In vivo imaging of the lung inflammatory response to *Pseudomonas aeruginosa* and its modulation by azithromycin

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Running title: *In vivo* monitoring lung inflammation

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ABSTRACT

Chronic inflammation of the airways is a central component in lung diseases and is frequently associated with bacterial infections. Monitoring the pro-inflammatory capability of bacterial virulence factors *in vivo* is challenging and usually requires invasive methods. We have evaluated the benefit of using a mouse model, transiently expressing the luciferase reporter gene under the control of an heterologous IL-8 bovine promoter, to detect and monitoring lung inflammation. This experimental animal model is based on the transient transduction of the IL-8 promoter, a gene representing a major player during inflammation, essential for leukocytes recruitment to the inflamed tissue and potentially an appropriate molecular read-out for monitoring the activation of inflammatory pathways caused by bacterial virulence factors. Lung inflammation in mice was induced using the culture supernatants from two *Pseudomonas aeruginosa* clinical strains, VR1 and VR2, isolated from patients affected by cystic fibrosis and showing different phenotypes in terms of motility, colony characteristics and biofilm production as well as pyoverdine, pyocyanine release. More interesting, the strains differ also for the presence in supernatants of metalloproteases, a family of virulence factors with known pro-inflammatory activity. *In vivo* imaging indicated that VR1 strain, releasing in its culture supernatant metalloproteases and other virulence factors, induced lung inflammation while the VR2 strain presented with a severely reduced pro-inflammatory activity. The bioluminiscence signal was clearly visible from 4 to 48 hours after supernatant instillation. The animal model was also used to test the anti-inflammatory activity of azithromycin, an antibiotic with demonstrated inhibitory effect on the synthesis of bacterial exoproducts. The inflammation signal in mice was in fact significantly reduced when bacteria grew in the presence of a sub-lethal dose of azithromycin causing inhibition of the synthesis of metalloproteases and other bacterial elements. The *in vivo* data were further supported by quantification of immune cells and cytokine expression in mouse broncho-alveolar lavage samples. The data presented indicate that the model is suitable to functionally monitor in real time the lung inflammatory response facilitating the identification of bacterial factors with pro-inflammatory

activity and the evaluation of the anti-inflammatory activity of old and new molecules for therapeutic use.

INTRODUCTION

Airway inflammation is a central component of a number of chronic lung diseases such as severe asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and bronchiectasis. Airway inflammation is characterized by edema, cellular infiltration, activated T lymphocytes and mast cells, increased airway secretions, and deposition of excess collagen [1, 2]. Frequently, the inflammation is associated with bacterial infections such as those caused by *Pseudomonas aeruginosa*, an opportunistic human pathogen involved in severe airway infections especially in patients suffering from CF and COPD [1, 3]. During the early onset of the lung infection, *P. aeruginosa* secretes a high number of virulence factors which are responsible for tissue damage and inflammation [4]. As the infection progresses, the bacterium switches off most of the virulence genes but synthesizes a biofilm matrix and becomes resistant to antibiotics causing a chronic disease frequently leading to respiratory failure and lung transplantation or death [4].

Therefore, it is mandatory to identify those factors and conditions causing lung cell damage and favoring the passage from an acute to a chronic bacterial infection by monitoring, for long times, the inflammation process. Furthermore, to avoid the onset of the chronic phase of the infection, it is important to treat *P. aeruginosa* infection during the acute phase using efficient antibiotic therapy and anti-inflammatory drugs.

By standard methods, the inflammation of the respiratory tract can be monitored by counting immunological markers recruited during the inflammatory process with sputum collection, a technique which provides poorly reliable results, or invasive sampling techniques such as bronchoscopy [5]. Animal models of acute and chronic lung infection have been used to study the bacterial behavior and for monitoring the host response in vivo [1, 6]. These models provide an important resource to identify essential bacterial genes for in vivo infection persistence and for the development and testing of new therapies [6, 7]. Recently, a mouse model, transiently expressing the luciferase reporter gene under the control of the bovine IL-8 promoter, has been described [8] and demonstrated to be suitable to functionally monitor in real time the lung inflammatory response

[8, 9, 10,11]. This small size experimental animal model is based on the transient transduction of the IL-8 promoter, a gene representing a major player during inflammation, essential for leukocytes recruitment to the inflamed tissue and an appropriate molecular read-out for monitoring the activation of inflammatory pathways [8]. Although mice do not have an IL-8 gene, mouse cell signaling and their transcriptional apparatus could specifically activate the bovine IL-8 gene promoter. Since lung disease manifestation in ruminants overlap with the majority of human lung disease manifestations, this model could be of great value to study human lung diseases too.

It has been shown that the *P. aeruginosa* strains isolated during the early phase of lung colonization had a pro-inflammatory capability higher than that induced by strains isolated during lung chronic colonization [12]. The pro-inflammatory effect, associated with the expression of IL-8 mRNA in CF airway epithelial cells, was shown to be associated to several *Pseudomonas* released proteins with proteolytic activity including members of the metalloprotease (MP) family [12]. Other virulence factors secreted by *Pseudomonas* such as exotoxins and exoenzymes, pyoverdine and pyocianine are also involved in tissue damage and inflammation. It has been suggested that azithromycin (AZM), an antibiotic used in CF patients, could elicit its anti-inflammatory activity by decreasing the synthesis of *Pseudomonas* exoproducts [13, 14, 15]. In fact, Molinari et al [13] have shown that sub-lethal doses of AZM strongly suppressed the synthesis of elastase, lecithinase, proteases and DNase, while in another study it has been shown that AZM reduced the production of bacteria virulence factors by inhibiting quorum-sensing [15].

In the present study, the IL-8/luciferase transgenic mouse model has been used *in vivo* to monitor the IL-8 mediated lung inflammation induced by *P. aeruginosa* secreted pro-inflammatory virulence factors and their modulation by azithromycin.

MATERIALS AND METHODS

Collection of bacterial cell-free supernatants

Two *P. aeruginosa* strains, VR1 and VR2, were isolated from sputum samples of cystic fibrosis (CF) patients followed at the Cystic Fibrosis Center in Verona, Italy. Written informed consent was obtained from the subjects enrolled in the study and approved by the Institutional Review Board of Azienda Ospedaliera Universitaria Integrata (AOUI) Verona as project 1612.

