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

3

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15

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17

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21 **Abstract**

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

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

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

## 41 Introduction

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

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

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

72 As the body's main entry portal for innocuous or harmful environmental antigens, the airways  
73 represent an attractive delivery route for drugs designed to treat respiratory diseases. We and others  
74 have recently demonstrated that the airway route is feasible for the delivery of Ab-based therapeutics  
75 [11-18]. The Abs induce a treatment response, and pass slowly into the systemic circulation [19-22].  
76 Furthermore, it has been shown that Ab-based therapeutics are sensitive to aerosolization but can  
77 retain their physical and immunological properties when delivered with specific devices and after the  
78 addition of specific excipients [16].

79 Overall, the results of preclinical studies have shown that inhaled Abs are therapeutically active and  
80 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.

## 91 **Materials and methods**

92

### 93 *Mice and reagents*

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

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

106

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

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

126

### 127 *Administration of the therapeutic Ab*

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

131 100 µg/animal) or its IgG2b isotype control (MPC11) were administered either via the caudal vein, into  
132 dorsal s.c. tissue, intraperitoneally (i.p.), or orotracheally using a MicroSprayer® aerosolizer (Penn-  
133 Century, USA), as described previously [21].

134

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

136 **At different time-points after *P. aeruginosa* infection, mice were euthanized using a lethal dose of**  
137 **ketamine/xylazine.** Bronchoalveolar lavage fluid was then collected by cannulating the trachea and  
138 washing the lung twice with 1.2 mL of PBS at room temperature. The lavage fluid was centrifuged at  
139 400 g for 10 min at 4°C, and the supernatant was stored at -20°C until analysis. The cell pellet was  
140 resuspended in PBS, and counted in a hemocytometer chamber. Cytospin samples were prepared  
141 using a cytocentrifuge (Shandon™ Cytospin 4™, ThermoFisher Scientific, USA; 1000 rpm for 10 min).  
142 After staining with RAL555 reagent (RAL Diagnostic, France), neutrophils, macrophages and  
143 lymphocytes were counted. **Lungs were homogenized for further analysis.**

144

#### 145 *Pulmonary permeability*

146 An *in vivo* pulmonary permeability assay was used to assess barrier function, as previously described  
147 [27]. Briefly, animals were injected intranasally with dextran-FITC (FD4; molecular mass: **4 kDa**;  
148 **Sigma, France; 10 mg/kg of body weight**) **30 min before drawing blood** (via retro-orbital puncture) - **at**  
149 **0 (uninfected controls), 3, 6 and 18 hours after infection** - and serum samples were prepared. The  
150 fluorescence intensity at 485/525 nm was measured using an Infinite M200 microplate reader (Tecan,  
151 Switzerland). The FITC concentrations were determined from standard curves generated by serial  
152 dilution of a stock FD4 solution. The FITC recovery was calculated by linear regression of the sample's  
153 fluorescence and taking account of the mouse's body weight.

154

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

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

162

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

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

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

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

173

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

175 The fate of free mAb166 was studied by population pharmacokinetic modelling using Monolix software  
176 (2018 R2 version, Lixoft, France). Several structural models were tested and compared using  
177 goodness-of-fit and residual plots, and other standard validation procedures. A two-compartment  
178 model was found to best describe the concentrations of free mAb166 measured in serum and BALF  
179 after airway, s.c., i.p. and i.v. administration. This approach also enabled us to estimate the fraction  
180 absorbed following airway and s.c. administration. The model's first compartment corresponds to the  
181 blood (as reflected by the serum), and the second corresponds to the airways (as reflected by BALF).  
182 The distribution between these two compartments was described by the first-order rate constants k<sub>12</sub>  
183 (for the transfer from the blood to the airway) and k<sub>21</sub> (for the transfer from the airway to the blood).  
184 The absorption rate following s.c. injection was described by the first-order constant k<sub>a</sub>. Elimination of  
185 mAb166 from the blood compartment was described by the first-order constant k<sub>10</sub>. The area under  
186 the concentration versus time curve (AUC) in blood or in airways was estimated between day 0 and  
187 day 3, in order to assess each animal's exposure to mAb166 as a function of the administration route.

