Journal of Antimicrobial Chemotherapy

Anti-*Pseudomonas aeruginosa* serotype O11 LPS immunoglobulin M monoclonal antibody panobacumab (KBPA101) confers protection in a murine model of acute lung infection

T. Secher[1]†, L. Fauconnier[1], A. Szade[2], O. Rutschi[3], S. C. Fas[3], B. Ryffel[1] and M. P. Rudolf[3]*

[1]UMR6218, CNRS, Orléans, France; [2]Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland; [3]Kenta Biotech AG, Bern, Switzerland

*Corresponding author. Kenta Biotech AG, Rehhagstrasse 79, CH-3018 Berne, Switzerland; Tel: +41-58-680-52-56; Fax: +41-58-680-52-51; E-mail: michael.rudolf@kentabiotech.com
†Present address: INRA and Ecole Nationale Vétérinaire, UMR1225 Host-Pathogen Interaction IHAP, Toulouse, France

Objectives: To investigate the *in vivo* efficacy in a murine pulmonary infection model of panobacumab (KBPA101), a human IgM monoclonal antibody directed against the O-polysaccharide moiety of *Pseudomonas aeruginosa* serotype O11, and to describe the anti-inflammatory effects in the lung as a consequence of the treatment.

Methods: We established an experimental murine model of acute pneumonia by intranasal administration of *P. aeruginosa* serotype O11. Mice were treated, after infection, with a single intravenous injection of panobacumab and panobacumab lung bioavailability was assessed. Inflammatory parameters such as pro-inflammatory cytokine production and neutrophil recruitment in broncho-alveolar lavage fluid (BALF) were measured and bacterial load in the lung was analysed.

Results: Panobacumab plays a significant role in addition to the host innate immune response, leading to improved control of pulmonary infection. The IgM antibody is able to reach the broncho-alveolar space and reduce the pulmonary bacterial load as well as lung inflammation in a dose-dependent manner. Furthermore, panobacumab treatment leads to enhanced neutrophil recruitment in BALF while reducing the host-derived production of pro-inflammatory mediators and lung injury.

Conclusions: These data provide evidence that panobacumab, an IgM-based immunotherapeutic, is highly efficacious in controlling acute lung infection by enhancing the natural innate immune response.

Keywords: acute pneumonia, pulmonary infections, anti-infective antibody, anti-inflammatory

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen associated with severe respiratory tract infections. The prevalence of *P. aeruginosa* infections has increased over the past decade and has become a major concern in morbidity and mortality in the hospital environment, especially in immunocompromised individuals.[1] *P. aeruginosa* is often a cause of ventilator-associated pneumonia (VAP) in patients with damaged airways from mechanical ventilation, trauma or antecedent viral infections, with a significantly higher attributable mortality compared with other pathogens.[2]

Treatment of *P. aeruginosa* infections is still a challenge due to its biochemical attributes,[3] including its poor outer membrane permeability and efflux properties. Furthermore, *P. aeruginosa* harbours chromosomal and/or plasmid-encoded genes mediating an intrinsic resistance to almost every class of antimicrobial agents.[4] Indeed, the prevalence of multidrug-resistant *P. aeruginosa* strains has increased over time and has become a major concern in the hospital environment.[5] Although antibiotics are necessary to clear pathogens from the circulation, the disrupted bacterial membrane can exacerbate the host inflammatory response,[6] as shown for ceftazidime.[7]

Upon infection with respiratory pathogens, the airway mucosal immune system rapidly develops a pro-inflammatory response, leading to production of inflammatory mediators both in aerial compartment and local tissue initiated by the recognition of pathogen-associated molecular patterns (PAMPs) by specific receptors such as the Toll-like receptors (TLRs) and triggered by the epithelial cells and the resident immune

cells.[8–10] Massive neutrophil recruitment to the site of infection is an essential mechanism to control invading pathogens[11] such as *P. aeruginosa*.[12] Complement-mediated opsonophagocytosis or complement-activated killing are the main pathways leading to pathogen eradication. These effector mechanisms are mainly mediated by IgM antibodies, which are the primary antibody-based immune reaction upon stimulation by bacterial antigens such as lipopolysaccharide (LPS).

Passive immunotherapy is recognized as a valuable addition to standard therapy against infectious disease, although it has not been extensively studied so far. Several groups have reported positive effects of anti-LPS,[13] anti-flagella[14] or anti-PcrV[15] antibodies during *P. aeruginosa* infections. Panobacumab is a human monoclonal antibody (MAb) of the IgM/κ isotype and is directed against the LPS O-polysaccharide *P. aeruginosa* serotype IATS (International Antigen Typing System) O11. It has been recently characterized *in vitro*[16] and its safety has been shown in humans.[17]

A significant survival benefit of prophylactic treatment of animals with panobacumab prior to bacterial infections has been demonstrated, mainly for disseminated burn wound infections.[16] Here we describe the effects of therapeutic treatment of pneumonia by panobacumab in a murine model of acute *P. aeruginosa* lung infection. We report here that MAb treatment directly targeted the lung compartment and is associated with enhanced neutrophil recruitment and bacterial clearance with subsequent attenuated lung inflammation. Mechanistically, we observed an increased *P. aeruginosa* opsonophagocytosis by neutrophils *in vitro*, which may explain the reduced bacterial load after lung infection. Furthermore, panobacumab treatment leads to an attenuated hyperinflammatory response associated with reduced levels of interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and keratinocyte-derived chemokine (KC).

Materials and methods

Mice and reagents

Adult female C57BL/6 (B6) mice (10–12 weeks old) were obtained from Janvier (Le Genest Saint-Isle, France). All mice were housed under specific-pathogen-free conditions at the Transgenose Institute (Centre National de la Recherche Scientifique, Orléans, France) and had access to food and water *ad libitum*. All animal experiments complied with the French government's ethical and animal experiment regulations.

Generation of panobacumab (KBPA101) has been described previously.[16] Panobacumab for injection was supplied as sterile, non-pyrogenic, PBS solution manufactured under good manufacturing practice (GMP) conditions (Kenta Biotech AG, Bern, Switzerland). Control antibody specific for *P. aeruginosa* serotype IATS O1 was generated through the same technology (manuscript in preparation) and purified MAb was supplied by Kenta Biotech. For necropsy analysis, mice were injected intravenously with 0.13, 0.4 and 1.2 mg/kg of panobacumab in 200 μL of PBS 2 h or 4 h after infection, with control animals receiving PBS instead. For a complementary survival study, the experimental setup was identical to the conditions for the necropsy analysis, albeit with a second intraperitoneal injection of panobacumab or PBS (as a control) 24 h after the infection.

