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**In a murine model of acute lung infection, airway administration of a therapeutic antibody confers greater protection than parenteral administration**

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**Running title:** Airway-administered antibody protects against pneumonia

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## Abstract

Due to growing antibiotic resistance, pneumonia caused by *Pseudomonas aeruginosa* is a major threat to human health and is driving the development of novel anti-infectious agents. Preventively or curatively administered pathogen-specific therapeutic antibodies (Abs) have several advantages, including a low level of toxicity and a unique pharmacological profile. At present, most Abs against respiratory infections are administered parenterally; this may not be optimal for therapeutics that have to reach the lungs to be effective. Although the airways constitute a logical delivery route for biologics designed to treat respiratory diseases, there are few scientific data on the advantages or disadvantages of this route in the context of pneumonia treatment. The objective of the present study was to evaluate the efficacy and fate of an anti-*P. aeruginosa* Ab targeting pcrV (mAb166) as a function of the administration route during pneumonia.

The airway-administered mAb166 displayed a favorable pharmacokinetic profile during the acute phase of the infection, and was associated with greater protection (relative to other delivery routes) of infected animals. Airway administration was associated with lower levels of lung inflammation, greater bacterial clearance, and recruitment of neutrophils in the airways.

In conclusion, the present study is the first to have compared the pharmacokinetics and efficacy of an anti-infectious Ab administered by different routes in an animal model of pneumonia. Our findings suggest that local delivery to the airways is associated with a more potent anti-bacterial response (relative to parenteral administration), and thus open up new perspectives for the prevention and treatment of pneumonia with Abs.

## Introduction

Pneumonia (whether either community- or hospital-acquired) is a leading cause of morbidity, quality-adjusted life year loss, and mortality in children, adults, and the elderly. Furthermore, an alarming rise in levels of antimicrobial resistance is compromising the effective prevention and treatment of pneumonia. The opportunistic pneumonia-causing pathogens *Pseudomonas aeruginosa* (*P. aeruginosa*) is often associated with devastating, acute disease, and chronic infections [1, 2]. *Pseudomonas aeruginosa* is one of the most prevalent infectious agents in intensive care units, and is the leading cause of ventilator-associated pneumonia in patients being treated for trauma or severe viral infections. Moreover, *P. aeruginosa*-associated lung infections are associated with significantly higher mortality than infections with other pathogens [3, 4]. The prevention and treatment of *P. aeruginosa* infections are challenging, in view of the bacterium's genetic and biochemical versatility and intrinsic resistance to antibiotics [5]. Overall, the data show that the significant increase in antibiotic resistance over the last 15 years has been associated with greater premature mortality [6].

The low number of novel antibiotics coming onto the market means that novel alternative or complementary anti-infectious strategies are urgently needed. One such strategy involves the use of therapeutic antibodies (Abs), which have already proven successful in the prevention or treatment of infectious lung diseases. Three anti-infectious Abs have been approved for clinical use: the humanized IgG1 palivizumab for the prevention of respiratory syncytial virus infection in high-risk infants, and the human IgG1 raxibacumab and the chimeric IgG1 obiltoxaximab for the prophylaxis and/or treatment of pulmonary anthrax. Furthermore, 12 Abs targeting respiratory pathogens (including 4 Abs against *P. aeruginosa*) are in clinical development [7].

In the clinic, most therapeutic antibodies (and, indeed, most therapeutic proteins) are administered parenterally, i.e. via the intravenous (i.v.), subcutaneous (s.c.) or intramuscular (i.m.) route. Oral administration is not indicated because therapeutic proteins are not usually formulated to resist acid denaturation in the stomach or proteolytic degradation in the rest of the gastrointestinal tract. Most Abs for the treatment of respiratory tract infections are administered i.v. [7]. However, this route is unlikely to be optimal for therapeutic designed to treat respiratory diseases; the few available literature data indicate that only a small fraction of the injected Ab reaches the lungs/airways [8-10]. Given that large amounts of Ab are usually required to protect against infections, an Ab's systemic distribution after i.v. injection might limit efficacy while increasing the risk of systemic toxicity (serum thickening, cytokine release syndrome).

As the body's main entry portal for innocuous or harmful environmental antigens, the airways represent an attractive delivery route for drugs designed to treat respiratory diseases. We and others have recently demonstrated that the airway route is feasible for the delivery of Ab-based therapeutics [11-18]. The Abs induce a treatment response, and pass slowly into the systemic circulation [19-22]. Furthermore, it has been shown that Ab-based therapeutics are sensitive to aerosolization but can retain their physical and immunological properties when delivered with specific devices and after the addition of specific excipients [16].

Overall, the results of preclinical studies have shown that inhaled Abs are therapeutically active and thus underpin the growing interest in the pulmonary/airway route [7, 23, 24]. Nevertheless, the

81 literature data on the advantages and disadvantages of parenteral versus local routes of anti-infective  
82 Ab administration are scarce. It is probably highly important to take account of situations in which the  
83 alveolus-capillary barrier is altered, since this might change the fate of and response to Abs. In the  
84 present study (performed in a murine model of acute pneumonia), we investigated the  
85 pharmacokinetics (PK) and protective activity of an archetypal anti-*P. aeruginosa* Ab as a function of  
86 its administration route. We found that airway administration of Ab was associated with a favorable PK  
87 profile, longer retention in the lung compartment, and a stronger anti-bacterial response by the host  
88 (characterized by enhanced neutrophil recruitment and bacterial clearance, and attenuated lung  
89 permeability). Local administration of Ab was also associated with a less intense acute inflammatory  
90 response, lower levels of pro-inflammatory cytokines.

## Materials and methods

### *Mice and reagents*

Adult male C57BL/6j (B6) mice (6 to 8 weeks old) were obtained from Janvier (France). All mice were housed under specific-pathogen-free conditions at the *PST Animaleries* animal facility (France) and had access to food and water *ad libitum*. All animal experiments complied with the current European legislative, regulatory and ethical requirements and were approved by the local animal care and use committee (reference: APAFIS#7608-2016111515206630).

mAb166 was generated using a PTA-9180 hybridoma (LGC Standards, France) and supplied as sterile, pyrogen-free solution in phosphate-buffered saline (PBS), in accordance with good manufacturing practice (BioXcell, USA). The IgG2b isotype control Ab MPC11 (European Collection of Authenticated Cell Cultures, UK), was generated using the same technique. After preliminary experiments, we decided to use a dose of 100 µg of mAb166 in a volume of 50 µL of PBS for airway administration or a volume of 100µL for i.v, i.p and s.c administration. The Ab was injected 2 hours before infection.

### *The model of acute P. aeruginosa infection*

*Pseudomonas aeruginosa* strain PA103 was kindly provided by Professor Teiji Sawa (Kyoto University, Japan). The colonies' uniformity was checked by plating on *Pseudomonas* isolation agar (PIA) plates. PA 103 has been transformed (using quadriparental mating) with a mini-Tn7T transposon that enables the constitutive expression of the *LuxCDABE* operon. The mice were infected with a freshly prepared inoculum. Starting from a frozen stock solution at 37°C, an overnight culture was prepared in 10 mL of Luria broth (LB) with shaking at 220 rpm. A 100 µL aliquot was then taken to start a fresh 10 mL culture in LB. The culture was stopped when an optical density (OD) of about 0.4 was achieved; this corresponded to a titer of about  $3 \times 10^8$  bacteria/mL. The bacteria were washed once in PBS diluted in saline, to obtain a titer of  $5 \times 10^5$  bacteria/40 µL. Each inoculum was then checked for accuracy (by direct plating on fresh PIA plates) and uniformity in a bioluminescence assay (ChemiDoc™, Bio-Rad, France). Mice were anaesthetized with 3% isofluorane. An operating otoscope fitted with an intubation speculum was used to maintain tongue retraction and visualize the glottis. A fiber optic wire, threaded through a 20 G catheter and connected to a fiber optic stylet (Harvard Apparatus, France) was inserted into the mouse's trachea. Correct intubation was confirmed using a lung inflation bulb, and 40 µL of the bacterial solution was applied through an ultrafine pipette tip. In all experiments, the animals' mortality and body weight were monitored daily. For ethical reasons, we sacrificed moribund animals and those having lost more than 25% of their initial body weight. The remaining mice were sacrificed 6 hours or 1, 3, 7, 14, 21 or 28 days after infection.

