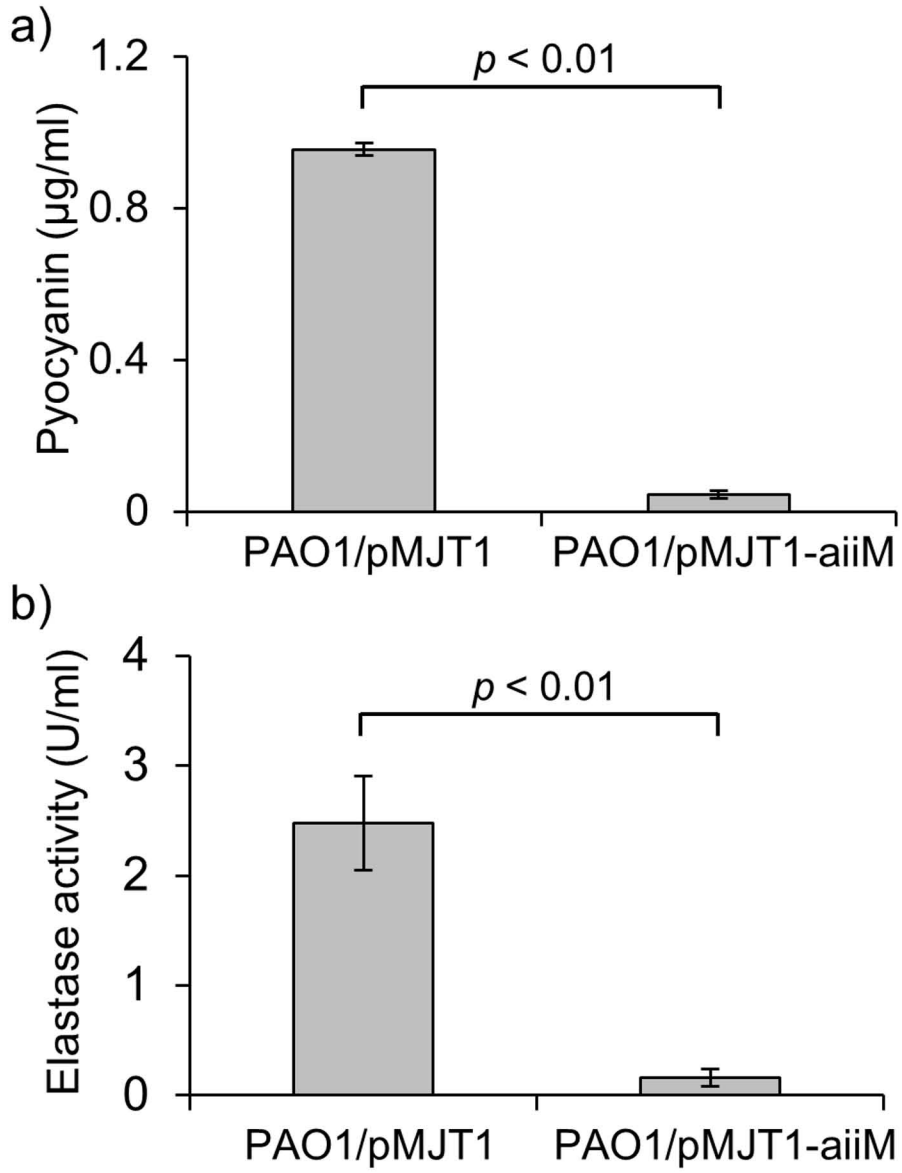
a)
1.2
0.8
0.4
0
Pyocyanin (µg/ml)
p < 0.01
PAO1/pMJT1
PAO1/pMJT1-aiiM
b)
4
3
2
1
0
Elastase activity (U/ml)
p < 0.01
PAO1/pMJT1
PAO1/pMJT1-aiiM

FIGURE 2.