188

#### 189 *Statistical analysis*

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



196 **Results**

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

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

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

222

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

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

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

242

#### 243 **mAb166's therapeutic efficacy against *P. aeruginosa* pneumonia depends on the** 244 **administration route**

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

261

#### 262 **The clearance of *P. aeruginosa* and neutrophil recruitment in the lungs depend on mAb166's** 263 **delivery route**

264 To gain insight into the mechanisms that contributed to the Ab's beneficial effect on *P. aeruginosa*-  
265 induced pneumonia, we analyzed the host's antibacterial responses 24 hours after infection. Mice  
266 treated parenterally with mAb166 exhibited a slightly (but not significantly) lower lung bacterial load  
267 (~0.2-0.4 log<sub>10</sub> cfu) than untreated controls, whereas delivery of mAb166 to the airways was  
268 associated with a significantly lower *P. aeruginosa* load in the lungs - about 1.3 log<sub>10</sub> cfu (Figure 4A).  
269 Beyond 24 hours, the *P. aeruginosa* load in surviving mice gradually diminished, and could not be  
270 detected 7 days post-infection (data not shown). Given that the exacerbation of pneumonia is  
271 intimately associated with lung remodeling and damage, we evaluated protein egress into the BALF as  
272 a surrogate marker for leakage of the alveolus-capillary barrier. Only animals treated with aerosolized  
273 mAb166 had significantly lower levels of protein in the BALF (Figure 4B), relative to untreated controls.  
274 In airway-treated animals, the blood-air barrier was therefore maintained for at least 7 days after the  
275 infection (Supplementary Figure 3A).

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

292

#### 293 **In mice treated with mAb166, the modulation of lung inflammation after *P. aeruginosa* infection** 294 **depends on the Ab's administration route**