### *Administration of the therapeutic Ab*

mAb166 is a murine monoclonal IgG2b,κ Ab against pcrV, a component of a type three secretion system (T3SS) expressed on the cell surface of *P. aeruginosa* and that translocates a wide array of toxins into the host cell's cytosol [25, 26]. Two hours before the infection, the anti-pcrV Ab (mAb166,

100 µg/animal) or its IgG2b isotype control (MPC11) were administered either via the caudal vein, into dorsal s.c. tissue, intraperitoneally (i.p.), or orotracheally using a MicroSprayer® aerosolizer (Penn-Century, USA), as described previously [21].

#### *Bronchoalveolar lavage, and organ sampling*

At different time-points after *P. aeruginosa* infection, mice were euthanized using a lethal dose of ketamine/xylazine. Bronchoalveolar lavage fluid was then collected by cannulating the trachea and washing the lung twice with 1.2 mL of PBS at room temperature. The lavage fluid was centrifuged at 400 g for 10 min at 4°C, and the supernatant was stored at -20°C until analysis. The cell pellet was resuspended in PBS, and counted in a hemocytometer chamber. Cytospin samples were prepared using a cytocentrifuge (Shandon™ Cytospin 4™, ThermoFisher Scientific, USA; 1000 rpm for 10 min). After staining with RAL555 reagent (RAL Diagnostic, France), neutrophils, macrophages and lymphocytes were counted. Lungs were homogenized for further analysis.

#### *Pulmonary permeability*

An *in vivo* pulmonary permeability assay was used to assess barrier function, as previously described [27]. Briefly, animals were injected intranasally with dextran-FITC (FD4; molecular mass: 4 kDa; Sigma, France; 10 mg/kg of body weight) 30 min before drawing blood (via retro-orbital puncture) - at 0 (uninfected controls), 3, 6 and 18 hours after infection - and serum samples were prepared. The fluorescence intensity at 485/525 nm was measured using an Infinite M200 microplate reader (Tecan, Switzerland). The FITC concentrations were determined from standard curves generated by serial dilution of a stock FD4 solution. The FITC recovery was calculated by linear regression of the sample's fluorescence and taking account of the mouse's body weight.

#### *Determination of the bacterial load in the lung*

Lung homogenates were prepared in 2 mL of PBS containing broad-spectrum protease inhibitors (Sigma, France), using a GentleMACS tissue homogenizer (Miltenyi Biotec, Germany). Tenfold serial dilutions of homogenates were plated on PIA plates. Plates were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere, and the colonies were counted after 24 hours. The colonies' uniformity was checked in a bioluminescence assay (ChemiDoc™, Bio-Rad, France). The homogenates were centrifuged at 800 g for 10 min at 4°C, and the supernatants were stored at -20°C until analysis.

#### *mAb166, cytokine, chemokine and protein assays*

mAb166 concentrations in broncho-alveolar lavage fluid (BALF) and lung homogenates were measured in an ELISA using purified recombinant pcrV as the coating antigen and HRP-conjugated goat anti-mouse IgG as the detection antibody (Jackson ImmunoResearch Laboratories, USA). The Ab-antigen complexes in the samples were dissociated with 300 mM acetic acid, and the solution was then neutralized with 1 M Tris pH 9, as described previously [28].

Concentrations of CXCL1, TNF-α, and IL-6 in BALF and lung homogenates were measured using specific ELISAs (Duoset Kit; R&D Systems, USA; limit of detection: at 50 pg/mL) according to the

manufacturer's instructions. Total BALF protein was determined using a BCA assay (ThermoFischer Scientific, France), according to the manufacturer's instructions (limit of detection: 5 µg/mL).

#### *Pharmacokinetic modeling of mAb166 in mice*

The fate of free mAb166 was studied by population pharmacokinetic modelling using Monolix software (2018 R2 version, Lixoft, France). Several structural models were tested and compared using goodness-of-fit and residual plots, and other standard validation procedures. A two-compartment model was found to best describe the concentrations of free mAb166 measured in serum and BALF after airway, s.c., i.p. and i.v. administration. This approach also enabled us to estimate the fraction absorbed following airway and s.c. administration. The model's first compartment corresponds to the blood as reflected by the serum), and the second corresponds to the airways (as reflected by BALF). The distribution between these two compartments was described by the first-order rate constants  $k_{12}$  (for the transfer from the blood to the airway) and  $k_{21}$  (for the transfer from the airway to the blood). The absorption rate following s.c. injection was described by the first-order constant  $k_a$ . Elimination of mAb166 from the blood compartment was described by the first-order constant  $k_{10}$ . The area under the concentration versus time curve (AUC) in blood or in airways was estimated between day 0 and day 3, in order to assess each animal's exposure to mAb166 as a function of the administration route.

#### *Statistical analysis*

Differences between experimental groups were determined in a one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-test, after confirmation that the data were normally distributed. The log-rank test was used for survival analysis. All statistical tests were performed with GraphPad Prism software (version 4.03 for Windows, GraphPad Software Inc., USA). All data are presented as mean  $\pm$  standard error of the mean (SEM). The threshold for statistical significance was set to  $p < 0.05$ .



## Results

### Acute lung infection by *P. aeruginosa* PA103 is associated with impairment of the alveolus-capillary barrier

We compared the standard parenteral routes used to administer Abs in the clinic (i.v. and s.c.) with local airway administration. Given that the i.p. route is often used to deliver drugs in preclinical studies, we also included it in our experiments.

To assess the PK, PD and safety profiles of the anti-*P. aeruginosa* Ab as a function of its administration route, we first established and characterized an acute pneumonia model (using the clinical isolate *P. aeruginosa* PA103) derived from that described by Frank et al. [26]. This model mimics the initial phases of airway colonization by *P. aeruginosa* in patients with ventilator-associated pneumonia or cystic fibrosis [29]. First, we determined the dose of PA103 needed to induce a robust (but not extreme) infection. As shown in Figure 1A, orotracheal administration of  $5 \times 10^6$  and  $5 \times 10^5$  cfu killed all the animals within 18 hours and 36 hours, respectively. However, all the mice infected with a dose of  $5 \times 10^4$  cfu survived for 7 days (Figure 1A). To characterize the time course of PA103-induced pneumonia, we measured the bacterial load in the lungs at 1, 3, 6 and 18 hours post-infection. The load increased significantly at 1 hour post-infection, rose further at 3 hours, and reached a plateau after 6 hours (Figure 1B). Discernible signs of infection (such as ruffled fur, a hunched posture, and motor impairment) appeared between 1 and 3 hours post-infection and gradually intensified over time. Surviving animals showed a noticeable reduction in signs of disease after 48 hours (data not shown). It is known that in the late stages of infection, *P. aeruginosa* can disrupt the pulmonary-vascular barrier and thus lead to systemic dissemination, which is associated with a poor clinical prognosis [30]. Accordingly, we observed a lasting, time-dependent increase in alveolar-capillary permeability after infection, as assessed *in vivo* by the passage of a fluorescent marker from the airways into the blood (Figure 1C). This increase in the pulmonary epithelium's permeability might have facilitated the observed time-dependent increases in the level (Figure 1D) and prevalence (Figure 1E) of *P. aeruginosa* translocation into the blood of infected animals.

### Passage and retention of an anti-*P. aeruginosa* Ab in the lung compartment depends on the administration route

To determine the impact of the presence of the antigen and pneumonia-associated inflammation on the fate of Abs after administration by different routes, we measured the free and total mAb166 concentrations in both the serum (Figure 2A) and BALF (Figure 2B) of animals infected with *P. aeruginosa*. During the acute phase of the infection (6 h, and 1 and 3 days post-infection), we found that the proportion of free mAb166 in the serum was higher for systemic administration than for delivery to the airways (Figure 2A). Conversely, the free mAb166 concentration in BALF was higher in animals treated locally (Figure 2B). When comparing free and total mAb166 levels in the serum (Supplementary Figure 1A) and the BALF (Supplementary figure 1B), we observed that a significant proportion of the Ab was complexed with bacteria in the systemically treated groups; this is doubtless explained by the greater level of bacteremia in these groups (Figures 1D and 1E). In both compartments, mAb166 could no longer be detected between 7 and 14 days after infection. A

compartmentalized PK analysis described the measured concentrations to a satisfactory extent (Table 1). The model suggested that 8% of the Ab was absorbed following airway administration. If one assumes that the entire dose reached the BALF, this means that 92% of mAb166 was able to reach pcrV in the airways following pulmonary administration. The animals' local exposure to mAb166 (as indicated by the airways/blood AUC ratio) was 10 times higher after airway administration than after other types of administration (Figure 2C).