Bacterial strains were grown overnight at 37°C in Bacto™ Tryptic Soy Broth (TSB, Becton, Dickinson and Company) with continuous agitation. The day after, *P. aeruginosa* cells were diluted in TSB to the concentration of $1x10^8$ CFU/ml (OD of 0.1 at 600 nm) and incubated in absence and in presence of 8 µg/ml of AZM, at 37°C for 16 hours with continuous agitation. The concentration chosen for this antibiotic is in the sub-minimum inhibitory concentration (sub-MIC) range for *P. aeruginosa*, and is consistent with the concentrations found in lungs of patients treated with this drug (16). By adding TSB, the cultures were normalized to an optical density of 0.2 OD at 600 nm. Culture supernatants (Sn) were collected by centrifugation (7000 x g, 30 min, 4°C) and filtered through a 0.22 µm Millipore filter to remove any remaining bacteria. Supernatants were concentrated to 30X using Amicon Ultra-15 30K (Millipore, Billerica, USA), then centrifuged at 27000 x g for 1 h at 4°C to remove cellular debris and finally sterilized by filtration through a Millipore 0.22 µm filter.

Phenotype characterization

Colony characterization:

P. aeruginosa VR1 and VR2 isolates were grown on LB agar plates for 24-48 h at 37°C and colony morphotypes were visually inspected for color, shape, edges regularity, and mucoidy.

Motility assays:

Swimming plates (1□% tryptone, 0.5□% NaCl and 0.3□% (w/v) agar) were point-inoculated from an overnight culture with a sterile toothpick and incubated at 37 °C for 24 h. The zone diameter was measured to assess swimming motility.

Swarming plates (0.5% peptone, 0.3% yeast extract, 0.5% NaCl, 0.5% D-glucose and 0.5% (w/v) agar) were point-inoculated from an overnight culture with a sterile toothpick and incubated at 37 °C for 24 h. Swarming motility was assessed by measuring the circular turbid zones formed by the bacterial cells migrating away from the point of inoculation.

Twiching motility was evaluated on Luria-Bertani (LB) 1% (w/v) agar plates. Overnight cultures were stabbed with a sterile toothpick through the agar layer to the bottom of the Petri dish. The plates were then incubated at 37 °C for 48 h. Twiching motility was examined by measuring the diameter of the halo formed in the plastic-agar interface.

Biofilm formation assay

Bacterial cells were grown at 37 °C in TSB-1% glucose until they reached the exponentially growing phase (OD_{650nm}=0.4). Exponentially growing cells were then diluted in TSB-1% glucose medium to reach 10^6 CFU/ml. Two hundred microliter of each cell suspension were used to inoculate sterile flat-bottomed polystyrene microtiter plates (CytoOne, Starlab) and plates were incubated aerobically at 37 °C without agitation for 48 h to allow biofilm formation. After incubation, the planktonic cells were aseptically aspirated, washed with sterile physiological solution and dried. For biofilm quantification, 100 µl of 1% methylene blue were added to each well and the plate maintained for 15 minutes at room temperature. The wells were subsequently slowly washed once with sterile and dried at 37 °C. The methylene blue bound to the biofilm was extract using 100 µl of 70% ethanol and the absorbance measured at 570 nm using “A3 Plate Reader” microplate reader (DAS Srl, Italy). All the mentioned experiment were performed in triplicate.

Virulence factor assays

The presence of virulence factors in culture supernatants was evaluated after growth of the bacterial strains with and without azithromycin.

Gelatin-zymography for analysis of the protease profile.

Five ml of 5X SDS sample buffer (5% SDS, 0.5 M Tris-HCl pH 6.8, 25% glycerol) were added to 20 µl of culture supernatants. The sample was run on a SDS-PAGE gel containing 1 mg/ml gelatin (Sigma-Aldrich) as described (12). The gel was washed twice (20 min per cycle) with 2.5% Triton X-100 at room temperature, then incubated in 200 ml of activation buffer (10 mM Tris-HCl, 1.25% Triton X-100, 5 mM CaCl2, 1 mM ZnCl2) overnight at 37 °C and finally stained with Coomassie Brilliant Blue G-250 in 20% methanol/10% phosphoric acid/10% ammonium sulfate and destained in water.

In Vitro Imaging Probe

A near infrared (NIR)-activatable "smart" probe (Perkin Elmer, Inc. Boston, MA, USA) was used for imaging protease activities. Specifically, MMPsense 750 FAST is a proteases activatable fluorescent *in vivo* imaging agent that is activated by key disease associated-proteinases such as MMPs including MMP-2, -3, -9 and -13 and bacterial proteases. Smart probes are optically silent in their inactivated state and becomes highly fluorescent following protease-mediated activation. The MMPsense 750 FAST probe was added to 50 µl of culture supernatants at the final concentration of 0.02 nmoles in a 96-well plate and an imaging system (IVIS, Caliper Life Sciences, Alameda, CA, USA) was used to quantify the time course of the fluorescence every 30 sec from 1 to 40 min.

Pyoverdine assay:

The production of pyoverdine by *P. aeruginosa* was measured spectrophotometrically by a modification of the method described by Meyer and Abdallah. Overnight cultures of the strains were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in 30 ml of King's B broth (low-iron medium) with and without 8 µg/ml AZM and grown at 37°C until they reached an OD_{600} of ≈2-3

(after 16 h). Cultures were normalized to OD_{600} of 0.2 in order to reproduce the conditions of supernatants used for mice stimulation. The absorbance of cell-free supernatants was measured at 405 nm. The concentration of pyoverdine was calculated by using the extinction coefficient ($1.9 \times 10^{-4}\ M^{-1}\ cm^{-1}$).

Pyocyanin assay:

The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution (Essar et al, 1990). Bacterial cultures were grown in LB medium with or without AZM following the same conditions described for pyoverdine assay. Five milliliters of cell-free supernatants were extracted with 3 ml of chloroform. The lower phase was mixed with 1 ml of 0.2 M HCl, and the absorbance of the resulting upper pink phase was measured at 520 nm (A_{520}). Concentration, expressed as μg of pyocyanin per ml of culture supernatant, was determined by multiplying the A520 value by 17.072.

Experimental Animals

Female inbred BalbC (7–8 week-old) mice were purchased from Harlan Laboratories Italy (San Pietro al Natisone, Udine, Italy). Prior to use, animals were acclimatized for at least 5 days to the local vivarium conditions (room temperature: 20–24°C; relative humidity: 40–70%; 12-h light-dark cycle), having free access to standard rodent chow and softened tap water. Animal experiments were conducted in compliance with national (Decreto Legislativo numero 26, 4 Marzo 2014) and international laws and policies (Guide for the Care and Use of Laboratory Animals) [17]. Animal studies were approved by the Institutional Animal Care and Use Committee at Chiesi Farmaceutici, Parma, Italy.