Acute model of P. aeruginosa lung infection

P. aeruginosa strain 2310.55 of serotype IATS O11 as well as *P. aeruginosa* serotype IATS O1 reference strain (ATCC 33348) were provided by Kenta

Biotech with certified titre and purity. The uniformity of the colonies was checked by plating on brain heart infusion (BHI) agar plates. The mice were infected with freshly prepared inoculum of the bacteria. An overnight culture in 10 mL BHI medium was prepared, starting from the frozen stock at 37°C and shaken at 150 rpm. Of this culture, 2.5 mL was taken to start a fresh 10 mL BHI culture. The culture was stopped when an OD of about 0.4 was reached (corresponding to a bacterial titre of about 2×10^8 bacteria/mL). The bacteria were washed once in PBS diluted in saline to obtain a titre of 10^6 bacteria/mL. Each inoculum was then checked for accuracy by plating directly on fresh BHI agar plates. Mice were anaesthetized with a low dose of intravenous ketamine/xylazine (1.25 mg/mL/0.5 mg/mL) and 40 μL of the bacterial solution or the corresponding vehicle solution (isotonic saline) was applied intranasally using an ultrafine pipette tip. Mortality and body weight of animals were monitored daily and in all experiments moribund animals or animals with a weight loss of more than 25% were sacrificed for ethical reasons.

Broncho-alveolar lavage and organ sampling

Broncho-alveolar lavage fluid (BALF) was collected 24 h after *P. aeruginosa* administration by cannulating the trachea under deep ketamine/xylazine anaesthesia and washing the lung twice with 1 mL saline at room temperature. The lavage fluid was centrifuged at 2000 rpm for 10 min at 4°C and the supernatant was stored at −80°C for analysis. The cell pellet was resuspended in PBS, counted in a haemocytometer chamber and cytospin preparations were made using a Shandon cytocentrifuge (1000 rpm for 10 min). The cells were stained with Diff-Quick (Dade Behring, Marburg, Germany) and counted for neutrophils and macrophages.

Lung bacterial load

Lung total weights were recorded after sacrifice and expressed as a percentage of the body weight. Lung homogenates were prepared in 2 mL of isotonic saline solution using a Dispomix tissue homogenizer (Medic Tools). Tenfold serial dilutions of homogenates were plated on BHI agar plates (Biovalley). Plates were incubated at 37°C and 5% CO_2 and the numbers of cfu were enumerated after 24 h.

Panobacumab, cytokine and chemokine determination

Panobacumab concentrations in BALF and serum were determined by ELISA using a mouse monoclonal anti-idiotypic antibody against panobacumab as described previously.[17] TNF-α, IL-6, IL-10 and keratinocyte-derived chemokine (KC or CXCL-1) concentrations in BALF and lung homogenates were measured by ELISA (Duoset Kit; R&D Systems) according to the manufacturer's instructions (with detection limits at 50 pg/mL).

Histology

Lungs were fixed in 10% buffered formalin (Shandon), dehydrated in ethanol and embedded in paraffin. Serial sections (3 μm) were stained with haematoxylin and eosin and all lung sections were evaluated by two independent observers for pathological changes, oedema, haemorrhage and cellular recruitment. Haemorrhage was scored as follows: 0, normal histomorphology; 1, minor haemorrhage localized in perivascular spaces; 2, moderate haemorrhage including peribronchiolar spaces; 3, extensive haemorrhage including lung parenchyma. Oedema was graded from 0 to 3 according to the ratio between blood vessel and perivascular space diameter. Cellular recruitment was graded from 0 to 3 according to its intensity. The histological score index was the sum of these three separate scores. At least two separate sections were assessed per lung.

In vitro phagocytosis assay

Murine bone marrow neutrophils were isolated from femurs in 2 mL of Hank's balanced salt solution (HBSS) (Invitrogen) without Ca^{2+} and Mg^{2+} and laid on top of a two-layer 3 mL Percoll gradient of 72% and 65% (Sigma–Aldrich) diluted in HBSS (100% Percoll was obtained by mixing nine parts of Percoll and one part of 10× HBSS) and centrifuged at 1100 **g** for 30 min at room temperature without braking. The enriched neutrophil fraction was recovered at the interphase. After washing twice with HBSS, 5×10^6 cells were obtained per mouse containing 95% of Gr-1-positive cells and counted using Trypan Blue. *P. aeruginosa* was freshly cultured to 2×10^8 bacteria/mL (OD of 0.4) and washed twice in HBSS. The bacterial pellet was resuspended in 1 mL of $NaCO_3$ (Sigma–Aldrich), 0.1 M, pH=9, then 100 μL of FITC (Sigma–Aldrich) at 10 mg/mL was added and incubated under agitation at room temperature for 1 h under light protection and then washed three times in HBSS. A total of 4×10^6 cells/mL were coincubated with bacteria at a multiplicity of infection (moi) of 1:100 and panobacumab at 0.1 and 1 mg/mL in pre-coated Eppendorf tubes with PBS-BSA 1%, fetal calf serum (FCS) 5%, for 0, 30 and 60 min at 37°C under agitation. The reaction was stopped by washing tubes at 4°C. FITC fluorescence was read on the FL-1 channel on an LSR-I cytometer after 0.2 mg/mL of Trypan Blue was added in order to quench the extracellular fluorescence.

Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by using one-way analysis of variance (ANOVA) followed by a Bonferroni post-test (which allows comparison of all pairs of groups). Log-rank test was used for survival analysis and Student's *t*-test was used for comparison between two groups. All tests were performed with GraphPad Prism, Version 4.03 for Windows (GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com). All data are presented as mean±standard error of the mean (SEM). A *P* value <0.05 was considered significant.

Results

P. aeruginosa clinical strain 2310.55, serotype O11 induces acute lung infection

First we established an acute pneumonia model in C57BL/6 mice with *P. aeruginosa* for the evaluation of treatment effects of a single dose of the human monoclonal anti-LPS serotype O11 IgM antibody, panobacumab. We titrated the amount of *P. aeruginosa* strain IATS O11 (derived from a clinical isolate) necessary for intense but non-lethal infection. Intranasal administration of 10^7 cfu of *P. aeruginosa* induced rapid death within the first 24 h. At the infectious dose of 10^6 cfu of *P. aeruginosa*, 80% of the mice survived after 48 h, but only 20% survived at 72 h or longer, and all mice infected at 10^5 cfu survived over the experimental period of 10 days (Figure 1a). The weight loss was maximal 3 days post-infection; up to 25% in the group with the inoculum size of 10^6 cfu. The mice that survived regained their initial weight (Figure 1b).