#### **mAb166's therapeutic efficacy against *P. aeruginosa* pneumonia depends on the administration route**

The use of an Ab from the same species as the experimental model may avoid confounding immunogenicity and nonspecific stimulation of the immune system. The murine mAb166 has been shown to prevent infection in both mice and rat lungs infected with *P. aeruginosa*, following either prophylactic administration or concurrent administration with the infectious agent [25, 26]. In our experiments, mAb166 was administered 2 hours before the dose of *P. aeruginosa*; this was done to avoid the generation of immune complexes that can arise during concomitant administration and thus obliterate PA103's virulence prior to contact with the host (Figure 3A). The Ab was delivered by each of the four tested routes, and the survival of the mice in each group was monitored for 1 week. To be noted, no additional death was observed during a 100-days follow-up. All mice treated with 100 µg of mAb166 exhibited displayed a higher survival rate (Figure 3B) than untreated mice. The survival rate after airway administration of mAb166 (80%) was significantly higher than after parenteral administration (40% to 55%) (Figure 3B). The mAb166 response was specific for PA103, and was not due to unrelated anti-inflammatory effect of IgG2b; the survival rate for mice treated with an irrelevant IgG2b isotype Ab was essentially the same as for untreated animals (Supplementary Figure 2). Taken as a whole, these data demonstrate that the airway administration of an anti-infectious Ab conferred a higher level of specific protection against acute pneumonia than parenteral administration.

#### **The clearance of *P. aeruginosa* and neutrophil recruitment in the lungs depend on mAb166's delivery route**

To gain insight into the mechanisms that contributed to the Ab's beneficial effect on *P. aeruginosa*-induced pneumonia, we analyzed the host's antibacterial responses 24 hours after infection. Mice treated parenterally with mAb166 exhibited a slightly (but not significantly) lower lung bacterial load (~0.2-0.4 log<sub>10</sub> cfu) than untreated controls, whereas delivery of mAb166 to the airways was associated with a significantly lower *P. aeruginosa* load in the lungs - about 1.3 log<sub>10</sub> cfu (Figure 4A). Beyond 24 hours, the *P. aeruginosa* load in surviving mice gradually diminished, and could not be detected 7 days post-infection (data not shown). Given that the exacerbation of pneumonia is intimately associated with lung remodeling and damage, we evaluated protein egress into the BALF as a surrogate marker for leakage of the alveolus-capillary barrier. Only animals treated with aerosolized mAb166 had significantly lower levels of protein in the BALF (Figure 4B), relative to untreated controls. In airway-treated animals, the blood-air barrier was therefore maintained for at least 7 days after the infection (Supplementary Figure 3A).

To determine which cellular components might be involved in the enhanced clearance of *P. aeruginosa*, we analyzed the BALF's immune cell counts 1 and 3 days post-infection. In all groups, total cell counts were higher in mAb166-treated mice than in untreated controls (Figure 4C). On day 1 post-infection, the cellular influx was significantly higher in animals treated with aerosolized mAb166 than animals in which mAb166 had been administered by other routes. Most of the recruited cells were neutrophils, which are essential for quickly controlling invading pathogens [31] (Figure 4D). On day 3 post-infection (i.e. after neutrophil exhaustion), monocytes/macrophages became preponderant for the mediation of neutrophil phagocytosis, inflammation resolution, and repair. As expected, pulmonary administration of mAb166 led to a significantly higher macrophage count in the BALF, relative to the other routes (Figure 4E). At later time points (i.e. in surviving animals), there were no intergroup differences (Supplementary Figures 3B, 3C and 3D). Although lymphocyte recruitment was similar in all groups at early time points (Figure 4F), we observed a significantly higher lymphocyte counts in the BALF of animals treated with aerosolized mAb166 21 and 28 days post-infection (Supplementary Figure 3E). Taken as a whole, our results suggest that the early, sustained presence of the Ab in the lung compartment post-infection enhances the lung's innate immune response to the pathogen, which in turn is correlated with better control of the bacterial load and a higher survival rate.

#### **In mice treated with mAb166, the modulation of lung inflammation after *P. aeruginosa* infection depends on the Ab's administration route**

Although inflammation is critical for the host's defense against invading pathogens, a protracted, uncontrolled reaction can cause tissue damage and organ dysfunction [32]. We therefore sought to determine the effect of mAb166 treatment on levels of inflammatory mediators in the BALF on day 1 after *P. aeruginosa* infection. Levels of the prototypal pro-inflammatory cytokines TNF $\alpha$  (Figures 5A and 5B) and IL-6 (Figures 5E and 5F) were significantly lower in mice treated with aerosolized mAb166 than in animals treated via other routes or in untreated controls. Low levels of CXCL1 (the main chemokine responsible for neutrophil recruitment) were observed in all mAb166-treated groups (Figures 5C and 5D) but the difference was greater in the lungs of animals that had received aerosolized mAb166 (Figure 5D). At later time points (i.e. in surviving animals), there were no intergroup differences (Supplementary Figure 4). Our results indicate that an enhanced innate immune response was associated with a weaker pro-inflammatory response, which protected the host against harmful lung dysfunction.

## Discussion

Although many different *P. aeruginosa* antigens have been investigated as parenterally administered immunotherapeutics or vaccine candidates (e.g. lipopolysaccharide, flagellin, and pilins), their efficacy remains limited [33, 34]. This may be because the parenteral administration route is not optimal. Since the main pathophysiological mechanisms underlying *P. aeruginosa* infection act within the lungs, matching the Ab's delivery route to the pathogenic target's location may produce a higher concentration in the vicinity of the target while limiting systemic exposure and reducing the likelihood of side effects. To the best of our knowledge, only a few literature studies have compared the local vs. parenteral delivery of anti-infectious Abs in the treatment of respiratory diseases [35, 36], and none of these characterized the Ab's PK/PD profiles in depth, **in pathophysiological conditions which impact on lung remodeling and inflammation and thereby may affect the fate of Ab**. Here, we showed for the first time that airway delivery of Ab is more efficacious than other delivery routes for containing and resolving *P. aeruginosa* pneumonia. There are several possible explanations for this greater efficacy.

**Firstly, we found that the anti-infectious Ab accumulated in the infected airways after local administration.** Most of the studies published to date characterized the fate of Ab at steady-state conditions, and found that IgG distribution to the airways was low after systemic delivery [8, 9, 17, 37] or even nasal delivery [35]. The lung inflammation and remodeling that occur during pneumonia might modulate this situation by increasing alveolus-capillary permeability, inducing proteolysis, modifying albumin homeostasis and thus altering the deposition, distribution and clearance of IgG. Accordingly, our PK model revealed a complex, infection-related mechanism of Ab distribution between the blood and the airways. Even though the mouse model of acute pneumonia likely corresponds to the worst possible scenario of alveolus-capillary barrier leakage and thus may be poorly representative of the clinic situation, the lungs' exposure to Ab was markedly higher after airway administration than after parenteral delivery. After local administration, the free mAb166 concentration was 10 times higher in the airways than in the blood. As previously shown under steady-state conditions or in models with an intact lung barrier [19, 30], we observed that airway-delivered Ab passed slowly and poorly into the systemic circulation and accumulated at high levels in the respiratory tract for a few days. It is possible that the rapid, efficacious control of the bacterial load and the lower level of inflammation associated with airway-delivered Ab might limit damage to the lung and this could explain (at least in part) the PK profile.

Indeed, protection against pneumonia after the airway delivery of mAb166 was associated with a lower bacterial load in the lung parenchyma. After airway delivery, the higher concentration of the Ab in the airways during the acute phase of the infection results in better access to the target antigen and thus better bacterial neutralization. The translocon protein pcrV is located within the T3SS' cap required for the injection of toxins into the host cell's cytosol [38], and is indispensable for *P. aeruginosa*'s virulence [39]. Thus, the Ab has a direct anti-infective action through antigen inhibition.

Moreover, Ab delivered through the airways provides greater protection against pneumonia by modulating the immune response. The clearance of *P. aeruginosa* from the airways results from the complex, coordinated involvement of many different cell types, including respiratory epithelial cells, resident and actively recruited myeloid cells, and lymphocytes. In the present study, we observed

greater accumulation of neutrophils in the BALF of animals treated with locally administered mAb166, when compared with parenterally treated mice and untreated mice. The early, massive recruitment of lung neutrophils is the main component of the innate immune system's response against *P. aeruginosa* infection [31], and is essential for controlling *P. aeruginosa* infections [40].