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

307 **Discussion**

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

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

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

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

347 greater accumulation of neutrophils in the BALF of animals treated with locally administered mAb166,  
348 when compared with parenterally treated mice and untreated mice. The early, massive recruitment of  
349 lung neutrophils is the main component of the innate immune system's response against *P.*  
350 *aeruginosa* infection [31], and is essential for controlling *P. aeruginosa* infections [40].  
351 In contrast, a protracted, uncontrolled inflammatory response is detrimental for the host, and  
352 eventually leads to multiple organ dysfunction [41]. Here, we showed, that 24 hours after the infection,  
353 the pulmonary administration of mAb166 was associated with lower levels of TNF, IL-6 and CXCL1  
354 production, relative to untreated and parenterally treated mice. At 6 hours TNF and CXCL1 production  
355 were similar between all treated groups (data not shown); CXCL1 decreasing over time only in  
356 pulmonary treated-animals. As previously shown, early production of CXCL1 preceding neutrophil  
357 recruitment is important to handle *P. aeruginosa* infection [40], while its accumulation correlates with  
358 deleterious inflammation [42, 43].  
359 These findings are in line with reports showing that although inflammation is important for controlling  
360 *P. aeruginosa* infection [40], its maintenance is correlated with a poor outcome [42, 43]. Thus, airway  
361 delivery of anti-*P. aeruginosa* Ab may help to better control lung inflammation – an effect that has  
362 proven to be of benefit in patients with cystic fibrosis [44].  
363 It should be noted that the pneumonia in this murine model is especially aggressive. Usually,  
364 untreated animals exhibit signs of severe inflammation within 6-8 hours of infection and die within 24-  
365 36 hours of infection. As previously reported [26], we gave the anti-infectious Ab prophylactically in  