***In Vivo* Gene Delivery**

The bIL-8-Luc plasmid (Department of Medical Veterinary Science, University of Parma, Italy) was obtained by sub-cloning the 2030 bp IL8 bovine promoter, amplified by PCR from Madin-Darby bovine kidney (MDBK; ATCC #CCL-22) genomic DNA and sub-cloned into the digested

pGL3basic vector (Promega) as previously described [8]. We applied *in vivo* JetPEI (Polyplus Transfection) as a carrier for delivering DNA to lung tissues. The DNA and JetPEI mix was formulated according to the product manual with a final N/P ratio of 7. Briefly, 40 μg of bIL-8–luc and 7 μL of JetPEI were both diluted into 200 μL 5% glucose. The two solutions were then mixed and incubated for 15 minutes at room temperature. The entire mixture was injected intravenously into BalbC mice and the expression of bIL-8–Luc was monitored through imaging with an IVIS imaging system.

***In Vivo* Bioluminescence Imaging**

Transfection per se causes a mild lung inflammatory response and bIL-8 activation that is detectable by BLI up to 3–4 days after DNA injection and disappears completely after 1 week.

One week after DNA delivery, the transient transgenic mice were injected intraperitoneally (i.p.) with luciferin (150 mg/kg) and bioluminescence imaging (BLI) was recorded in order to check the baseline activation of the IL-8 pathway. Briefly, 10 and 15 min following luciferin injection, mice were lightly anesthetized with isoflurane and images were obtained using the IVIS imaging system: an average of photons emitted from the chest of the mice was quantified using Living Image® software (Caliper Life Sciences, Alameda, CA, USA). Data were expressed as mean folds of induction (FOI) over the baseline activation of each mouse. The following day, mice were intratracheally challenged with 10X supernatants and BLI was recorded at 4, 24, and 48 hours.

Bronchoalveolar Lavage and Cytokine Determination

Twenty-four hours after intratracheal challenge, animals were weighted, anaesthetized with isoflurane and sacrificed by bleeding from the abdominal aorta for bronchoalveolar lavage fluid (BALF) collection, performed as previously described [8]. BALF supernatants were frozen at -80 °C for simultaneous quantitation of multiple cytokines/chemokines using a Bio-Plex™ Cytokine Assay Kit (Bio-Rad Laboratories, Segrate, Milano, Italy).

The cell pellet was suspended in 0.2 ml of PBS and total and differential cell counts were obtained using an automated cell counter (Dasit XT 1800J, Cornaredo; Milano, Italy).

Reagents

In vivo JetPEI DNA transfection reagent (Polyplus Transfection) was obtained from Euroclone (Milan, Italy), D-luciferin was obtained from Perkin Elmer Inc. (Boston, MA,USA). Azithromycin was from Pfizer (Italy).

Data analysis

Experimental values were expressed as the mean and standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance followed by Dunnett's t test (* $p < 0.05$, ** $p < 0.01$).

RESULTS

Phenotypic characterization of *Pseudomonas aeruginosa* clinical isolates

We hypothesized that one or more virulence factors released by *P. aeruginosa* in the lung of CF patients during infection might have a role as pro-inflammatory factors. Specifically and in addition to other released bacterial products, we considered tested the role of metalloproteases it has been demonstrated in CF airway epithelial cells that the pro-inflammatory role is mainly associated to *Pseudomonas* released proteins with proteolytic activity [12]. We have therefore selected two clinical *P. aeruginosa* strains, VR1 and VR2, isolated from two different patients and showing significant differences as regards phenotypic characteristics and presence of metalloproteases and other virulence factors in culture supernatants. The phenotypic characterization of the two strains is shown in Table 1. VR1 and VR2 differentiated for the production of pyoverdin and pyocyanin

which are released only in the VR1 supernatant, the capability of moving by swimming, swarming and twitching detectable in VR1 but non in VR2, the production of biofilm which is more evident in VR1 with respect to VR2 and by the level of protease activity, well detectable in the VR1 supernatant and very reduced in the VR2 one.

Culture supernatants were prepared from the two different bacterial strains by grown them in a culture medium in the absence (SnVR1 and SnVR2) and in the presence of a sub-MIC dose of azithromycin (SnVR1+AZM and SnVR2+AZM). The inhibitory action of AZM on the expression of *Pseudomonas* virulence factors is clearly shown in Table 1. The specific inhibitory effect of AZM on metalloprotease synthesis was also demonstrated by testing supernatants for the presence of proteolytic activity with a proteases activable fluorescent probe (Figure 1) and by zymography (Figure 2)

In vivo monitoring of lung inflammation induced by _P. aeruginosa_ culture supernatants on IL-8 transiently transgenic mice

In vivo monitoring of lung inflammation after intra-tracheal challenge with *P. aeruginosa* SnVR1 and SnVR2 at 1X, 3X, 10X and 30X concentrations was conducted by in vivo imaging in bIL-8 luc transient transgenic mice. Administration of the 10X SN concentration was sufficient to induce the maximal increase in BLI signal. In fact, the use of the 30X Sn preparation did not translate into a higher inflammation signal, indicating a saturation of the system at lower concentration (10X) (Figure 3A). For this reason, the rest of the experiments were conducted at 10X concentration. SnVR1 pro-inflammatory activity was clearly detectable as soon as 4 hours post instillation, although the BLI signal reached the highest peak at 24 hours (FOI 5.11±1.07) and was still detectable at 48 hours (Figure 3B). On the contrary, the SnVR2 did not induce detectable inflammation in the mouse lung at any time point. The BLI signal induced by the challenge with SnVR1+AZI was significantly low (FOI 1.58±0.21) after 24 hours with respect to the response

induced by SnVR1 obtained from the same bacterial strain grown without the antibiotic (Figure 3B).