In contrast, a protracted, uncontrolled inflammatory response is detrimental for the host, and eventually leads to multiple organ dysfunction [41]. Here, we showed, that 24 hours after the infection, the pulmonary administration of mAb166 was associated with lower levels of TNF, IL-6 and CXCL1 production, relative to untreated and parenterally treated mice. At 6 hours TNF and CXCL1 production were similar between all treated groups (data not shown); CXCL1 decreasing over time only in pulmonary treated-animals. As previously shown, early production of CXCL1 preceding neutrophil recruitment is important to handle *P. aeruginosa* infection [40], while its accumulation correlates with deleterious inflammation [42, 43].

These findings are in line with reports showing that although inflammation is important for controlling *P. aeruginosa* infection [40], its maintenance is correlated with a poor outcome [42, 43]. Thus, airway delivery of anti-*P. aeruginosa* Ab may help to better control lung inflammation – an effect that has proven to be of benefit in patients with cystic fibrosis [44].

It should be noted that the pneumonia in this murine model is especially aggressive. Usually, untreated animals exhibit signs of severe inflammation within 6-8 hours of infection and die within 24-36 hours of infection. As previously reported [26], we gave the anti-infectious Ab prophylactically in order to prevent the excessive loss of animals. Although the prevention of pneumonia may be useful in people at a high risk of *P. aeruginosa* respiratory infections (such as mechanically ventilated patients), a therapeutic approach would have greater clinical relevance (i.e. the initiation of specific immunotherapy following confirmation of the *P. aeruginosa* infection). In the light of our present results, it is tempting to speculate that therapeutic administration through the airways may enable better control of *P. aeruginosa* infection; however, this will need to be confirmed in further research.

After decades of (mis)use of broad-spectrum antibiotics, antibiotic-resistant pathogens have become a real threat for human health and have made the management of pneumonia even more challenging [45]. *Pseudomonas aeruginosa* is on the World Health Organization's priority list of antibiotic-resistant bacteria, and infection-control experts and researchers are making concerted efforts to fight this deadly pathogen [46]. As shown in the present study, passive immunotherapy with an aerosolized anti-pcrV Ab might be a valuable way of preventing and possibly treating *P. aeruginosa* pneumonia. A PEGylated fragment of mAb166 for i.v. administration was developed by KaloBios Pharmaceuticals Inc. and has been tested in a Phase IIa study in mechanically ventilated patients infected with *P. aeruginosa* [47]. However, the Ab did not to meet the primary clinical endpoint [48]. Based on our findings, airway administration and a prophylactic clinical regimen (rather than therapeutic) might be more effective.

In conclusion, we demonstrated that prophylactic administration of anti-*P. aeruginosa* Ab to the lungs led to a more efficacious antibacterial and host response than conventional parenteral administration did. Our findings open up perspectives for the development of novel, inhaled, Ab-based prophylactics

386 and therapeutics, which may facilitate the development of novel, inhaled, Ab-based prophylactics and  
387 therapeutics for use against respiratory infections.

388 **Competing interests statement**

389 The authors have no competing interests to declare.

390

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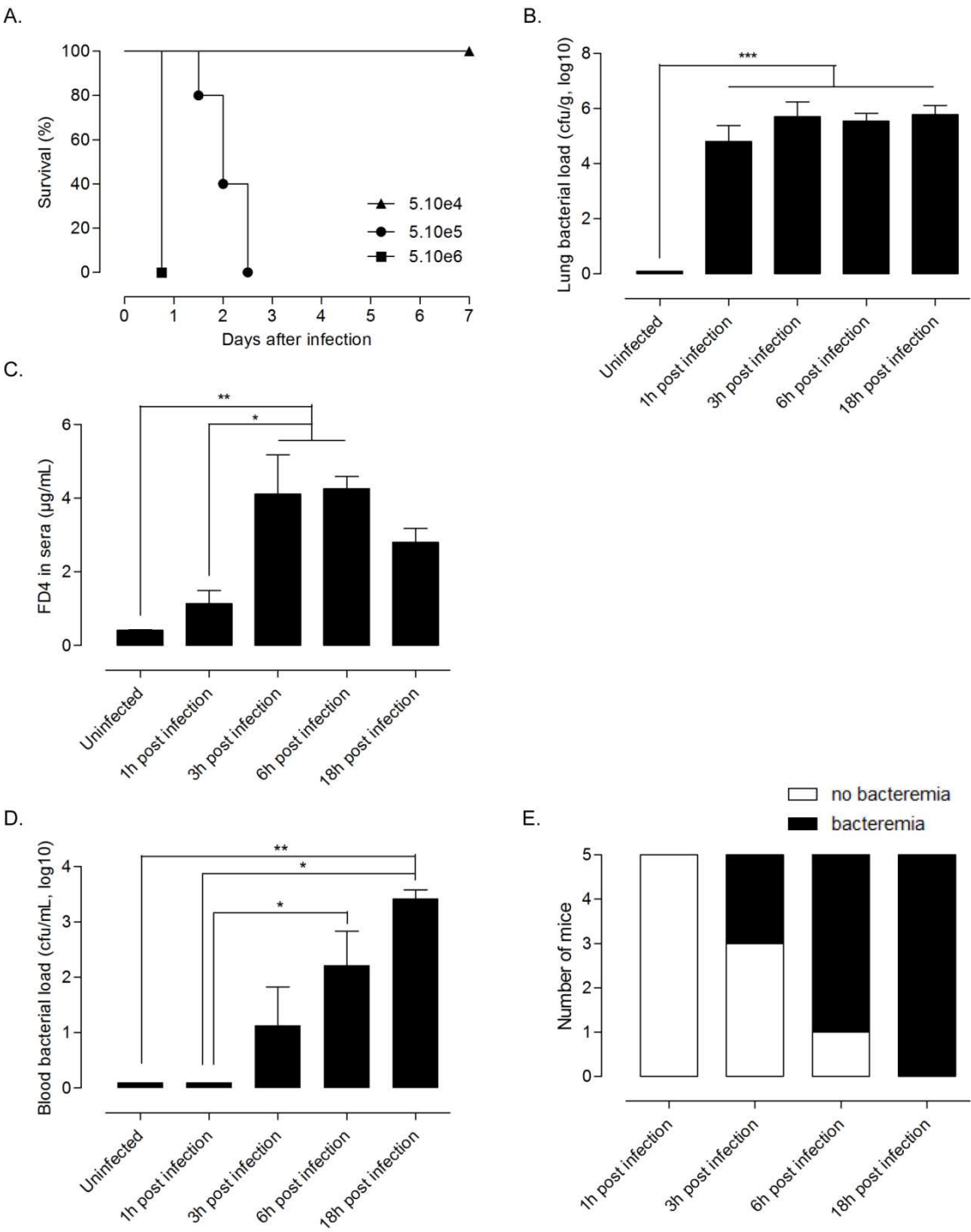
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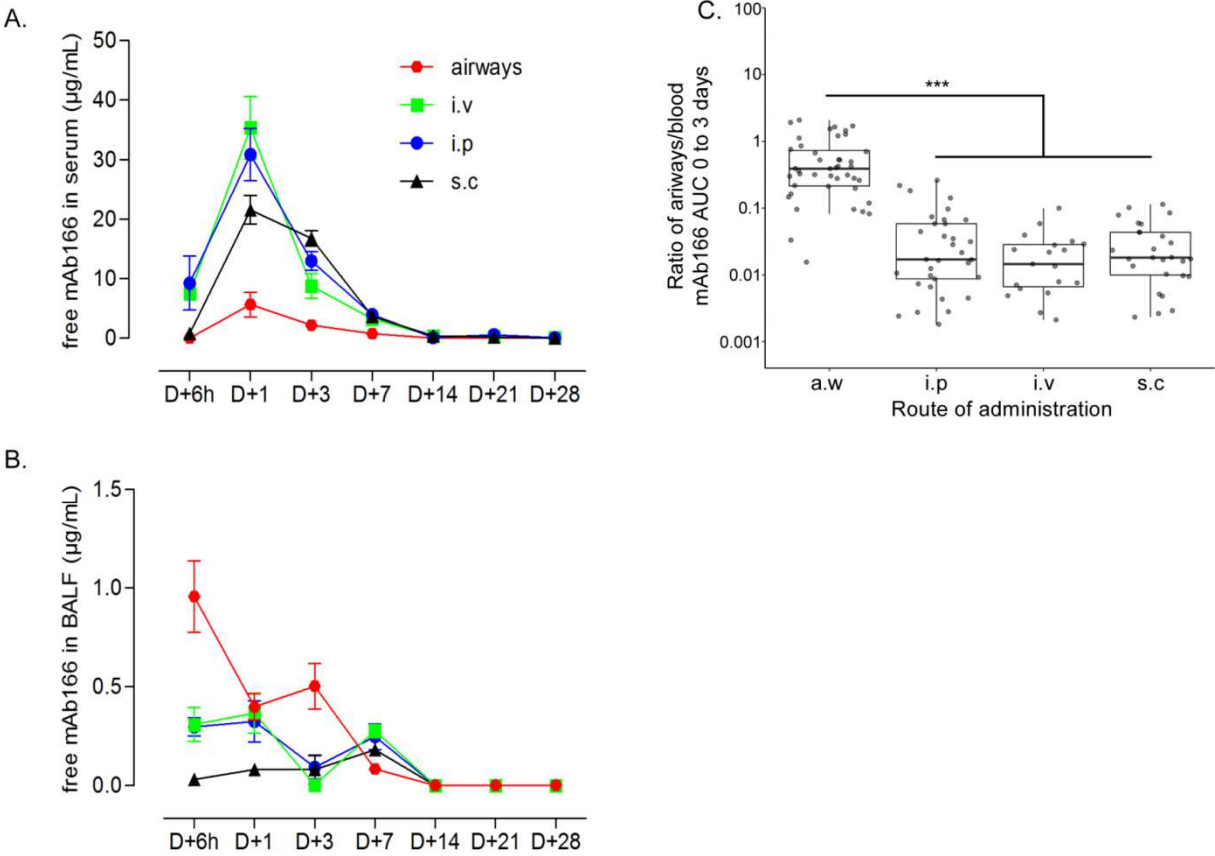
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550 **Figures**  
551 **Figure 1**