As we were interested in the dynamics of the lung infection, a kinetic study was performed based on the previous data with the inoculum size of 10^6 cfu, with determination of bacterial load as a marker for infection progression and lung weight as a surrogate marker for lung inflammation at 2 h, 4 h or 24 h
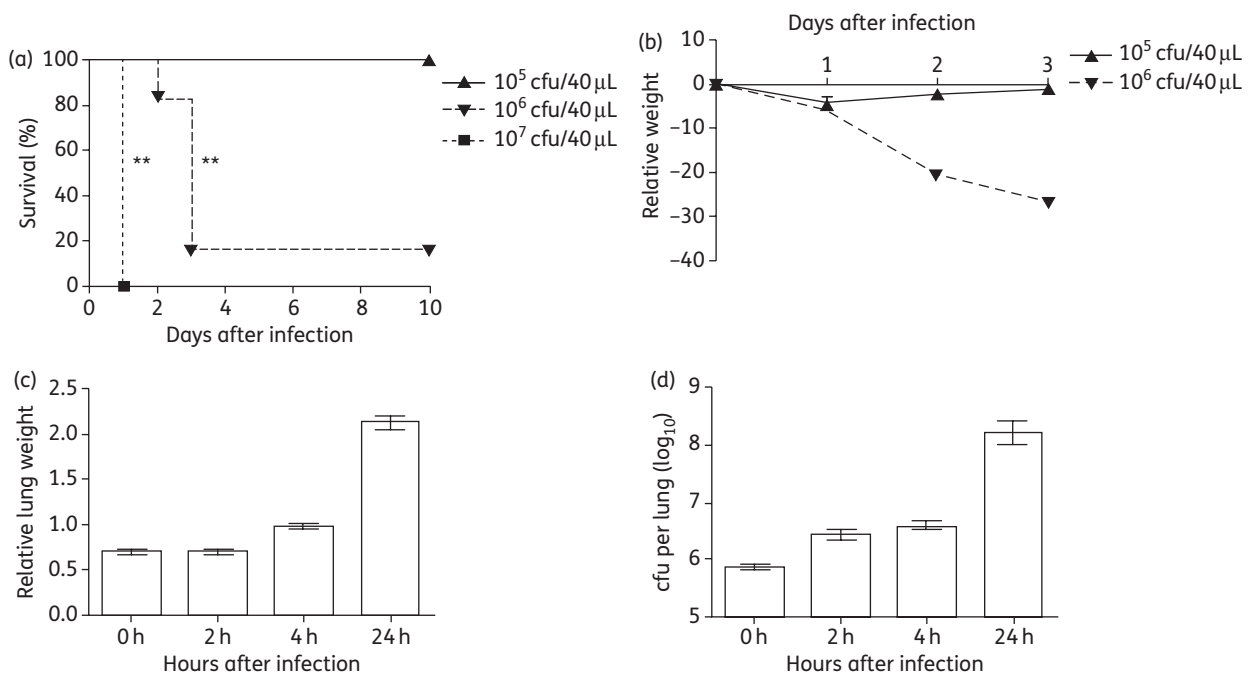
Figure 1. *Pseudomonas aeruginosa* clinical strain 2310.55, serotype O11 induces acute lung infection. B6 mice received intranasal instillation of 40 μL of *P. aeruginosa* strain 2310.55 (10^5, 10^6 or 10^7 cfu). Survival rates (a) and body weight (b) were monitored for 10 days after infection. B6 mice received intranasal instillation of 40 μL of *P. aeruginosa* (10^6 cfu) and lung weights (c) and lung cfu (d) were recorded at the time of infection (0 h) or 2, 4 and 24 h after the infection. Groups of five mice were used and mean values±SEM are shown (log-rank analysis, **$P < 0.01$ comparing 10^6 and 10^7 groups to 10^5 group). The results are representative of three independent experiments.

post-infection. Starting at 2 h post-infection, we observed a significant increase in bacterial load, which increased further over 24 h. An increase in lung weight was noticed starting 4 h post-infection, with an increase over 24 h correlating with the bacterial load (Figure 1c and d). The clinical investigation listed discernible symptoms such as ruffled fur, hunched posture and motor impairment. These symptoms appeared between 4 h and 6 h after infection and gradually increased over time. Surviving animals had a noticeable improvement in clinical symptoms after 48 h, which was paralleled by a reduction in lung bacterial load that reached complete clearance after 10 days (data not shown). In summary, at an inoculum of 10^6 cfu of *P. aeruginosa*, robust lung inflammation with a massive increase in lung weight and bacterial load after 2 h was observed. Therefore the 24 h timepoint and the 10^6 cfu infectious dose were chosen for further experiments.

Panobacumab is detected both in the serum and airway upon infection

The primary site of *P. aeruginosa* infection within the lung is the aerial compartment. Tissue penetration of antibodies, especially of the high molecular weight IgM isotype, has been assumed to be very inefficient in healthy animals.[18,19] However, we hypothesized that inflammation influences epithelial integrity and thus panobacumab might reach the site of infection with sufficient efficiency to enhance bacterial clearance. To test this hypothesis we analysed the antibody concentration in both serum as well as BALF of control animals versus *P. aeruginosa*-infected animals treated with panobacumab.

Panobacumab was detected at approximately the same level in the serum of both infected and non-infected mice after intravenous injection with 0.4 mg/kg of panobacumab (Figure 2a). Upon *P. aeruginosa* infection, the presence of panobacumab could also be demonstrated in the BALF of infected mice (Figure 2b), whereas no panobacumab was detected in the BALF of uninfected animals. Therefore the data demonstrate the inability of IgM antibodies to cross into the broncho-alveolar space under normal conditions. However, damage to the

epithelial barrier of the lung during pneumonia[20] facilitated the access of panobacumab to the broncho-alveolar compartment, which may allow for modulation of the host immune response towards the bacterial infection.

Therapeutic and specific effect of panobacumab on P. aeruginosa infections in mice

As previously demonstrated, the passive immunization of animals with panobacumab had a significant benefit on the survival of animals if administered concomitantly with the bacterial challenge and could reduce the dissemination of *P. aeruginosa* after pulmonary infections.[16] Nevertheless, exacerbation of pneumonia is frequently caused by a massive inflammatory response. Therefore we tested in our animal model whether panobacumab could modulate the outcome of *P. aeruginosa*-induced pneumonia by reducing the bacterial load as well as by modulation of lung weight, which served as a surrogate marker for the reduction of pulmonary oedema and inflammation. Indeed, treatment of infected animals with 0.4 mg/kg panobacumab resulted in a significant decrease in lung weight (Figure 3a) and a decrease in bacterial load of about 1.5 $\log_{10}$ cfu (Figure 3b). However, there was no statistically significant difference in lung weights between the treatment regimens of 2 h and 4 h post-infection. Collectively these data suggest that the therapeutic application of panobacumab monoclonal antibody, even 4 h post-infection, is beneficial for the host. Thus in all subsequent experiments, treatment was initiated 4 h post-infection.