Recruitment of inflammatory cells and cytokine activation induced by *P. aeruginosa* culture supernatants

Twenty-four hours after mice stimulation with *P. aeruginosa* products, BALF was recovered from IL-8 transgenic animals treated with both VR1 and VR2 supernatants in order to compare their effect on cell recruitment and cytokine expression. SnVR1, containing a series of important virulence factors, significantly stimulated total white blood cells (WBC) and neutrophils recruitment (respectively $6.52x10^3$±0.44 and $4.19x10^3$±0.43cells/µl) and the expression of cytokines IL-1β, TNF-α, IL-17, RANTES, KC, IL 12 (p70) and IL 12 (p40) (Figures 4 and 5). SnVR2 profile differed from the one of SnVR1, being the effect of SnVR2 lower with respect to both inflammatory cells recruitment (WBC 2.39±0.23$x10^3$ and neutrophils 1.70±.0.22 $x10^3$ cells/µl) and expression of the cited cytokines. SnVR2 showed a comparable effect on the release of RANTES and IL 12 (p40).

BALF was recovered also from IL-8 transgenic mice stimulated with SnVR1+AZM and SnVR2 +AZM in order to evaluate the effect of the antibiotic on the pro-inflammatory activity of both preparations. The challenge with SnVR1+AZM stimulated at a significant lower level WBC and neutrophils recruitment (respectively 4.07±0.39$x10^3$ and 2.05±03.4 $x10^3$ cells/µl) (Figure 4) and the expression of the cytokines IL-1β, IL-17, RANTES, KC and IL-12 (p70) but not that of TNF-α and IL 12 (p40) in comparison with animals treated with SnVR1 (figure 5). A significant difference between the stimulation caused by SnVR2 and SnVR2+AZM was not observed except for RANTES and IL12-(p40) level (Figure 5).

CONCLUSIONS

The mechanisms and mediators that drive the induction and progression of chronic inflammation and altered lung function in a series of human diseases are not completely understood and this has severely hampered the development of effective treatments [1, 2, 3]. Although most of the animal models do not exactly mimic human lung diseases and each model has its own benefits and deficiencies, animal modelling systems that accurately reflect human disease pathophysiology continue to be essential for the development and validation of pathogenic aspects and new therapies. In most mammalian models, TNFα, IL-1β, and IL-8 are central components of a complex cytokine network that initiates, amplifies, and sustains the inflammatory response in tissue [18]. Available evidence also supports the importance of this network in coordinating acute inflammatory responses within the lung. The mouse model used in this study, transiently transgenized with a luciferase reporter gene under the control of the IL-8 bovine promoter, can be easily employed for monitoring the human lung inflammation due to the high similarity existing between respiratory diseases in ruminants and humans without the high costs and demands in terms of maintenance of ruminant models [8].

In the present study, the IL8/luciferase mouse model has been applied to the *in vivo* monitoring of lung inflammation induced by virulence factors released by *Pseudomonas aeruginosa* and to the evaluation of the anti-inflammatory action of AZM as an inhibitor of the synthesis of bacterial factors involved in pathogenicity. Data here presented show that a *P. aeruginosa* clinical strain, isolated from early lung colonization in a CF patient, synthesized flagella and biofilm and produced and released pyocyanin and pyoverdine and proteins with metallo-protease activity. The synthesis and release of these virulence factors significantly decreased when bacteria were grown in the presence of AZM. The pro-inflammatory effect of strain VR1 has been shown in IL-8/luciferase transiently transgenic mice by applying in vivo imaging and further confirmed by the increase in WBC and neutrophils recruitment and cytokine levels in the airways of transgenized mice. On the contrary, the supernatant from bacteria grown in the presence of AZM, a condition associated with

a much lower production of virulence factors, stimulated the inflammatory response only at a very reduced level. Similar low levels of IL-8 promoter activation was observed when the supernatant of the *P. aeruginosa* VR2 strain lacking the specific virulence factors, was instilled in the airways of IL-8/luciferase transgenized mice.

These data support the notion that the clinical benefits associated to AZM treatment in CF patients might be at least in part due to the lowering of the exoproduct synthesis induced by the antibiotic in bacterial cells.

Data obtained in this study demonstrate that the model is suitable to functionally monitor in real time the lung inflammatory response to bacterial products with pro-inflammatory activity and to confirm and better understand the anti-inflammatory effect of azithromycin, an antibiotic frequently used in cystic fibrosis. The non invasive nature of this mouse model and the possibility for bIL-8-luc transiently transgenized mice to be stimulated with bacterial products for long times [8] enables the monitoring of a biological process longitudinally in the same mouse and represents an obvious advance for functional as well as pharmacological studies. The model might be adapted and applied to study the pathogenesis of lung inflammatory diseases such as cystic fibrosis, to identify bacterial/nonbacterial factors with pro-inflammatory activity and to predict the possible therapeutic effect of known and new molecules with a presumptive anti-inflammatory action.

Acknowledgements

This work was supported by Fondazione Fibrosi Cistica project number 18/2013 and Lega Italiana Fibrosi Cistica (Italian Cystic Fibrosis League) through Veneto Branch - Associazione Veneta Lotta contro la Fibrosi Cistica Onlus

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Figure legends

Figure 1. Protease activity of cell-free supernatants from *Pseudomonas aeruginosa* grown in the presence and the absence of azithromycin.

MMPsense 750 FAST was added to supernatants from VR1 and VR2 strains grown in presence or absence of AZM (SnVR1 ± AZM and SnVR2 ± AZM) concentrated 1X, 10X and 30X. IVIS imaging system has been used to quantify the time course of the fluorescence. Data were expressed as induction folds over baseline (bacterial growth medium TSB). Results are reported as mean ± SEM and significance attributed when $P<0.05$ (*) or $P<0.01$(**).

Figure 2. Analysis of metalloproteases activity in VR1 and VR2 bacterial lysates and culture supernatants. MPs activity in lysates (2A) and supernatants (2B) from VR1 and VR2 *Pseudomonas aeruginosa* strains grown with and without azithromycin, as shown by zymography assay.

Figure 3.- In vivo imaging of lung inflammation induced by *P. aeruginosa* culture supernatants on IL-8 transiently transgenic mice

A) Representative images of mice (n = 3 per group) transiently transgenized with bIL-8-Luc and intratracheally instilled with bacterial cell-free 1X, 3X, 10X and 30X supernatants from VR1. The growth medium TSB was used as a control. Mice were monitored at 4, 24 and 48 hours post stimulation by BLI drawing a region of interest (ROI) over the chest.