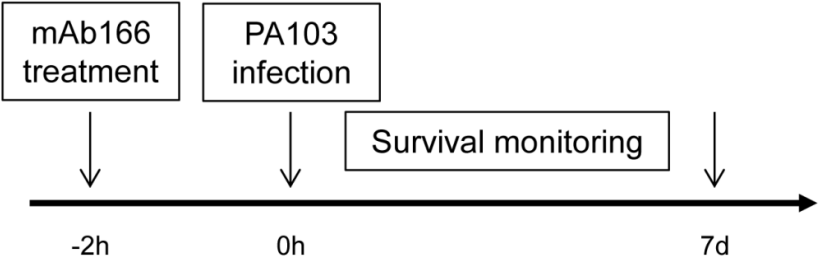


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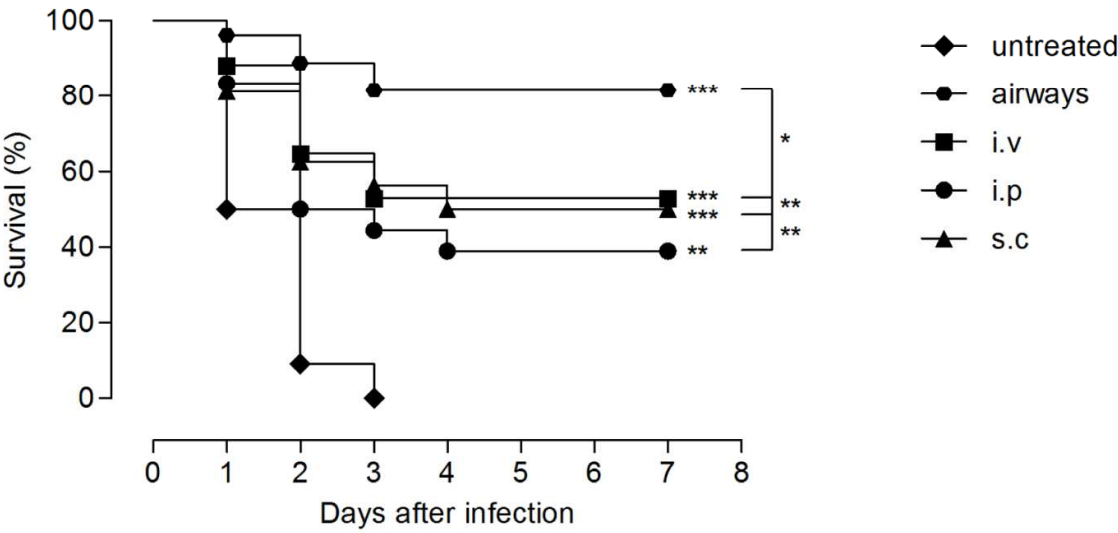


556 **Figure 3**

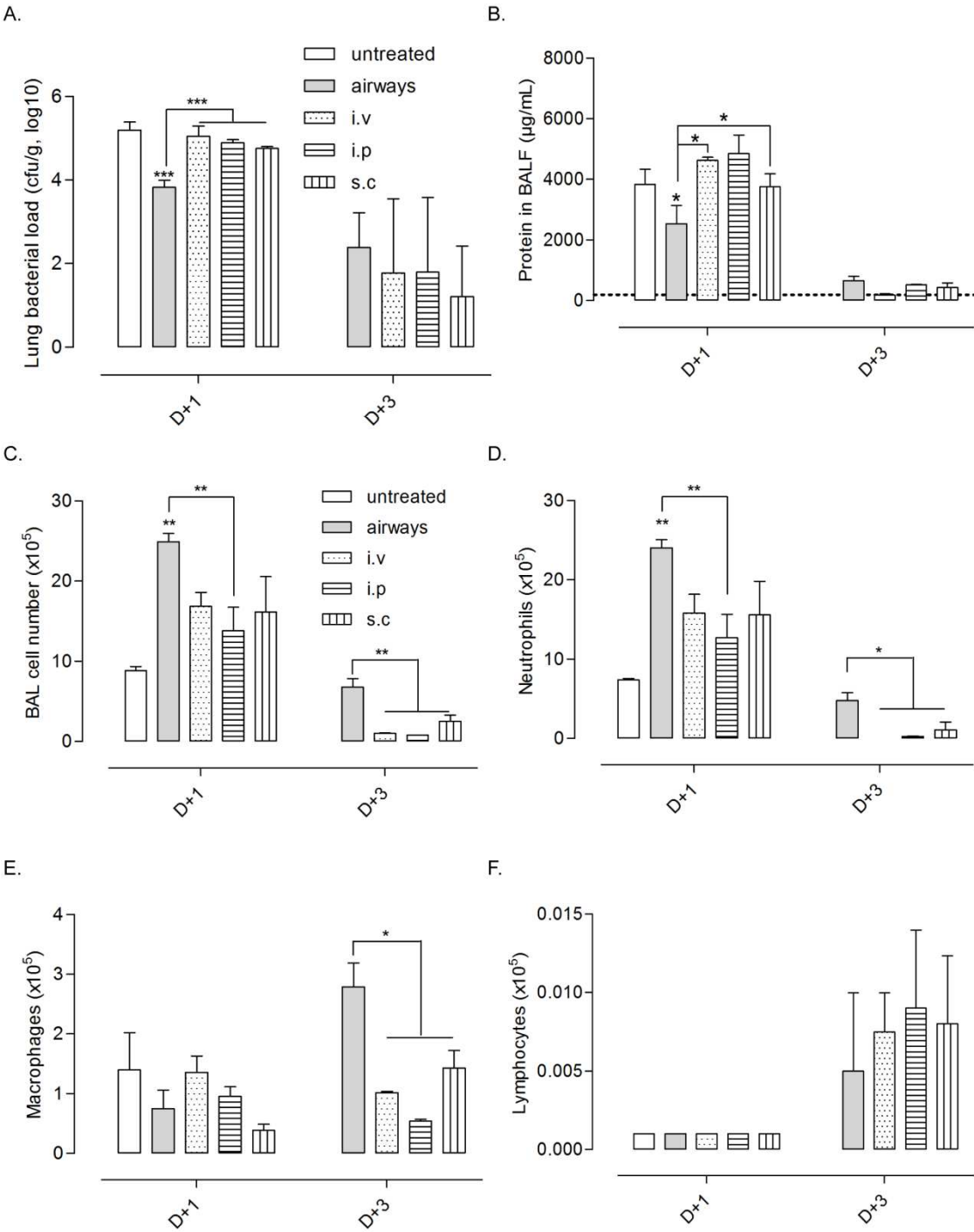
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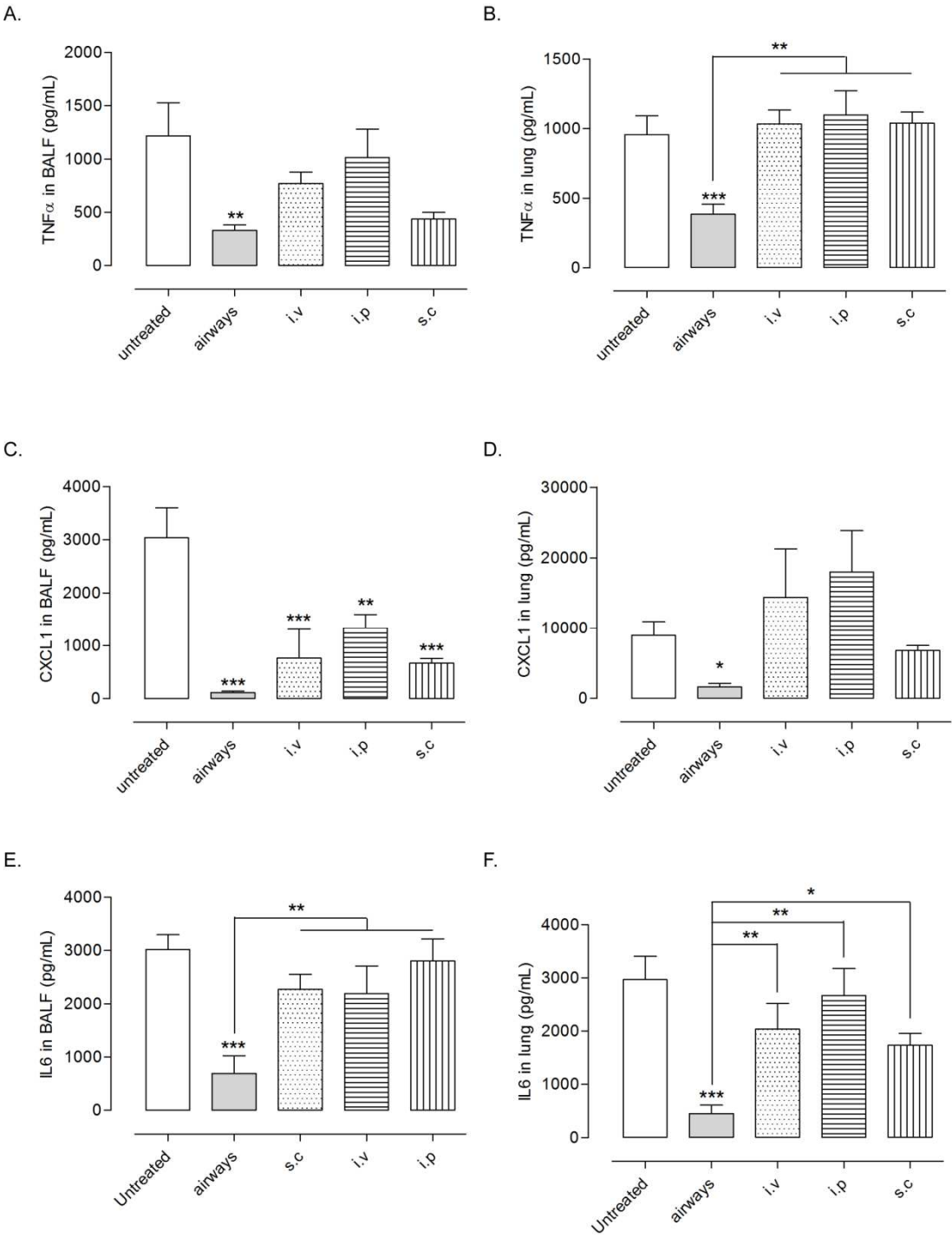