In a second set of experiments, we established a dose–response effect with serial dilutions of panobacumab. Additional dosing steps were 1.2 mg/kg (which is the dose used in clinical testing of panobacumab, representing tripling the established dose of 0.4 mg/kg) and 0.13 mg/kg (representing a third of the established dose). We observed a significant reduction in lung weight for the two higher treatment groups of 0.4 mg/kg and 1.2 mg/kg, but not 0.13 mg/kg when compared with the untreated controls (Figure 3c). Yet there was no additional dose effect observed by increasing the dose from 0.4 to 1.2 mg/kg. Similar results were obtained when analysing the bacterial load
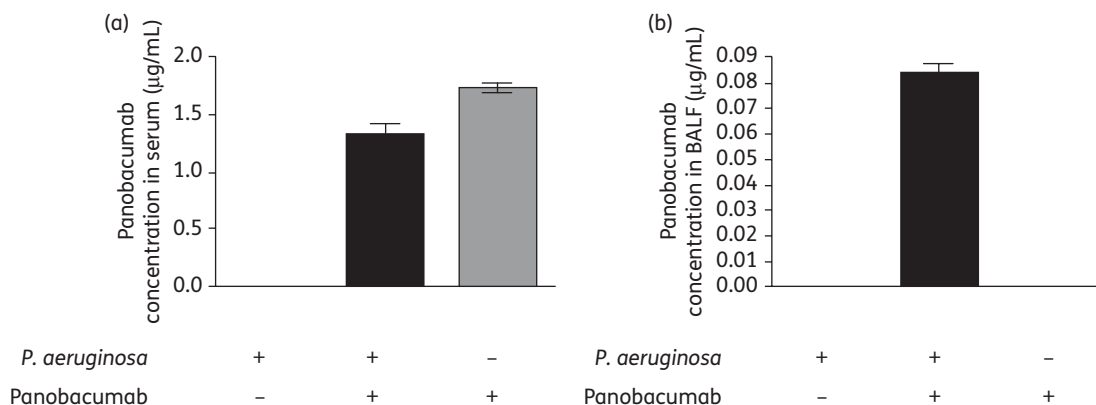
Figure 2. Panobacumab is detected both in the serum and aerial compartment upon infection. B6 mice received intranasal instillation of 40 μL of *P. aeruginosa* strain 2310.55 (10^6 cfu) or saline solution. Panobacumab at 0.4 mg/kg or saline solution was given intravenously 4 h after infection. The concentrations of panobacumab in serum (a) and BALF (b) were determined 24 h after infection. Groups of five mice were used and mean values ± SEM are shown. The results are representative of two independent experiments.

in the lung of panobacumab-treated versus untreated animals. The dose of 0.4 mg/kg demonstrated a significant reduction of the bacterial load by 1.2 $\log_{10}$ cfu, whereas no significant improvement was observed by increasing the dose from 0.4 to 1.2 mg/kg (Figure 3d). Therefore systemic treatment by a single infusion of panobacumab at a dose of 0.4 mg/kg or higher can significantly reduce lung bacterial load as well as lung inflammation. In addition, we observed an important delay of more than 24 h in mortality post-infection and a minor but significant survival benefit of panobacumab administration using the same experimental setup of intranasal challenge with *P. aeruginosa* (Figure 3e), with the only exception being a second dosing after 24 h post-challenge. The dose of 0.4 mg/kg was chosen for the survival experiment based on previous experience with panobacumab in survival experiments, where the dosing was sufficient for a significant survival benefit.[16]

In order to test if the observed effects are specific for panobacumab targeting *P. aeruginosa* serotype O11 and not due to a non-specific anti-inflammatory effect of IgM antibodies, an additional monoclonal human IgM antibody targeting serotype O1 was used as a control and additionally panobacumab was used as a control in an acute lung infection with *P. aeruginosa* of serotype O1. In both experiments a significant effect on lung weight and bacterial load was observed for the antibody specific for the serotype of the *P. aeruginosa* strains used for infection, e.g. panobacumab showed a potent reduction in both lung weight (Figure 3f) and bacterial load (Figure 3g) in the O11 challenge model, but not an IgM MAb specific for the O1 serotype. In contrast, no effect was observed on lung weight (Figure 3h) and bacterial load (Figure 3i) for panobacumab in the O1 challenge model. Thus we concluded that the observed anti-inflammatory effects were due to a specific interaction of panobacumab with the LPS of serotype O11 and not due to a non-specific anti-inflammatory effect of the IgM antibody.

Enhanced neutrophil recruitment and bacterial clearance in the lungs of panobacumab-treated mice

Having shown that panobacumab reaches the site of infection and has a significant beneficial effect, we wanted to elucidate which cellular components could be involved in the enhanced clearance of bacteria. Protection from pneumonia is associated with enhanced cell recruitment in BALF. It is known that recruitment of neutrophils to the site of infection is an essential mechanism to control invading pathogens.[21] Therefore we analysed the recruitment of cells to the lung (BALF) in panobacumab-treated or untreated animals in our model. Upon infection, panobacumab-treated mice (0.4 mg/kg) presented with a greater number of total cells (Figure 4a), especially neutrophils (Figure 4b), in the BALF compared with control PBS-treated mice with pulmonary infection, while macrophage/monocyte recruitment was comparable between the groups (Figure 4c). Thus neutrophil recruitment significantly increased in the presence of panobacumab. The effect was accompanied by a significant bacterial load reduction of 1.3 $\log_{10}$ cfu/lung (Figure 4e) as well as a significant reduction in lung inflammation as assessed by lung weight determination (Figure 4d). Thus the presence of monoclonal MAb panobacumab at the site of the infection enhances the lung innate immune response to *P. aeruginosa*,

which in turn is correlated with better control of bacterial growth and reduction of bacterial load upon panobacumab treatment.

The enhanced clearance of bacteria at the site of infection might be due to improved uptake/bactericidal capabilities of migrated neutrophils in the lung in the presence of panobacumab. To address this issue we performed an *in vitro* uptake/killing assay of mouse neutrophils with panobacumab-treated bacteria. FITC-labelled *P. aeruginosa* were incubated in the presence of neutrophils and panobacumab, and the intracellular load of FITC-labelled *P. aeruginosa* (after Trypan Blue quenching of extracellular fluorescence) was assessed by flow cytometry immediately or 30 and 60 min after incubation (Figure 4f). Since panobacumab in the dose range of 0.05 to 1.5 ng/mL has demonstrated opsonophagocytosis activity *in vitro* using a human cell line,[16] we chose a similar dose range of 0.1 to 1 ng/mL to be tested with murine cells. As shown in Figure 4(f), increasing concentrations of panobacumab significantly augmented the quantity of intracellular FITC-labelled *P. aeruginosa* over time, confirming the potential of panobacumab treatment to enhance the uptake of bacteria by murine neutrophils.