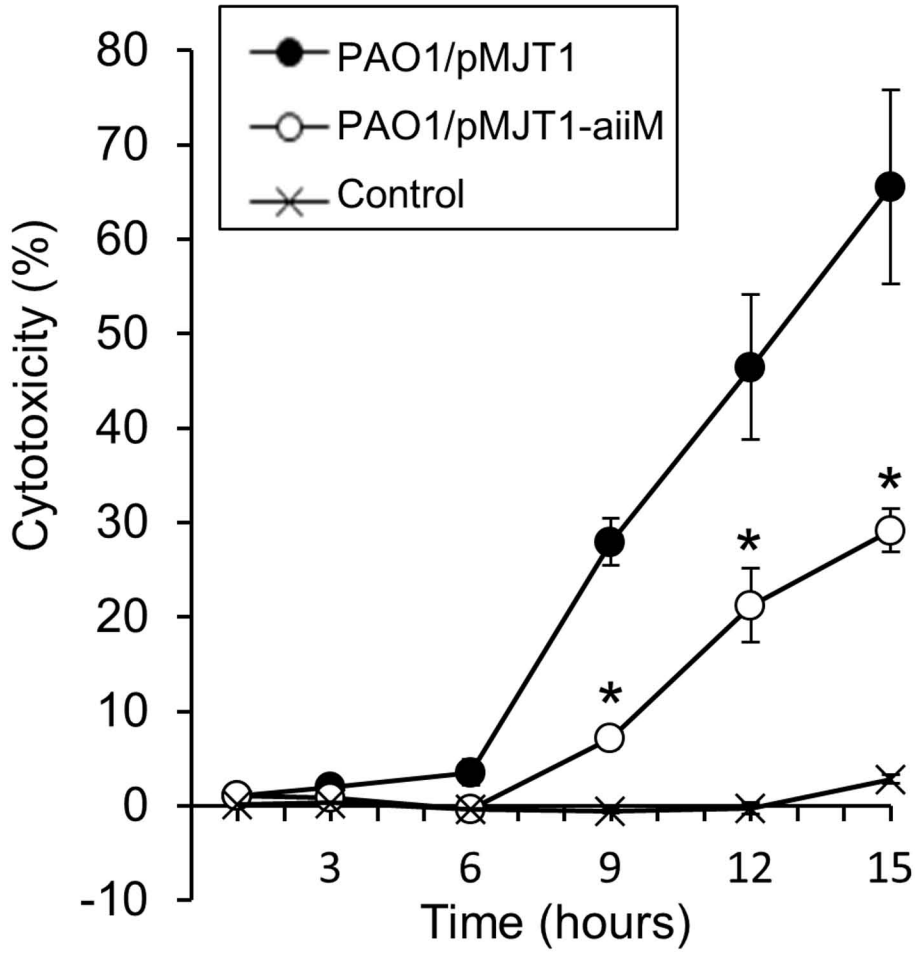
PAO1/pMJT1
PAO1/pMJT1-aiiM
Control
Cytotoxicity (%)
80
70
60
50
40
30
20
10
0
-10
3
6
9
12
15
Time (hours)
*
*
*

FIGURE 3.