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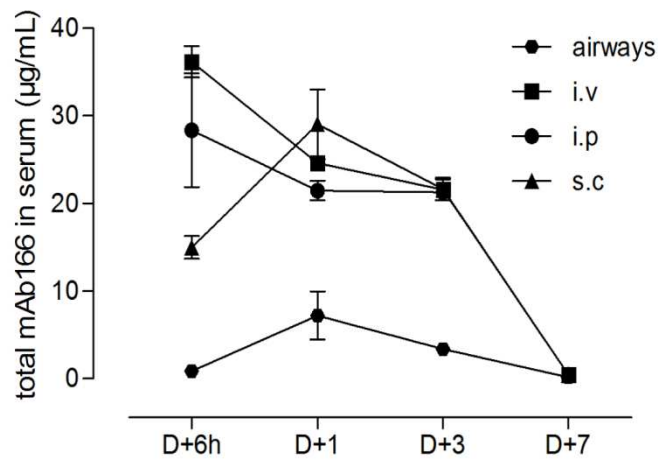
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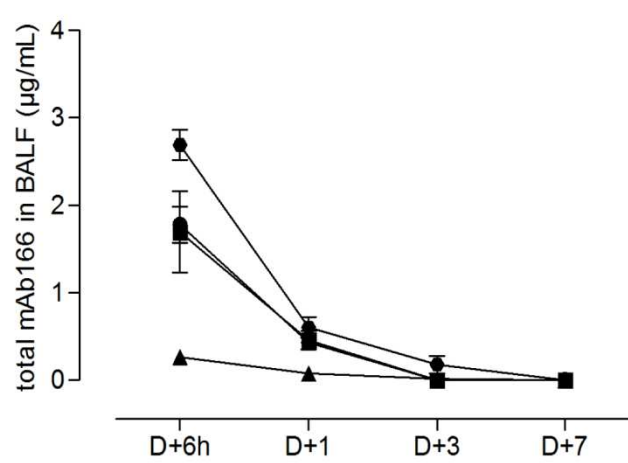


565 **Supplementary Figure 1**

A.



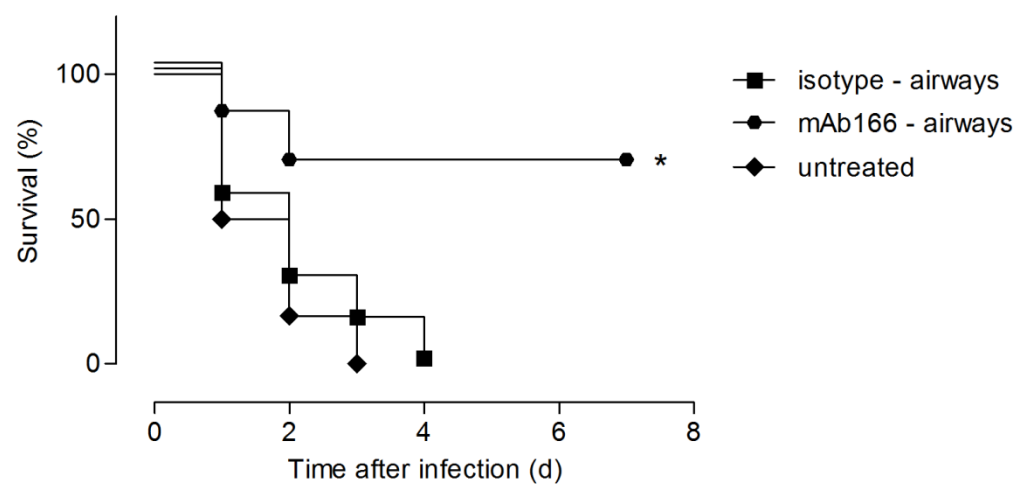
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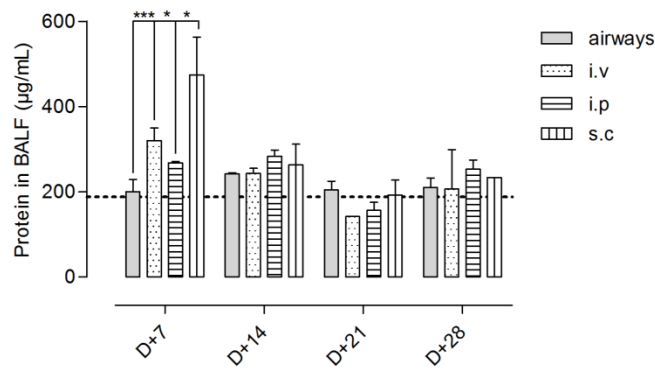