Reduced lung inflammatory mediators production and lung injury in panobacumab-treated mice

There are occasions when the host inflammatory response is exaggerated and protracted.[22] Therefore we investigated the effect of local levels of inflammatory mediators upon *P. aeruginosa* infection in BALF (Figure 5, upper row) and lung homogenates (Figure 5, lower row) 24 h post-infection. The pro-inflammatory cytokines IL-6 (Figure 5a and b) and TNF (Figure 5e and f), as well as KC (Figure 5c and d), which is the functional murine homologue to IL-8, were significantly lower in BALF as well as the lung compartment of panobacumab-treated animals, whereas levels of IL-10 were not significantly different in treated versus control animals (data not shown). These data indicate that despite the increase in neutrophils to the broncho-alveolar space of infected animals, cytokine and chemokine production induced by *P. aeruginosa* infection can be reduced by therapeutic panobacumab administration.

Microscopic examinations of the lungs of infected animals revealed focal inflammation with distinct alveolar congestion, cellular infiltrates around bronchi and capillaries, microthrombi in small vessels and oedema (Figure 6a and b). These parameters were reduced in panobacumab-treated mice, which was confirmed by a semi-quantitative assessment of microscopic lung sections that revealed a protective trend in lung injury, cell recruitment and oedema in panobacumab-treated mice compared with the untreated controls (Figure 6c). Taken together, panobacumab treatment leads to reduced inflammation signals and less lung damage during *P. aeruginosa* infection.