B) Representative images of mice (n = 8 per group) transiently transgenized with bIL-8-Luc and intratracheally instilled with bacterial cell-free, 10X supernatants from VR1 and VR2 strains grown in presence or absence of AZM (VR1 ± AZM and VR2 ± AZM). The growth medium TSB was used as a control. Mice were monitored at 4, 24 and 48 hours post stimulation by BLI drawing a region of interest (ROI) over the chest.

Data are also presented as light intensity quantification of the ROI using the LivingImage software. The experiment was repeated 3 times and each point represents the mean ± standard error of 8 animals. Data were expressed as folds of induction (FOI) over baseline activity of each mice and statistical differences were tested by One Way ANOVA followed by Dunnet's post hoc test for group comparisons. Results are reported as mean ± SEM and significance attributed when $P<0.05$ (*) or $P<0.01$(**).

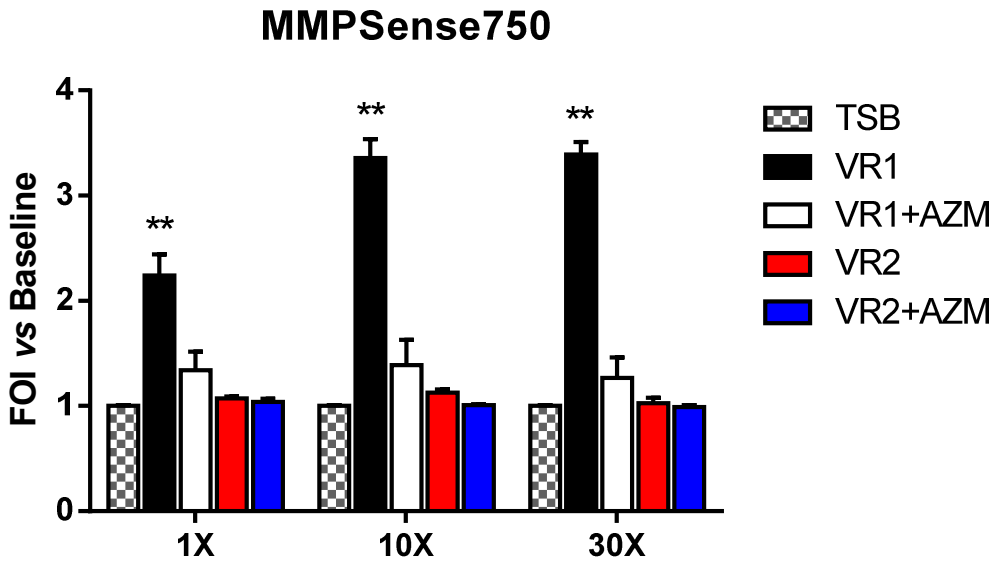
Figure 4.- Immune cell recruitment during lung inflammation caused by *P. aeruginosa* supernatants. Cellular infiltration into the lung of mice intratracheally instilled with bacterial cell-free 10X supernatants from VR1 and VR2 strains grown in presence or absence of AZM (VR1 ± AZM and VR2 ± AZM). TSB is the bacterial growth medium and was used as a control. The amount of white blood cells and neutrophils found in BALF was expressed as number of cells per µl at 24 hours post treatment. The experiment was repeated 3 times and each point represents the mean ± standard error of 8 animals. Results are reported as mean ± SEM and significance attributed when $P<0.05$ (*) or $P<0.01$(**).

Figure 5. Cytokines concentration in mice bronchial lavage fluid after stimulation with *P. aeruginosa* culture supernatants. Cytokine levels in BALF of IL-8 transgenized mice 24 hours after intratracheally challenge with bacterial cell-free supernatants from VR1 and VR2 strains grown in presence or absence of AZM (VR1 ± AZM and VR2 ± AZM). Data were expressed as folds of induction (FOI) over the control (bacterial growth medium TSB) The experiment was repeated 3

466 times and each point represents the mean ± standard deviation of 8 animals. Results are reported as
467 mean ± SEM and significance attributed when $P < 0.05$ (*) or $P < 0.01$(**).
468
469
470

Figure 1

A)


MMPSense750
FOI vs Baseline
0
1
2
3
4
**
**
**
1X
10X
30X
TSB
VR1
VR1+AZM
VR2
VR2+AZM

B)