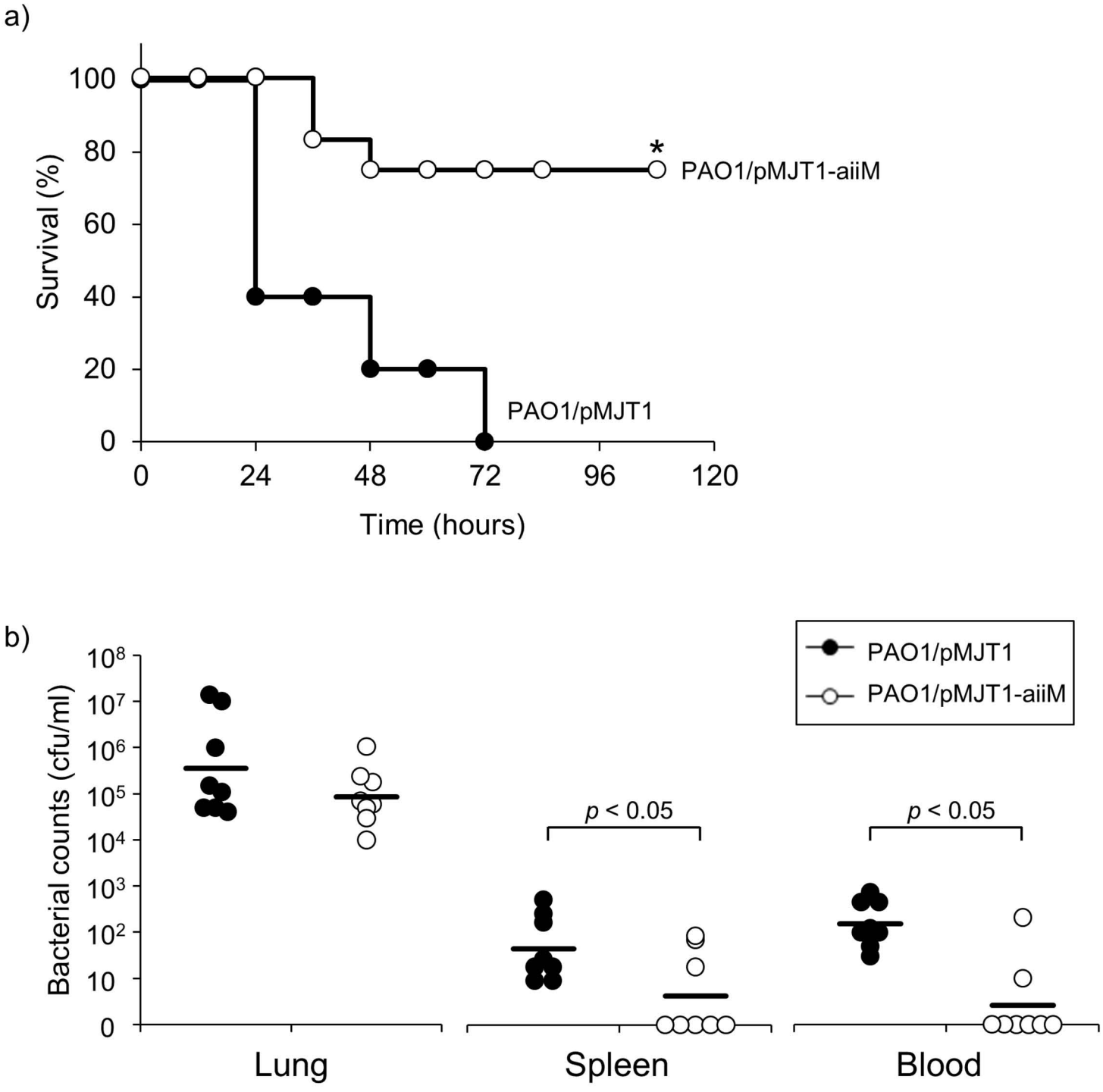
a)
100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120
Time (hours)
*
PAO1/pMJT1-aiiM
PAO1/pMJT1
b)
Bacterial counts (cfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
PAO1/pMJT1
PAO1/pMJT1-aiiM
$p < 0.05$
$p < 0.05$
Lung
Spleen
Blood

FIGURE 4.