Discussion

In this study we demonstrated that therapeutic administration of an anti-*P. aeruginosa* serotype O11 LPS immunoglobulin M monoclonal antibody, panobacumab, may protect mice from lethal pneumonia.
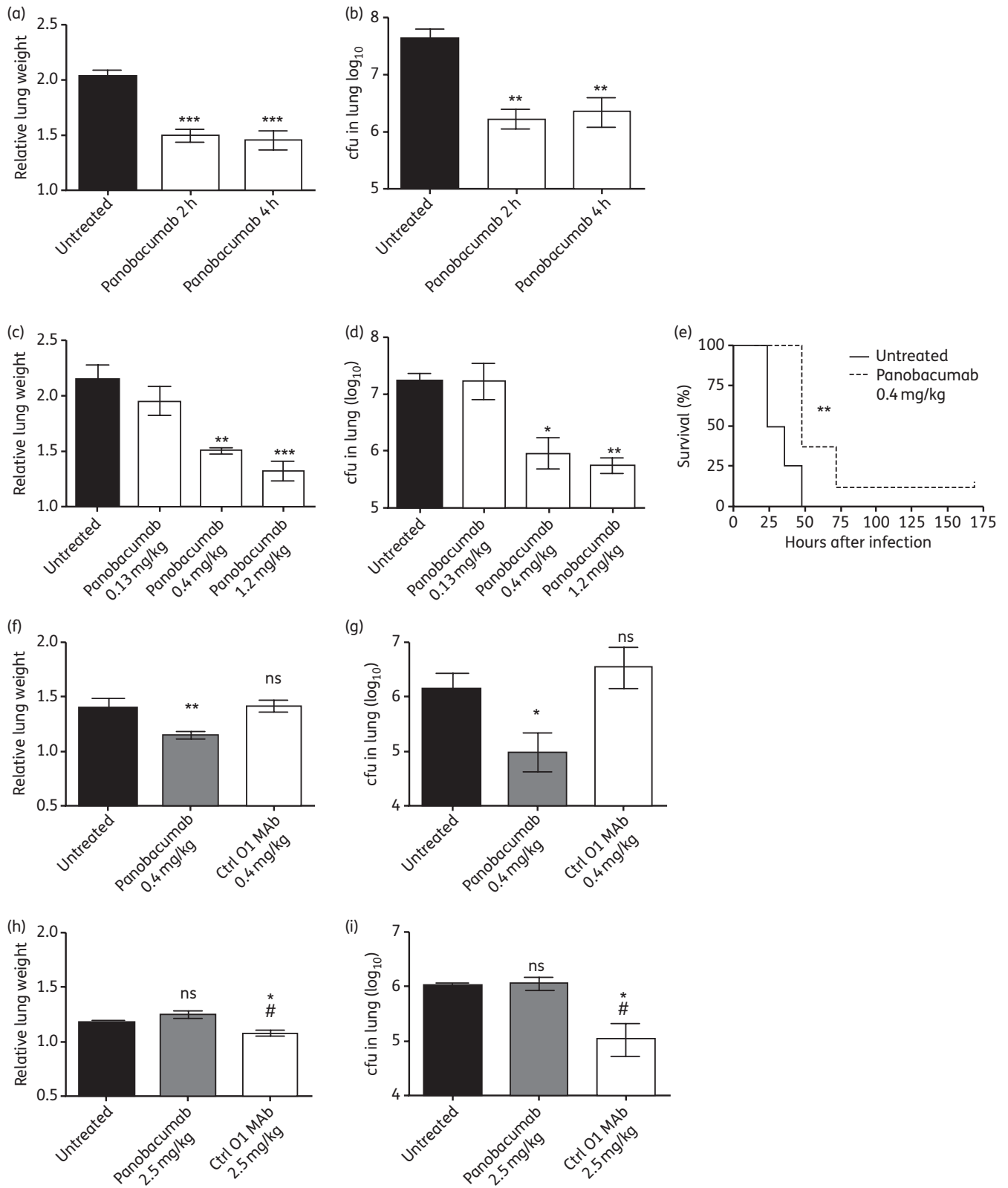
Figure 3. Therapeutic and specific effect of panobacumab on *P. aeruginosa*-infected mice. (a, b) B6 mice received intranasal instillation of 40 μL of *P. aeruginosa* strain 2310.55 (10^6 cfu). Panobacumab was given intravenously at 0.4 mg/kg, 2 and 4 h after the infection. Lung weights (a) and lung cfu (b) were recorded 24 h after the infection. (c, d) B6 mice received intranasal instillation of 40 μL of *P. aeruginosa* (10^6 cfu). Panobacumab was given
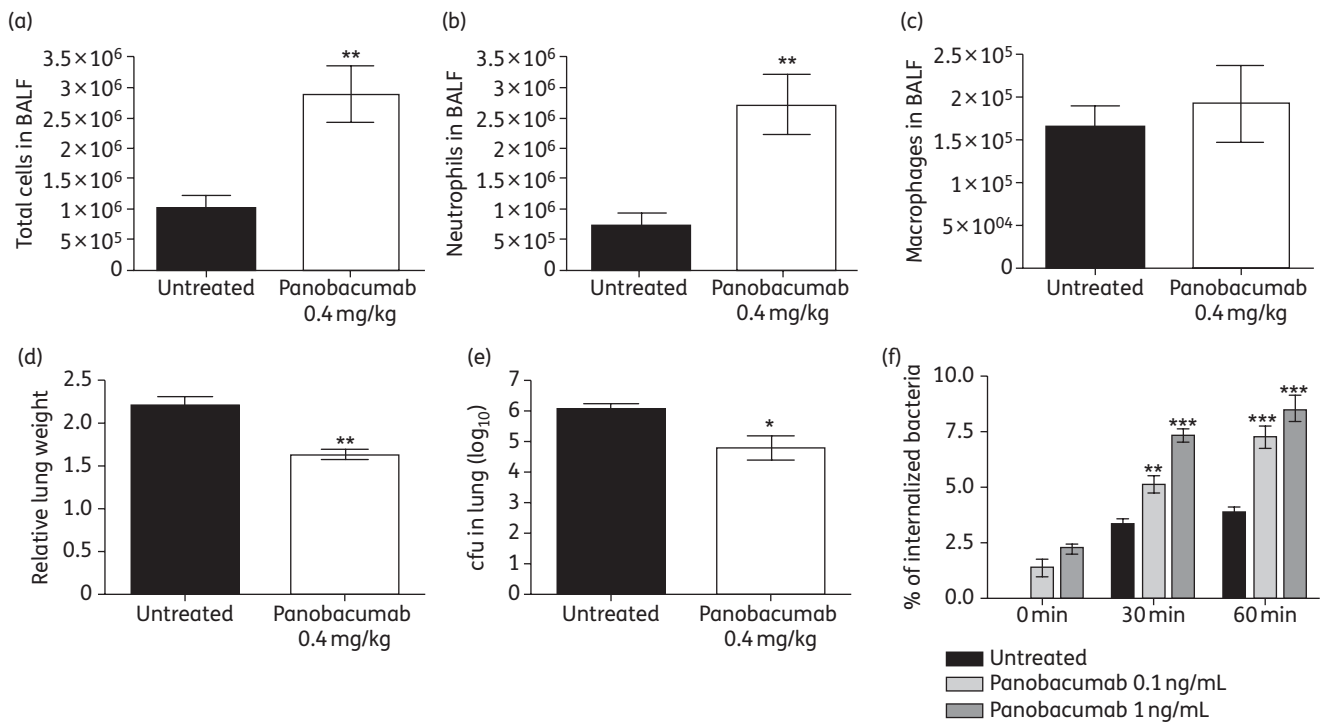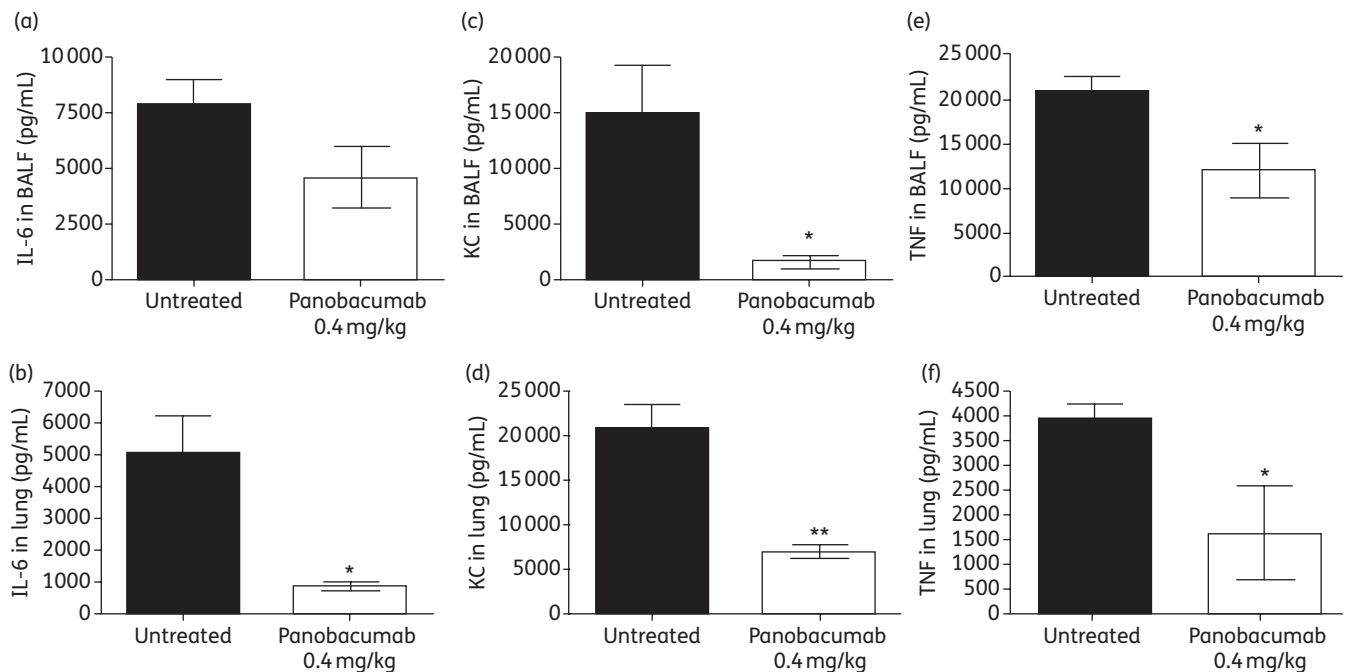
Figure 5. Reduced lung inflammatory mediator production in panobacumab-treated mice. B6 mice received intranasal instillation of 40 μL of *P. aeruginosa* strain 2310.55 (10^6 cfu). Panobacumab was given intravenously at 0.4 mg/kg 4 h after the infection. The concentration of IL-6 (a and b), KC (c and d) and TNF (e and f) in BALF and lung homogenates were determined 24 h after infection. Groups of five mice were used and mean values ± SEM are shown (Student's *t*-test; *$P < 0.05$, **$P < 0.01$, comparing panobacumab to untreated group). The results are representative of three independent experiments.

It is important to note that the untreated animals usually show signs of severe infection and mortality between 24 and 48 h post-infection. In order to prevent the loss of control animals while still having substantial amounts of panobacumab in the system [half-life of panobacumab is 18.9 h in the mouse (A. W. Zürcher, CSL Behring, Switzerland; unpublished data)], it was decided to evaluate the effects of panobacumab treatment on lung tissue and innate immune functions at 24 h post-infection.