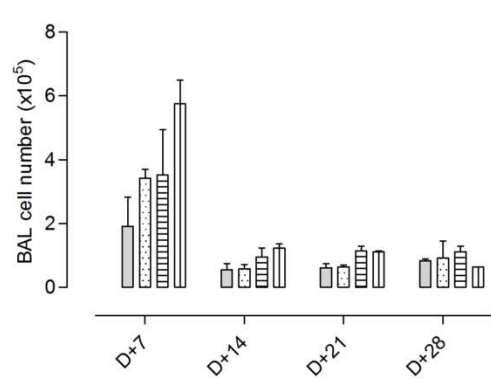
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571 **Supplementary Figure 3**

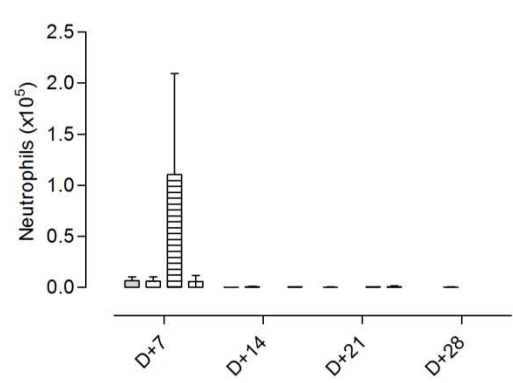
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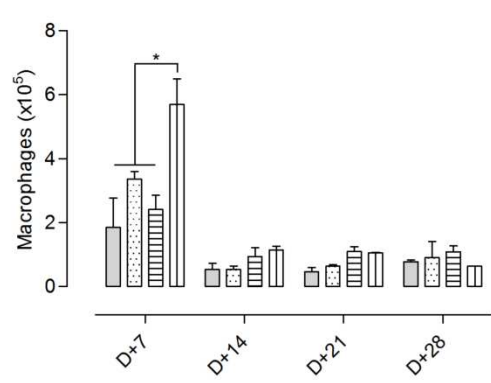
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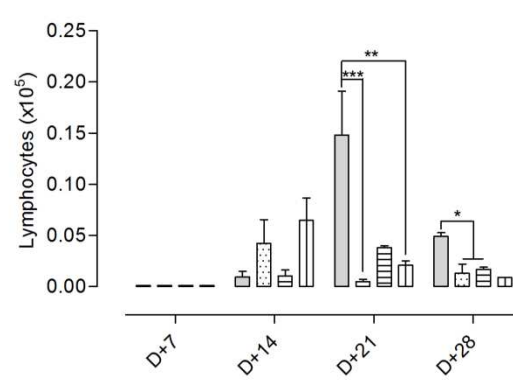
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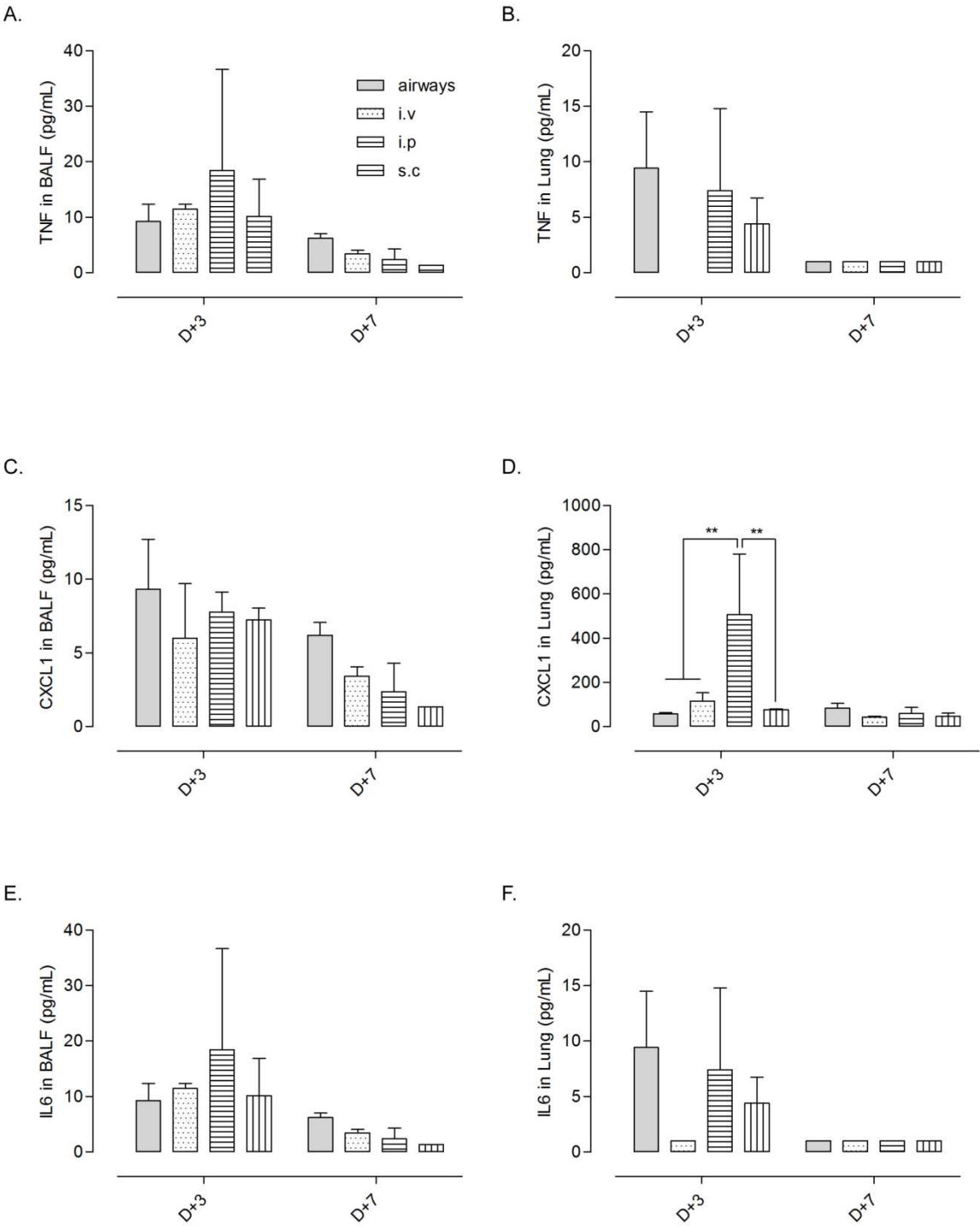


E.



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**Table 1**

parameter	$\theta$	CV%	P-value (Wald test)
$V_1$ (mL)	5,57	89	
$\beta_{V1(\text{Weight})}$	3,94		0,0002
$\beta_{V1(\text{Infected})}$	-1,00		0,0181
$k_{10}$ (day <sup>-1</sup> )	0,46	14	
$\beta_{k10(\text{Weight})}$	-1,07		0,0040
$k_{13}$ (day <sup>-1</sup> )	0,06	78	
$k_{31}$ (day <sup>-1</sup> )	0,62	78	
$F_{AW}$ (-)	0,08	42	
$V_B$ (mL)	5,61	22	
$k_{a(SC)}$ (day <sup>-1</sup> )	0,54	81	
$\beta_{kaSC(\text{Weight})}$	-6,98		0,0006
$F_{SC}$ (-)	0,63		

Where estimated parameters are:  $V_1$  and  $V_B$ : Serum and BALF volumes of distribution, respectively.  $F_{AW}$ ,  $F_{SC}$ : Proportion of the dose that reach the serum after AW and SC route, respectively.  $k_{10}$ : Constant of elimination from the serum.  $k_{13}$ ,  $k_{31}$ : constants of distribution to and from the peripheral volume of distribution, respectively.  $k_a$  (SC): constant of absorption after s.c. route.  $\beta_{V1}$  (Weight),  $\beta_{k10}$  (Weight),  $\beta_{kaSC}$  (Weight) : Parameters quantifying the influence of the weight on  $V_1$ ,  $k_{10}$  and  $k_a$  (SC), respectively.  $\beta_{V1}$  (Infected): Influence of the infected status on the serum volume of distribution  $V_1$ . -  $\theta$  is the population parameter, CV% is the coefficient of variation.

## Figure Legends

Figure 1: *P. aeruginosa* strain PA103 induces acute lung infection and increases the permeability of the capillary-alveolar barrier.