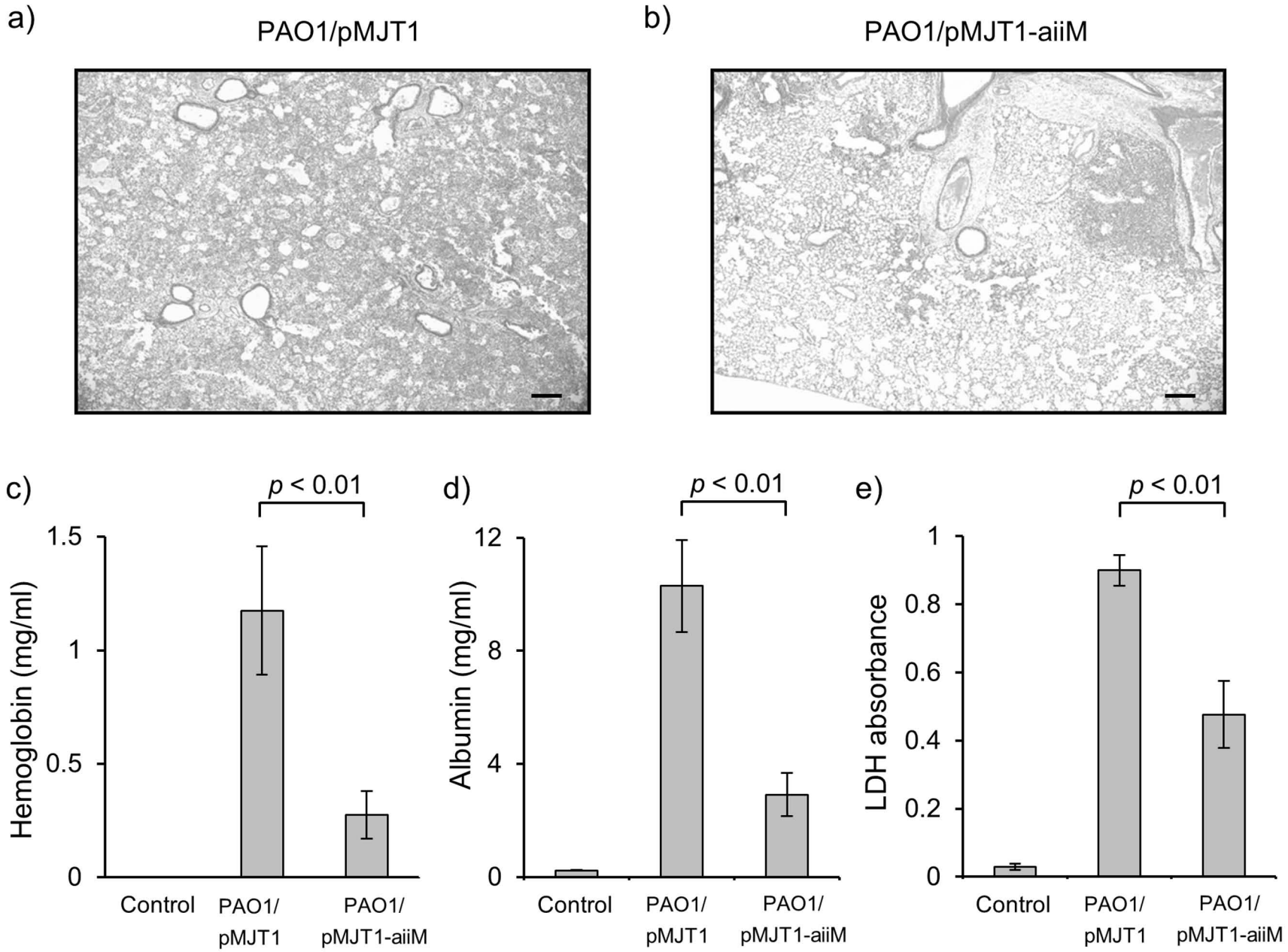
a)
PAO1/pMJT1
b)
PAO1/pMJT1-aiiM
c)
p < 0.01
Hemoglobin (mg/ml)
1.5
1
0.5
0
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM
d)
p < 0.01
Albumin (mg/ml)
12
8
4
0
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM
e)
p < 0.01
LDH absorbance
1
0.8
0.6
0.4
0.2
0
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM

FIGURE 5.