Since the breakthrough of monoclonal antibody production in 1975, only a few antibodies, and even fewer IgM antibodies, have been used in human therapy. Due to its pentameric nature, IgM possesses high avidity and causes agglutination or clumping, a mechanism that facilitates the clearance of pathogens. As panobacumab is of the IgM isotype, with an apparent molecular weight of 945 kDa, tissue penetration and localization of the antibody is crucial for efficacy. Nevertheless, tissue penetration of immunoglobulins is tightly controlled and previous reports have observed a low tissue penetration of IgM antibodies in healthy animals.[23,24] Although high molecular weight compounds such as macrolide antibiotics reach pulmonary tissue, their physicochemical characteristics are entirely different from biologics. Experimental data indicate that inflammatory cells actually do act as transporting vehicles for macrolides from plasma to epithelial surfaces,[25] whereas IgM remains in the plasma of healthy animals, a result that was also confirmed by our own data. Nevertheless, we clearly established that panobacumab reaches the aerial compartment of diseased animals due to the tissue damage or inflammatory responses caused by the infection.

We showed that the observed effect of panobacumab is strictly dependent on the appropriate *P. aeruginosa* serotype, since our data clearly demonstrate that panobacumab no longer displays efficacy when applied in an infection model with a different *P. aeruginosa* serotype. As there is no indication of cross-reactivity of panobacumab to murine tissue or deposition in the kidneys,[16] the observed effects are clearly dependent on the interaction of panobacumab with its antigen.

We observed that lung inflammation was less pronounced in the presence of panobacumab compared with untreated controls. Indeed, upon infection, BALF and lung levels of IL-6, TNF-α and KC were significantly reduced under panobacumab treatment. An up-regulation of these pro-inflammatory mediators is often associated with life-threatening infection by Gram-negative bacteria or the presence of endotoxin in the lung environment and thus is a major cause of severe airway diseases. In pneumonia, pathogen-mediated inflammation is essential for host defence, whereas uncontrolled activation of leucocytes and lung-resident cells can lead to exacerbated tissue injury. Our results indicate that the reduction of bacterial cells by panobacumab during the treatment of lung infection also resulted in reduced levels of pro-inflammatory mediators induced by the presence of *P. aeruginosa*. These data were similar to those observed in RvE1-treated mice undergoing *Escherichia coli* infection and lung injury, which presented lower levels of pro-inflammatory cytokines and chemokines.[26] Therefore the present study established a correlation between panobacumab-induced cytokines and bacterial load reduction.
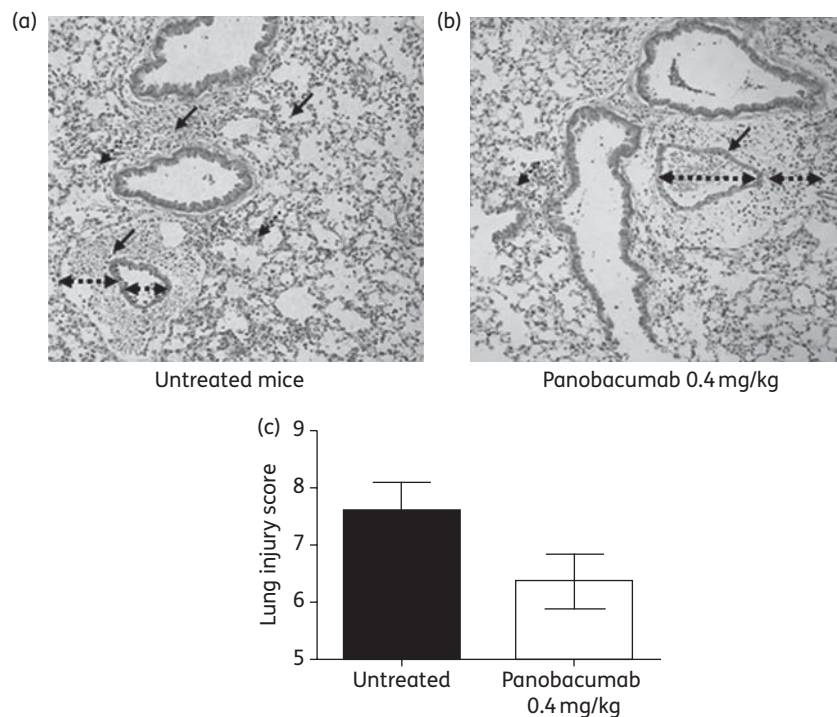
Figure 6. Reduced lung injury in panobacumab-treated mice. B6 mice received intranasal instillation of 40 μL of *P. aeruginosa* strain 2310.55 (10^6 cfu). Panobacumab was given intravenously at 0.4 mg/kg 4 h after infection. Lung tissues were histologically examined 24 h after the infection. Lung sections from untreated (a) and panobacumab-treated (b) mice revealed increased neutrophil influx (continuous arrows), haemorrhage (dotted arrows) and oedema (double-headed arrows). Lung injury score was recorded in treated mice compared with untreated mice (c). All sections were stained with haematoxylin and eosin and observed at ×20 magnification. Groups of five mice were used and mean values ± SEM are shown. The results are representative of two independent experiments.

Massive lung neutrophil recruitment is a major component of the protective host response to *P. aeruginosa* pneumonia and appears to be more important than the contribution of other immune cell types.[18] In the present study, increased neutrophil accumulation in the BALF was correlated with better control of the bacterial load in panobacumab-treated mice. Neutrophil recruitment is dependent on the production of chemotactic chemokines. Among them, the CXC family, including KC (CXCL-1) or IL-8 (CXCL-8), has been known for years to be involved in human and animal models of airway diseases.[19,21,27] We observed that KC production with *P. aeruginosa* was no different in treated and untreated animals at 12 h post-infection (at around 6–8000 pg/mL, data not shown), but over the next few hours treated animals had significantly lower KC values, whereas control animals showed higher levels of KC at 24 h post-infection (see Figure 5). These findings are in accordance with those from Sun *et al.*[28] confirming that early production of KC and neutrophil recruitment are important for combating *P. aeruginosa* infection, whereas neutrophil accumulation correlates with deleterious inflammation.[29,30] Although the time-course was not determined for other cytokines at 12 h, we can speculate that other pro-inflammatory cytokines follow a similar trend, since we observed overall an attenuated lung injury in treated animals versus controls.

Numerous *P. aeruginosa* virulence factors contribute to its pathogenicity by altering host immune defences. Among them, host tissue architecture, tight junctions and opsonophagocytosis are highly impaired.[31] Neutrophils and macrophages are involved in *P. aeruginosa* clearance by antibody-mediated or complement-mediated opsonophagocytosis. In the present study we observed a positive effect of panobacumab treatment of *P. aeruginosa* on mouse neutrophil phagocytosis *in vitro*. The extrapolation of these *in vitro* results, clearly establishing a positive effect of panobacumab on neutrophil-mediated phagocytosis, to an experimental *in vivo* model is always uncertain. The fact that the panobacumab concentration in BALF was 60-fold higher than its half-maximal activity concentration leads us to assert that panobacumab was able to cross into the broncho-alveolar space where it might be in close contact with the necessary effector cells. Nevertheless, the local concentrations at the actual site of infection might be even higher.