366 order to prevent the excessive loss of animals. Although the prevention of pneumonia may be useful in  
367 people at a high risk of *P. aeruginosa* respiratory infections (such as mechanically ventilated patients),  
368 a therapeutic approach would have greater clinical relevance (i.e. the initiation of specific  
369 immunotherapy following confirmation of the *P. aeruginosa* infection). In the light of our present  
370 results, it is tempting to speculate that therapeutic administration through the airways may enable  
371 better control of *P. aeruginosa* infection; however, this will need to be confirmed in further research.  
372 After decades of (mis)use of broad-spectrum antibiotics, antibiotic-resistant pathogens have become a  
373 real threat for human health and have made the management of pneumonia even more challenging  
374 [45]. *Pseudomonas aeruginosa* is on the World Health Organization's priority list of antibiotic-resistant  
375 bacteria, and infection-control experts and researchers are making concerted efforts to fight this  
376 deadly pathogen [46]. As shown in the present study, passive immunotherapy with an aerosolized  
377 anti-pcrV Ab might be a valuable way of preventing and possibly treating *P. aeruginosa* pneumonia. A  
378 PEGylated fragment of mAb166 for i.v. administration was developed by KaloBios Pharmaceuticals  
379 Inc. and has been tested in a Phase IIa study in mechanically ventilated patients infected with *P.*  
380 *aeruginosa* [47]. However, the Ab did not to meet the primary clinical endpoint [48]. Based on our  
381 findings, airway administration and a prophylactic clinical regimen (rather than therapeutic) might be  
382 more effective.  
383 In conclusion, we demonstrated that prophylactic administration of anti-*P. aeruginosa* Ab to the lungs  
384 led to a more efficacious antibacterial and host response than conventional parenteral administration  
385 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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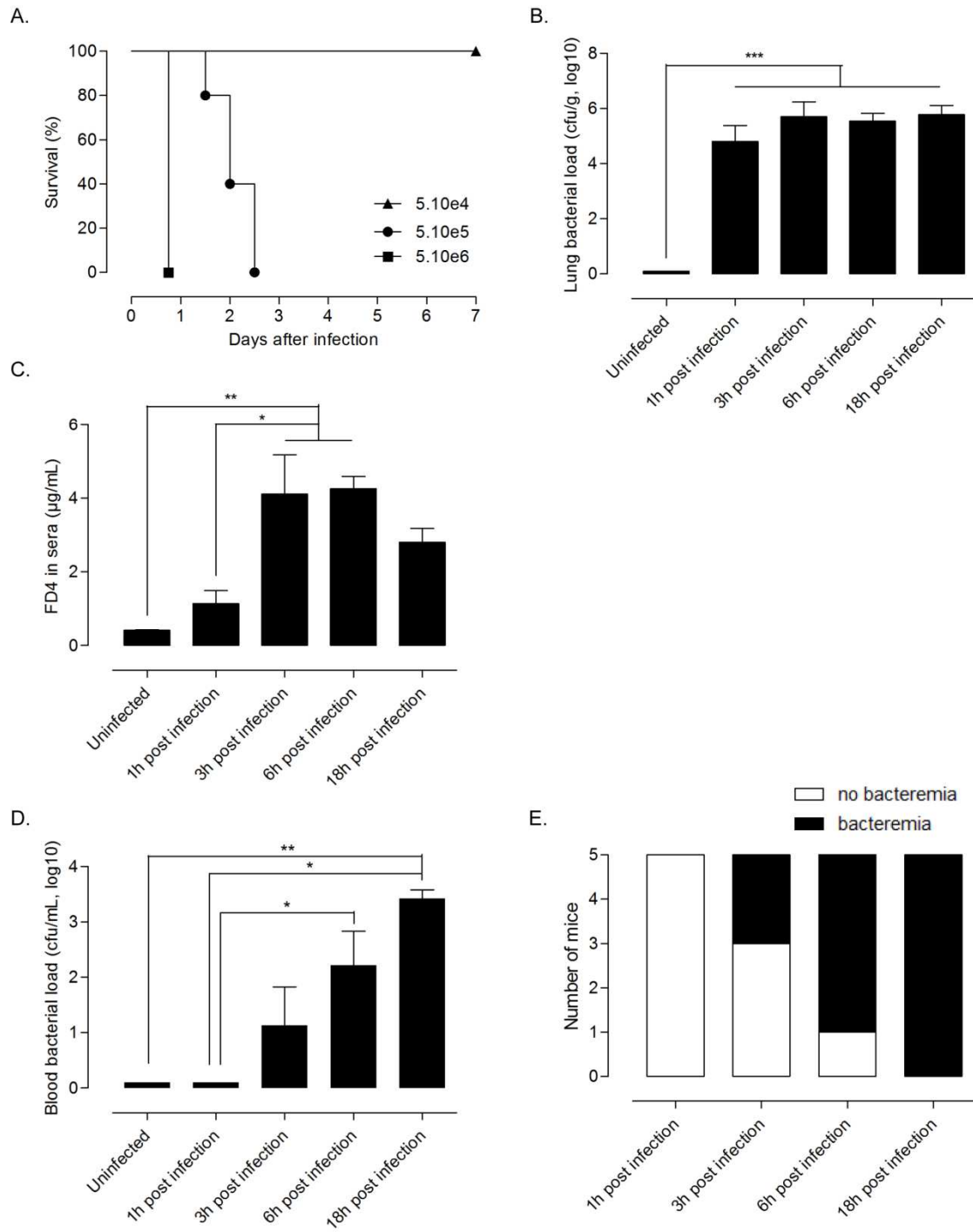
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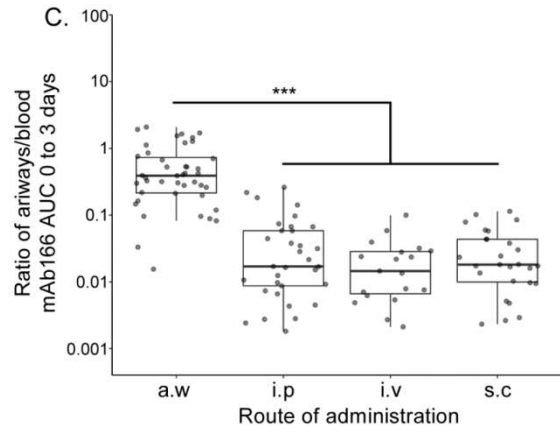
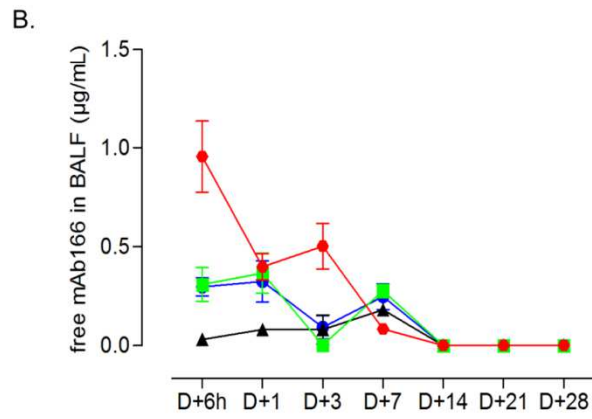
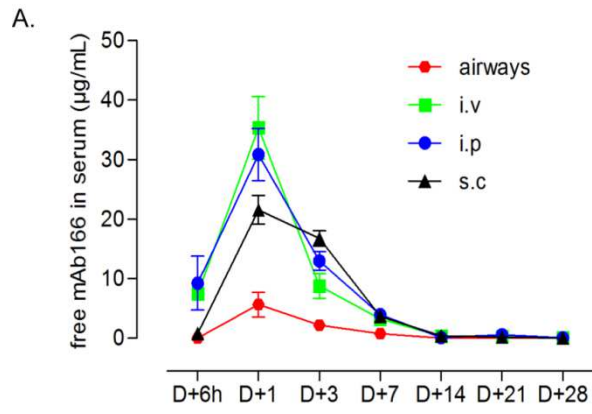
550 **Figures**