C57/BL6J (B6) mice received a 40  $\mu$ L orotracheal instillation of *P. aeruginosa* strain PA103 ( $5 \times 10^4$ ,  $5 \times 10^5$  or  $5 \times 10^6$  cfu). Survival (A) was monitored for 7 days after infection. In another experiment, B6 mice received a 40  $\mu$ L orotracheal instillation of *P. aeruginosa* ( $5 \times 10^5$  cfu) and were injected intranasally with dextran-FITC 30 min before analysis. The bacterial load (cfu) in the lung (B), the translocation of 4 kDa dextran-FITC (FD4) into blood (C), and the bacterial load in the blood (cfu) (D) were measured 0, 1, 3, 6 and 18 hours after the infection. The proportion of mice with *P. aeruginosa* in the blood (E) was evaluated. The data are quoted as the mean  $\pm$  SEM. \*, \*\* and \*\*\*:  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, in a one-way ANOVA with Newman-Keuls's correction for multiple comparisons. The results are representative of two independent experiments (n=5-10 mice for each).

Figure 2: Kinetics of and exposure to mAb166 in the serum and the airways after PA103 infection, as a function of the route of administration.

B6 mice received 100  $\mu$ g of mAb166 via the pulmonary (airway), intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal instillation of 40  $\mu$ L of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). The total mAb166 concentrations in serum (A) and in BALF (B) were determined 6 h and 1, 3, 7, 14, 21 and 28 days after the infection, using an ELISA. The data are quoted as the mean values  $\pm$  SEM.

Using estimated pharmacokinetic parameters for day 0 and day 3 (Table 1), the airways/blood (C) AUC ratios were determined. The lower and upper hinges correspond to the interquartile range (IQR, the first and third quartiles). The upper whisker extends to the largest value no further than  $1.5 \times$  IQR from the hinge. The lower whisker extends from the hinge to the smallest value no further than  $1.5 \times$  IQR from the hinge. The results correspond to 7 pooled, independent experiments (n=12-27 mice for each).

Figure 3: Airway administration of a therapeutic antibody is associated with greater resistance against *P. aeruginosa* pneumonia.

B6 mice were treated (or not) with 100  $\mu$ g of mAb166 via the pulmonary (airway), intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal instillation of 40  $\mu$ L of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). Survival was monitored for 7 days after infection. \*, \*\* and \*\*\*:  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, in a log-rank test). The results correspond to 7 pooled, independent experiments (n=12-27 mice for each).

Figure 4: Enhanced *P. aeruginosa* clearance and neutrophil recruitment in the lungs of airway-treated mice.

B6 mice were treated (or not) with 100  $\mu$ g of mAb166 via the pulmonary (airway), intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal instillation of 40  $\mu$ L of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). The bacterial load (cfu) in the lungs (A)

was measured 24 and 72 hours post-infection. Total protein (B), the total cell count (C), and counts of neutrophils (D), macrophages (E) and lymphocytes (F) in the BALF were evaluated 24 h post-infection. The data are quoted as the mean  $\pm$  SEM. \*, \*\* and \*\*\*:  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, in a one-way ANOVA with Newman-Keuls's correction for multiple comparisons. The results are representative of three independent experiments (n=5-10 mice for each).

#### Figure 5: Lower levels of lung inflammation in mice treated with aerosolized mAb166 after *P. aeruginosa* infection.

B6 mice were treated (or not) with 100  $\mu$ g of mAb166 via the pulmonary (airway), intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal instillation of 40  $\mu$ L of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). The concentrations of TNF (A and B), CXCL1 (C and D) and IL-6 (E and F) in BALF and lung homogenates were determined 24 hours after the infection. The data are quoted as the mean  $\pm$  SEM. \*, \*\* and \*\*\*:  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, in a one-way ANOVA with Newman-Keuls's correction for multiple comparisons. The results are representative of three independent experiments (n=5-10 mice for each).

#### Table 1: mAb166 compartmentalized PK model

Where estimated parameters are: V1 and VB: Serum and BALF volumes of distribution, respectively. FAW, FSC: Proportion of the dose that reach the serum after AW and SC route, respectively. k10: Constant of elimination from the serum. k13, k31: constants of distribution to and from the peripheral volume of distribution, respectively. Ka (SC): constant of absorption after s.c. route.  $\beta$ V1 (Weight),  $\beta$ k10 (Weight),  $\beta$ kaSC (Weight): Parameters quantifying the influence of the weight on V1, k10 and ka (SC), respectively.  $\beta$ V1 (Infected): Influence of the infected status on the serum volume of distribution V1. -  $\theta$  is the population parameter, CV% is the coefficient of variation.

#### Supplementary Figure 1: Kinetics of free mAb166 in the serum and the airways after PA103 infection, as a function of the route of administration.

B6 mice received 100  $\mu$ g of mAb166 via the pulmonary (airway), intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal instillation of 40  $\mu$ L of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). The free mAb166 concentrations in serum (A) and in BALF (B) were determined 6 h and 1, 3, 7, 14, 21 and 28 days after the infection, using an ELISA. The data are quoted as the mean values  $\pm$  SEM.

#### Supplementary Figure 2: Airway administration of a therapeutic antibody is associated with greater resistance against *P. aeruginosa* pneumonia.

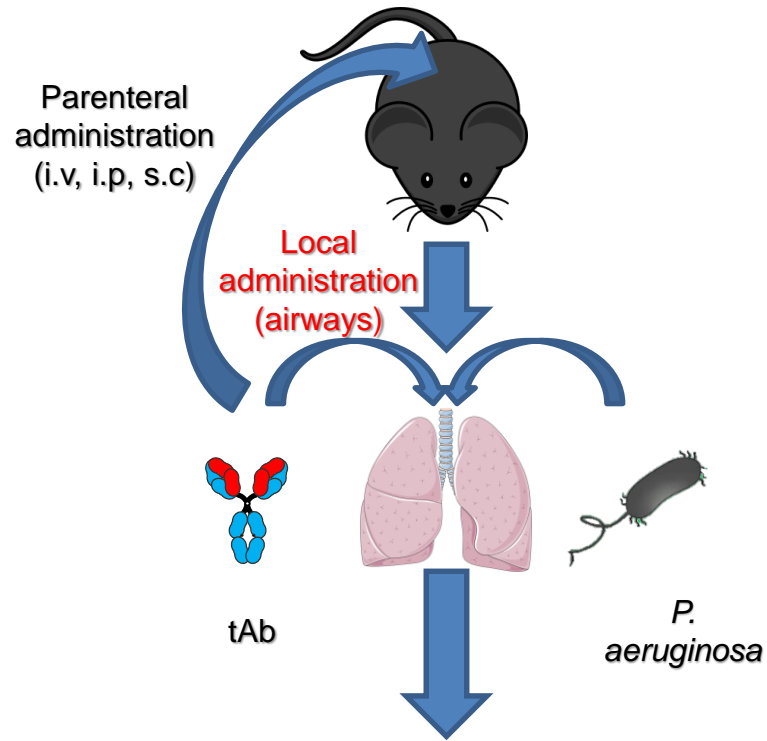
B6 mice were treated (or not) with 100  $\mu$ g of mAb166 or an IgG2b isotype control via the pulmonary route 2 hours before being infected by orotracheal instillation of 40  $\mu$ L of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). Survival was monitored for 7 days after infection. \*:  $p < 0.05$  in a log-rank test. The results are representative of 1 experiment (n= 5-8 mice).

#### Supplementary Figure 3: Late leukocyte recruitment in the lungs of *P. aeruginosa*-infected mice.

B6 mice were treated (or not) with 100 µg of mAb166 via the pulmonary (airway), intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal instillation of 40 µL of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). Total protein (A), the total cell count (B) and counts of neutrophils (C), macrophages (D) and lymphocytes (E) in the BALF were evaluated 7, 14, 21 and 28 days after the infection. The data are quoted as the mean  $\pm$  SEM. \*, \*\* and \*\*\*:  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, in a one-way ANOVA with Newman-Keuls' correction for multiple comparisons. The results are representative of four independent experiments (n=5-10 for each).

Supplementary Figure 4: Late lung inflammation in the lungs of *P. aeruginosa*-infected mice.

B6 mice were treated (or not) with 100 µg of mAb166 via the pulmonary (airway), intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal instillation of 40 µL of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). The concentrations of TNF (A and B), CXCL1 (C and D) and IL-6 (E and F) in BALF and in lung homogenates were determined 3 and 7 days after the infection. The data are quoted as the mean  $\pm$  SEM. \*, \*\* and \*\*\*:  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively, in a one-way ANOVA with Newman-Keuls' correction for multiple comparisons. The results are representative of four independent experiments (n=5-10 for each).



### Local vs Parenteral