In conclusion, we demonstrated that systemic treatment with panobacumab enhances the clearance of *P. aeruginosa* and attenuates lung inflammation in the experimental model of acute lung infection. Moreover, the indirect effect of panobacumab in inhibiting local hyperinflammation during an ongoing infection was established and correlated with decreased lung injury and neutrophil recruitment, resulting in enhanced survival. We clearly established the efficacy of panobacumab treatment of respiratory *P. aeruginosa* infection by augmenting the natural innate immune response.

Transparency declarations

Authors R. O., F. S. C. and R. M. P. are employees of Kenta Biotech AG and own stock in Kenta Biotech AG. All other authors: none to declare.

References

1 Niederman MS. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *Am J Respir Crit Care Med* 2005; **171**: 388–416.

2 Chastre J, Fagon JY. Ventilator-associated pneumonia. *Am J Respir Crit Care Med* 2002; **165**: 867–903.

3 Rello J, Diaz E. Pneumonia in the intensive care unit. *Crit Care Med* 2003; **31**: 2544–51.

4 El Solh AA, Alhajhusain A. Update on the treatment of *Pseudomonas aeruginosa* pneumonia. *J Antimicrob Chemother* 2009; **64**: 229–38.

5 Friedland I, Gallagher G, King T et al. Antimicrobial susceptibility patterns in Pseudomonas aeruginosa: data from a multicenter Intensive Care Unit Surveillance Study (ISS) in the United States. *J Chemother* 2004; **16**: 437–41.

6 Hopkin DA. Frapper fort ou frapper doucement: a Gram-negative dilemma. *Lancet* 1978; **2**: 1193–4.

7 Trautmann M, Zick R, Rukavina T et al. Antibiotic-induced release of endotoxin: in-vitro comparison of meropenem and other antibiotics. *J Antimicrob Chemother* 1998; **41**: 163–9.

8 Campodonico VL, Gadjeva M, Paradis-Bleau C et al. Airway epithelial control of Pseudomonas aeruginosa infection in cystic fibrosis. *Trends Mol Med* 2008; **14**: 120–33.

9 Hippenstiel S, Opitz B, Schmeck B et al. Lung epithelium as a sentinel and effector system in pneumonia-molecular mechanisms of pathogen recognition and signal transduction. *Respir Res* 2006; **7**: 97.

10 Taylor PR, Martinez-Pomares L, Stacey M et al. Macrophage receptors and immune recognition. *Annu Rev Immunol* 2005; **23**: 901–44.

11 Haziot A, Ferrero E, Kontgen F et al. Resistance to endotoxin shock and reduced dissemination of Gram-negative bacteria in CD14-deficient mice. *Immunity* 1996; **4**: 407–14.

12 Cripps AW, Dunkley ML, Clancy RL et al. Pulmonary immunity to Pseudomonas aeruginosa. *Immunol Cell Biol* 1995; **73**: 418–24.

13 Akiyama M, Oishi K, Tao M et al. Antibacterial properties of Pseudomonas aeruginosa immunotype 1 lipopolysaccharide-specific monoclonal antibody (MAb) in a murine thigh infection model: combined effects of MAb and ceftazidime. *Microbiol Immunol* 2000; **44**: 629–35.

14 Oishi K, Sonoda F, Iwagaki A et al. Therapeutic effects of a human antiflagella monoclonal antibody in a neutropenic murine model of *Pseudomonas aeruginosa* pneumonia. *Antimicrob Agents Chemother* 1993; **37**: 164–70.

15 Shime N, Sawa T, Fujimoto J et al. Therapeutic administration of anti-PcrV F(ab′) (2) in sepsis associated with Pseudomonas aeruginosa. *J Immunol* 2001; **167**: 5880–6.

16 Horn MP, Zuercher AW, Imboden MA et al. Preclinical *in vitro* and in vivo characterization of the fully human monoclonal IgM antibody KBPA101 specific for *Pseudomonas aeruginosa* serotype IATS-O11. *Antimicrob Agents Chemother* 2010; **54**: 2338–44.

17 Lazar H, Horn MP, Zuercher AW et al. Pharmacokinetics and safety profile of the human anti-Pseudomonas aeruginosa serotype O11 immunoglobulin M monoclonal antibody KBPA-101 in healthy volunteers. *Antimicrob Agents Chemother* 2009; **53**: 3442–6.

18 Mizgerd JP. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin Immunol* 2002; **14**: 123–32.

19 Goodman RB, Strieter RM, Martin DP et al. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996; **154**: 602–11.

20 Grassme H, Kirschnek S, Riethmueller J et al. CD95/CD95 ligand interactions on epithelial cells in host defense to Pseudomonas aeruginosa. *Science* 2000; **290**: 527–30.

21 Harada A, Sekido N, Akahoshi T et al. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol* 1994; **56**: 559–64.

22 Meduri GU. The role of the host defence response in the progression and outcome of ARDS: pathophysiological correlations and response to glucocorticoid treatment. *Eur Respir J* 1996; **9**: 2650–70.

23 Hart TK, Cook RM, Zia-Amirhosseini P et al. Preclinical efficacy and safety of mepolizumab (SB-240563), a humanized monoclonal antibody to IL-5, in cynomolgus monkeys. *J Allergy Clin Immunol* 2001; **108**: 250–7.

24 Kazmierowski JA, Durbin WA, Reynolds HY. Kinetics of immunoglobulin transport into canine bronchial secretions. *Proc Soc Exp Biol Med* 1976; **152**: 493–8.

25 Drusano GL. Infection site concentrations: their therapeutic importance and the macrolide and macrolide-like class of antibiotics. *Pharmacotherapy* 2005; **25**: 150S–8S.

26 Seki H, Fukunaga K, Arita M et al. The anti-inflammatory and proresolving mediator resolvin E1 protects mice from bacterial pneumonia and acute lung injury. *J Immunol* 2010; **184**: 836–43.

27 Tsai WC, Strieter RM, Mehrad B et al. CXC chemokine receptor CXCR2 is essential for protective innate host response in murine Pseudomonas aeruginosa pneumonia. *Infect Immun* 2000; **68**: 4289–96.

28 Sun L, Guo RF, Newstead MW et al. Effect of IL-10 on neutrophil recruitment and survival after Pseudomonas aeruginosa challenge. *Am J Respir Cell Mol Biol* 2009; **41**: 76–84.

29 Qiu Y, Zhu J, Bandi V et al. Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003; **168**: 968–75.

30 Muhlebach MS, Stewart PW, Leigh MW et al. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. *Am J Respir Crit Care Med* 1999; **160**: 186–91.

31 Bainbridge T, Fick RB Jr. Functional importance of cystic fibrosis immunoglobulin G fragments generated by Pseudomonas aeruginosa elastase. *J Lab Clin Med* 1989; **114**: 728–33.