551 **Figure 1**



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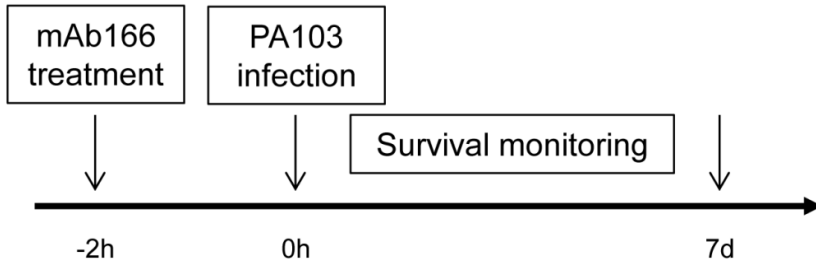
553 **Figure 2**



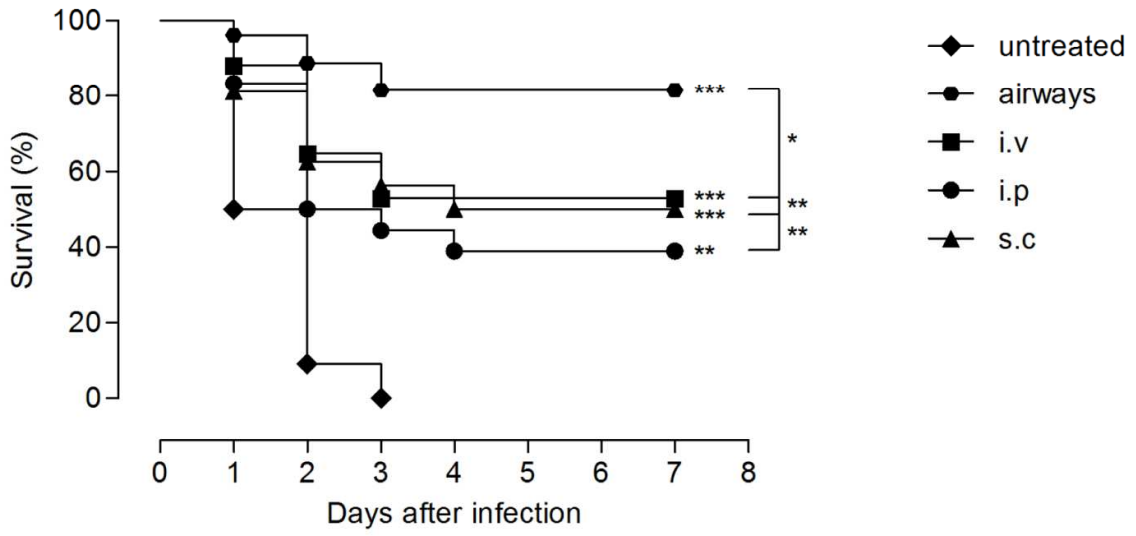
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556 **Figure 3**

A.