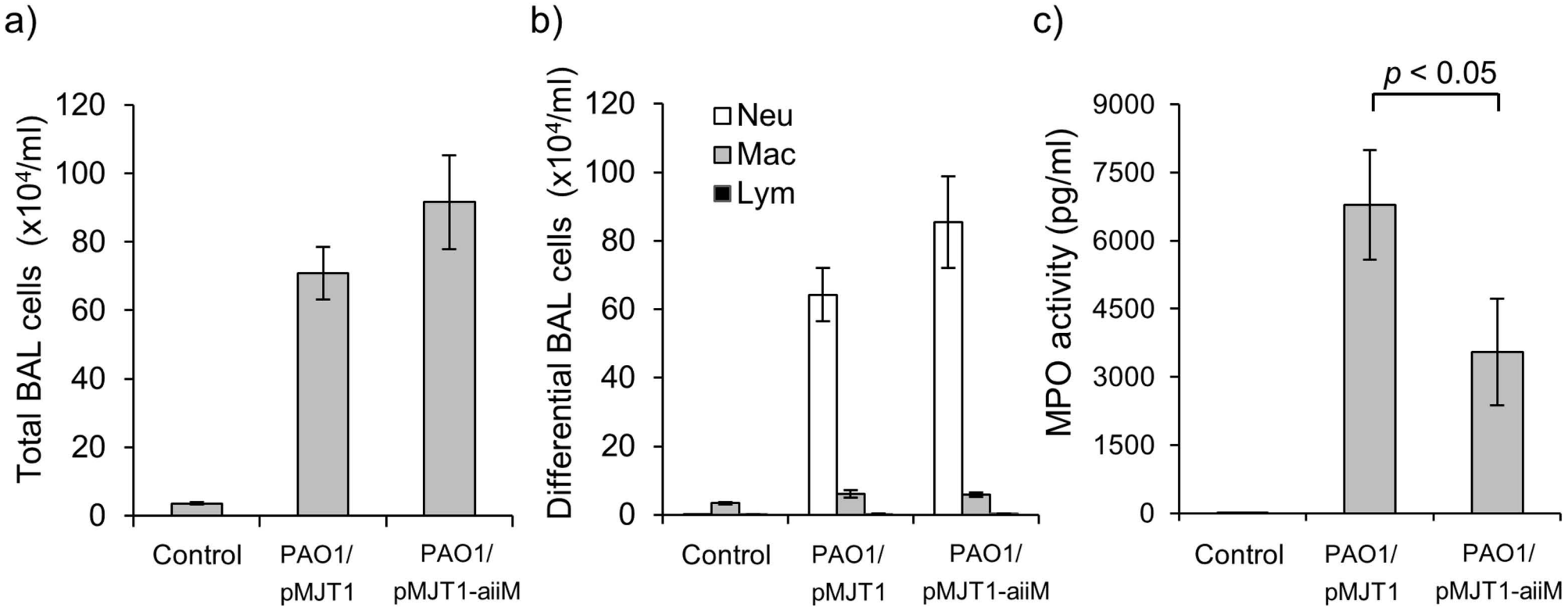
a)
Total BAL cells (x10⁴/ml)
120
100
80
60
40
20
0
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM
b)
Differential BAL cells (x10⁴/ml)
Neu
Mac
Lym
c)
p < 0.05
MPO activity (pg/ml)
9000
7500
6000
4500
3000
1500

FIGURE 6.