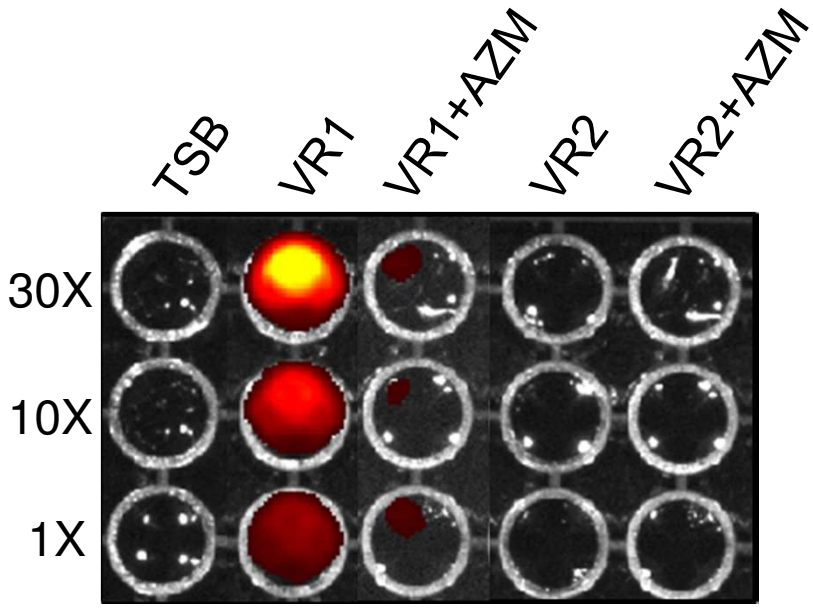

TSB
VR1
VR1+AZM
VR2
VR2+AZM
30X
10X
1X

Figure 2

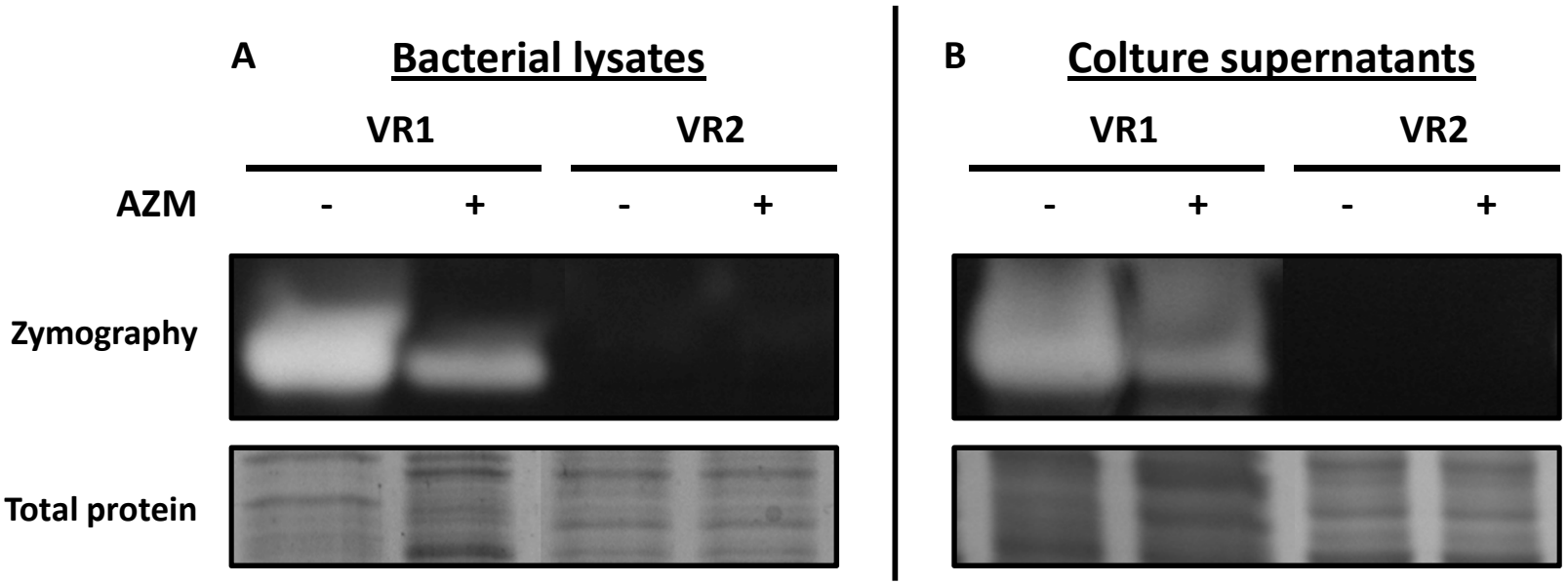
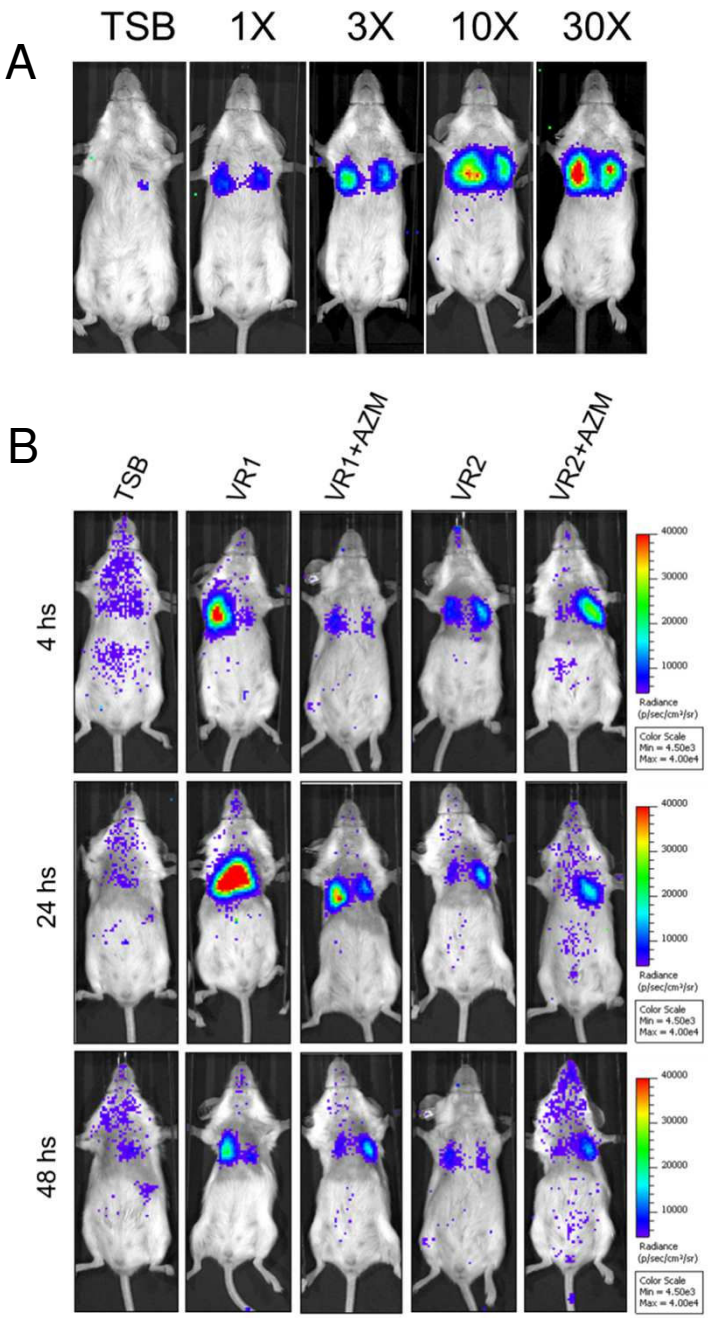
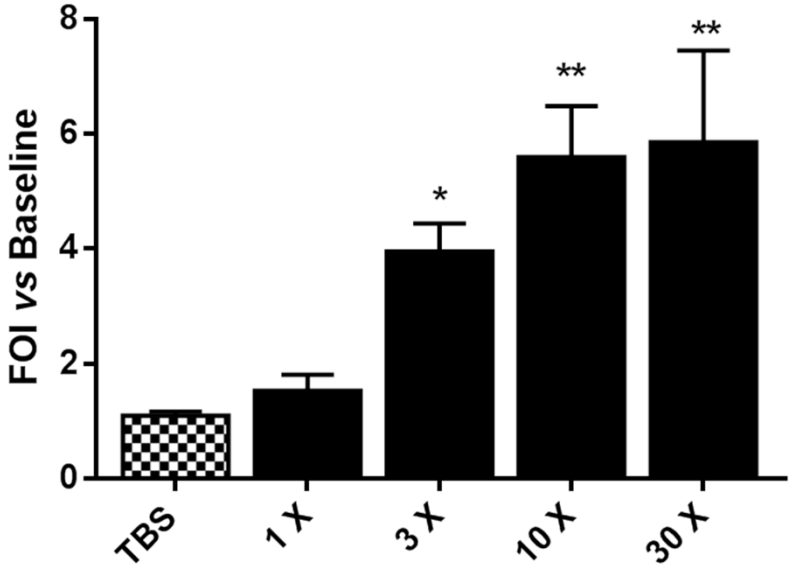