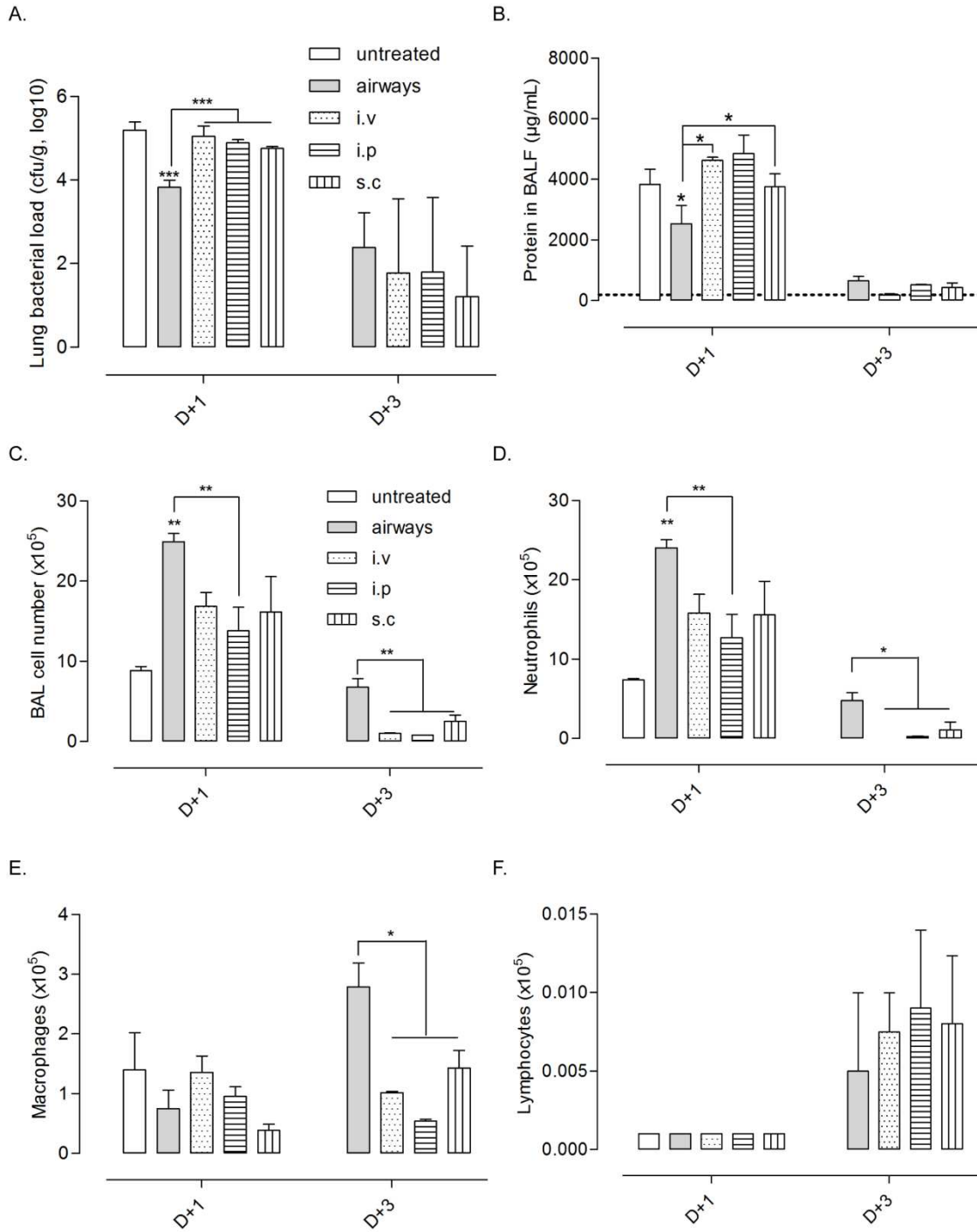


B.



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559 **Figure 4**

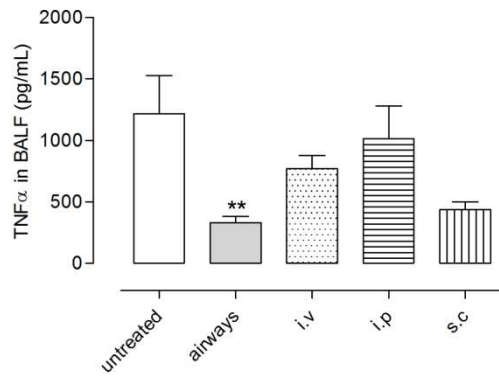


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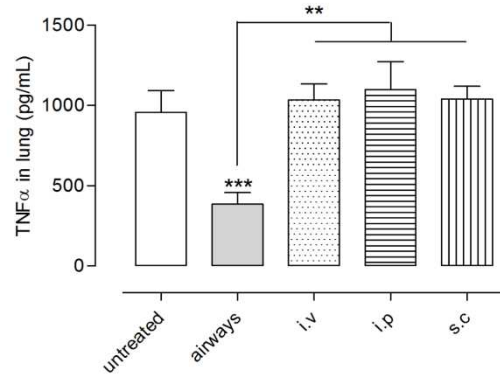
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562 **Figure 5**