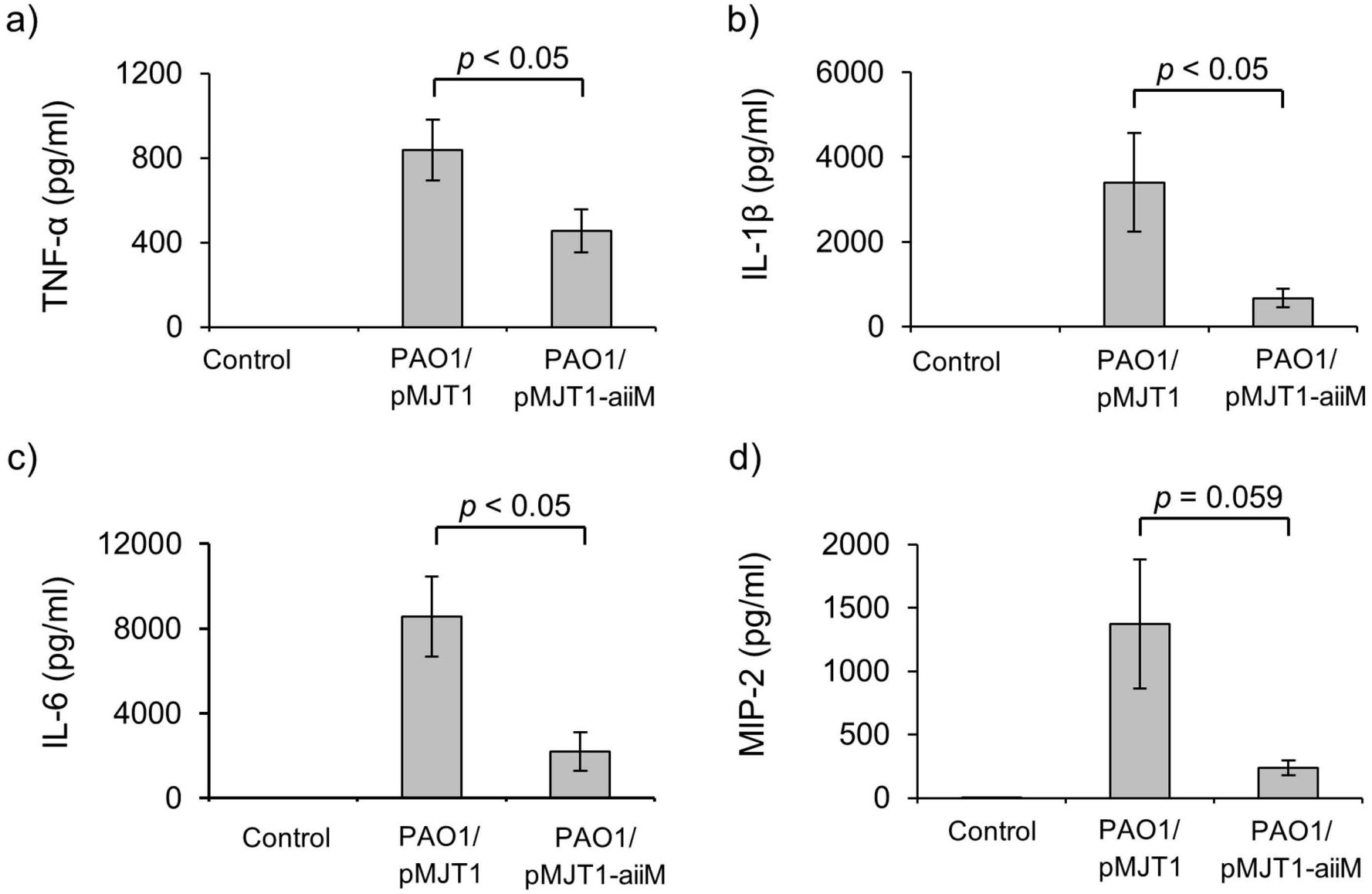
a)
TNF-α (pg/ml)
1200
800
400
0
p < 0.05
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM
b)
IL-1β (pg/ml)
6000
4000
2000
0
p < 0.05
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM
c)
IL-6 (pg/ml)
12000
8000
4000
0
p < 0.05
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM
d)
MIP-2 (pg/ml)
2000
1500
1000
500
0
p = 0.059
Control
PAO1/ pMJT1
PAO1/ pMJT1-aiiM

FIGURE 7.