A
Bacterial lysates
VR1
VR2
AZM
-
+
-
+
Zymography
Total protein
B
Colture supernatants
VR1
VR2
-
+
-
+

Figure 3


A
TSB
1X
3X
10X
30X
B
TSB
VR1
VR1+AZM
VR2
VR2+AZM
4 hs
24 hs
48 hs
Radiance (p/sec/cm²/sr)
Color Scale Min = 4.50e3 Max = 4.00e4


FOI vs Baseline
TBS
1 X
3 X
10 X
30 X
*
**
**

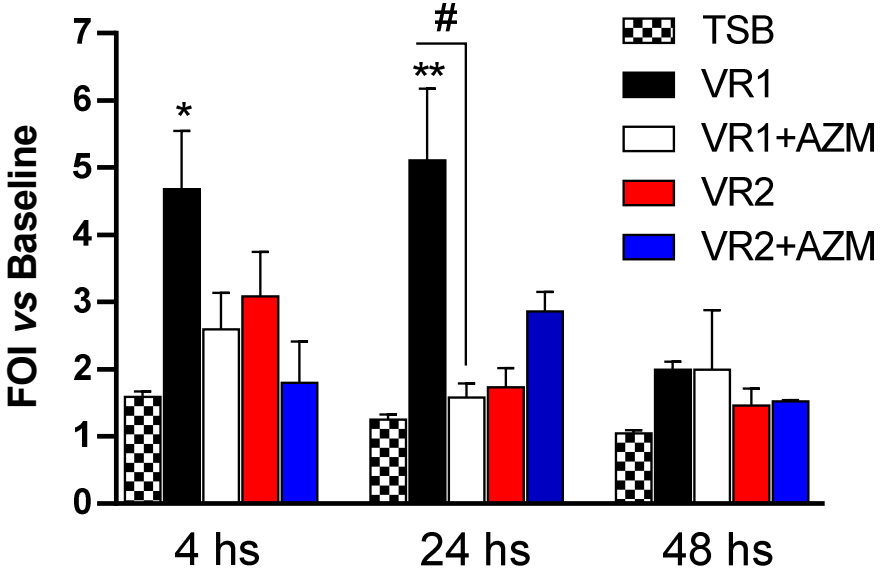
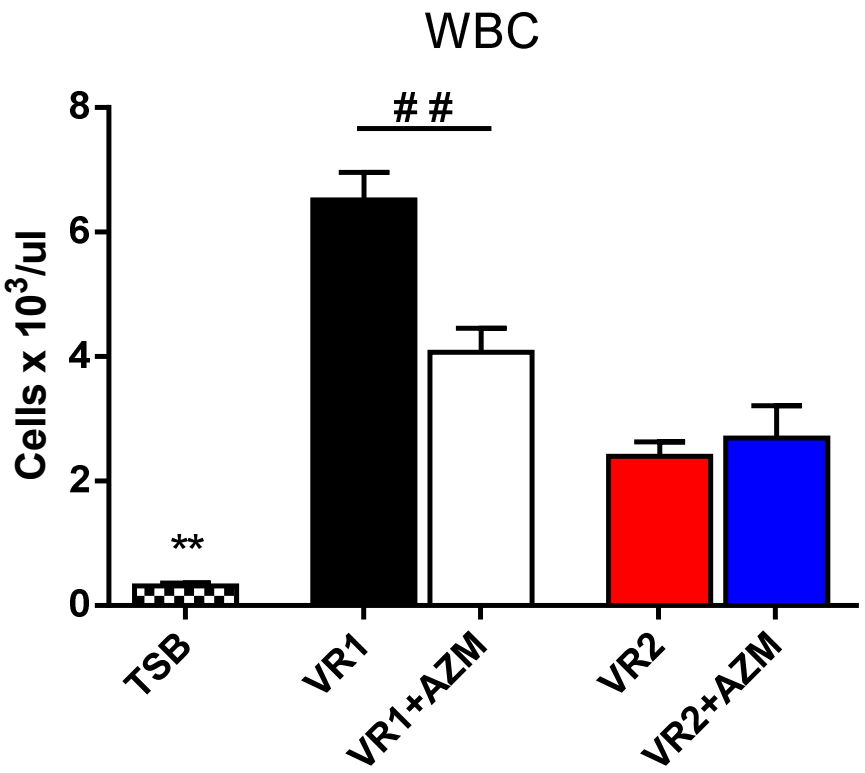

FOI vs Baseline
TSB
VR1
VR1+AZM
VR2
VR2+AZM
4 hs
24 hs
48 hs
*
**
#

Figure 4

A)


WBC
Cells x 10^3/ul
8
6
4
2
0
##
**
TSB
VR1
VR1+AZM
VR2
VR2+AZM

B)