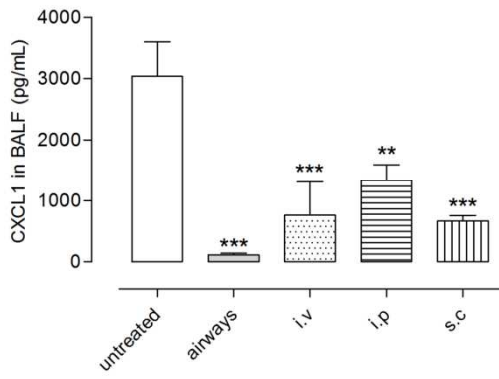
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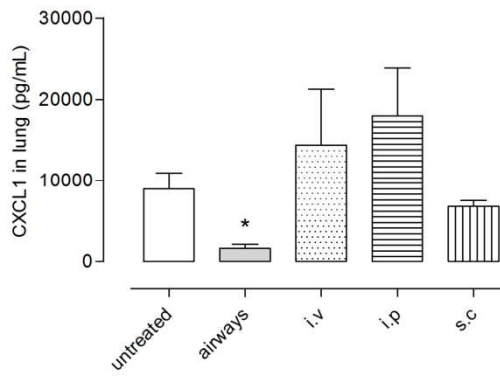
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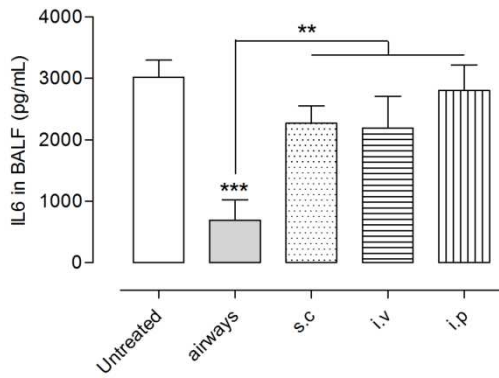
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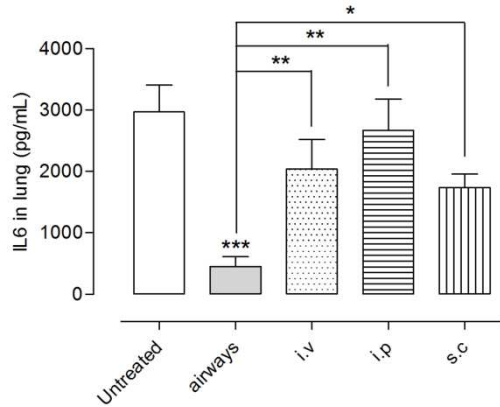
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E.



F.

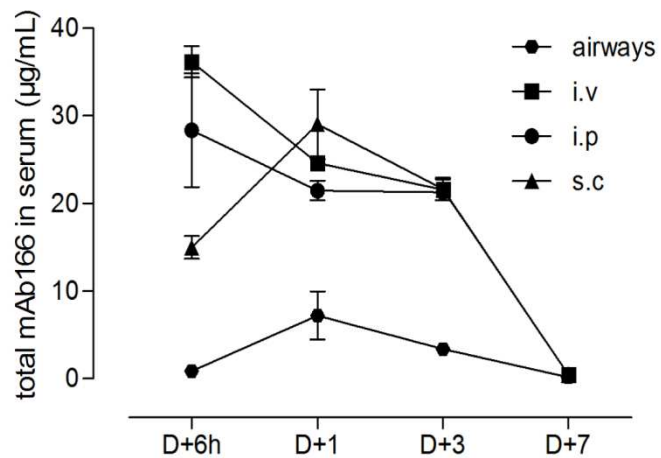


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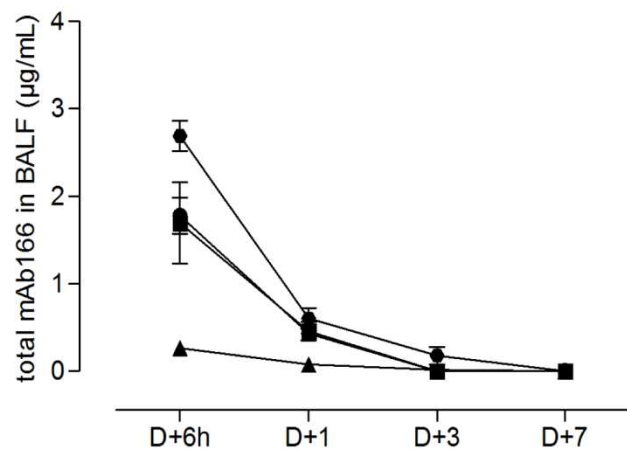
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565 **Supplementary Figure 1**

A.



B.