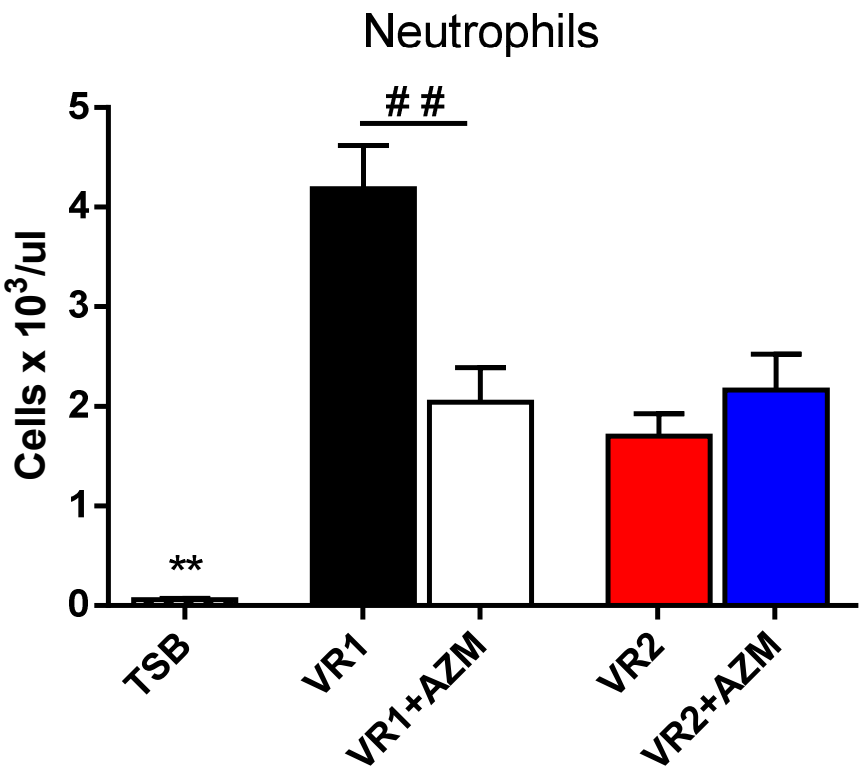

Neutrophils
Cells x 10^3/ul
5
4
3
2
1
0
##
**
TSB
VR1
VR1+AZM
VR2
VR2+AZM

Figure 5

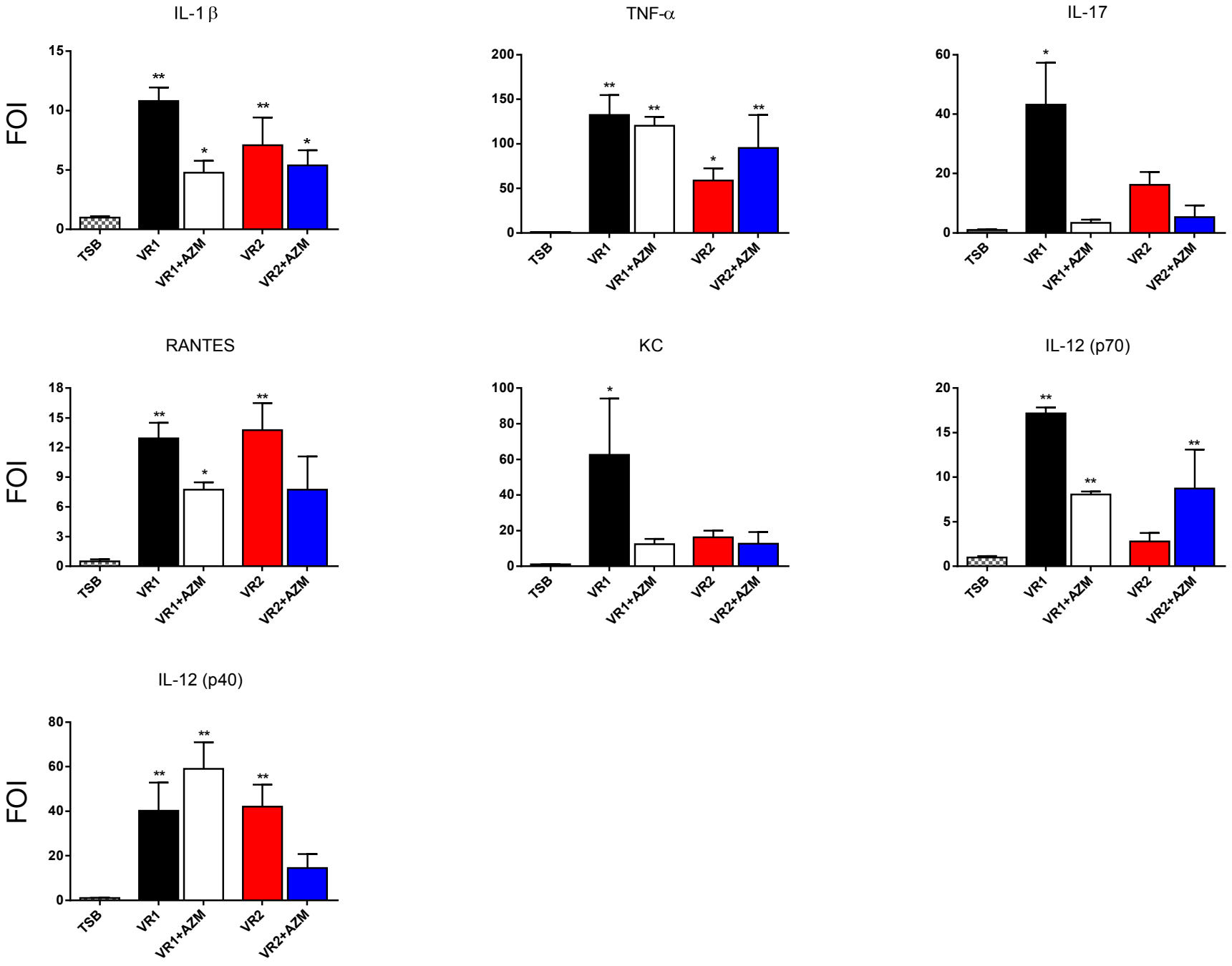

IL-1 β
TNF-α
IL-17
RANTES
KC
IL-12 (p70)
IL-12 (p40)
FOI
TSB
VR1
VR1+AZM
VR2
VR2+AZM

Figure 6

Virulence factors and phenotypes		***P. aeruginosa* strain VR1**	***P. aeruginosa* strain VR1 + AZM**	***P. aeruginosa* strain VR2**	***P. aeruginosa* strain VR2 + AZM**
Metallo-proteases		+++	+	-	-
Pyocianin	µg/ml	0,29	0,16	0,12	0,13
	Relative conc. (OD550/OD600)	0,048	0,026	0,020	0,022
Pyoverdine	µg/ml	2,63	0,00	0,13	0,06
	Relative conc. (OD550/OD600)	0,103	0,00	0,005	0,002
Colony phenotype	Colour	Green	Light brown	Light brown	Light brown
	Mucoidy	No	No	Yes	Yes
	Edges	Jagged	Jagged	Regular	Regular
	Morphology	Swarming	Swarming	Round	Round
Biofilm production		+++	ND	++	ND
Twiching (□ cm)		0,8	0,7	0,6	0,8
Swimming (□ cm)		2,3	1,2	0,5	0,5
Swarming (□ cm)		1,6	1,2	0,3	0,4