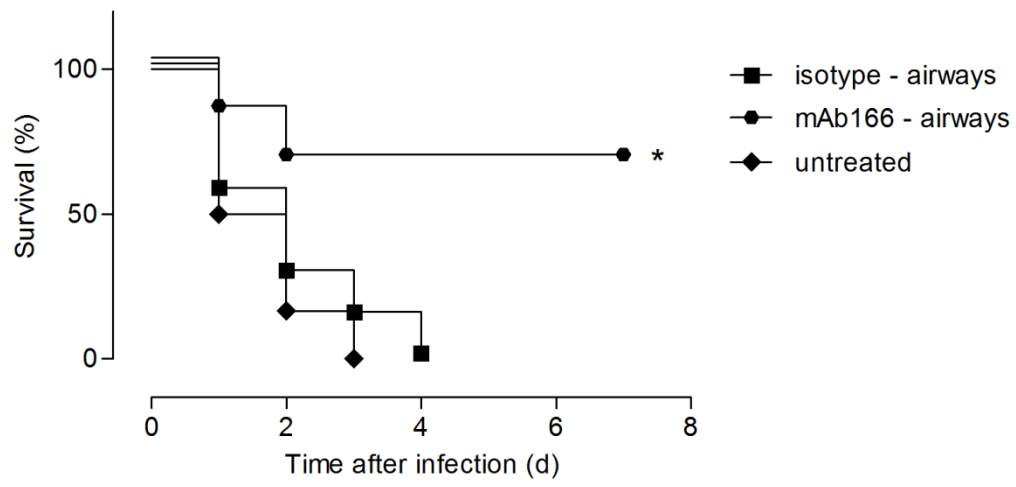


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568 **Supplementary Figure 2**

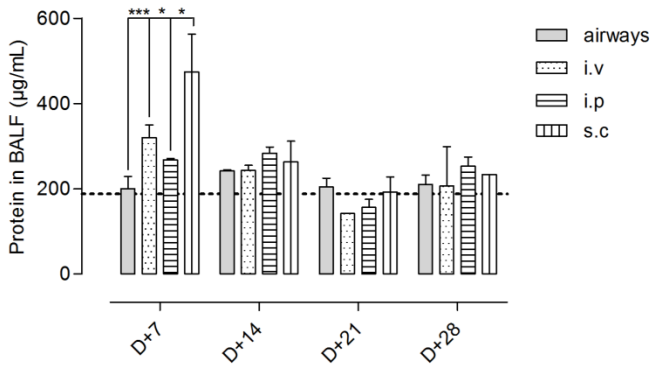


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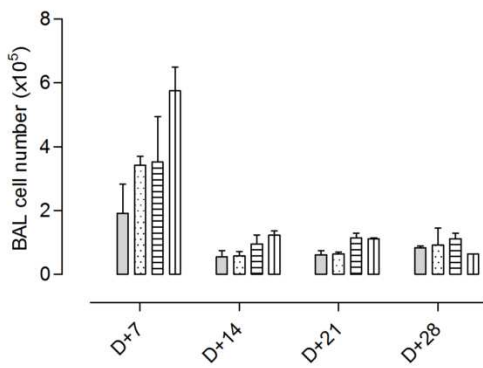
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571 **Supplementary Figure 3**

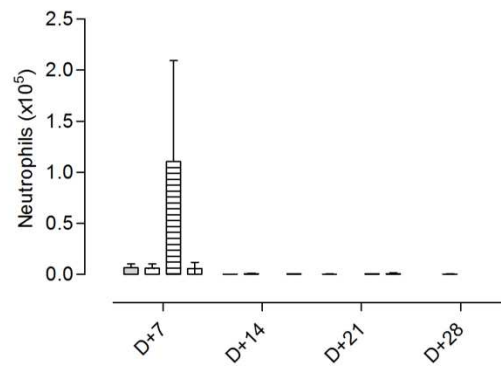
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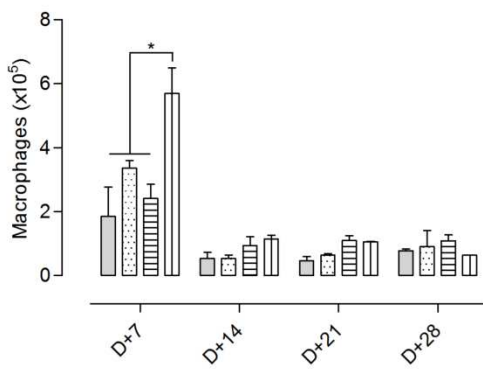
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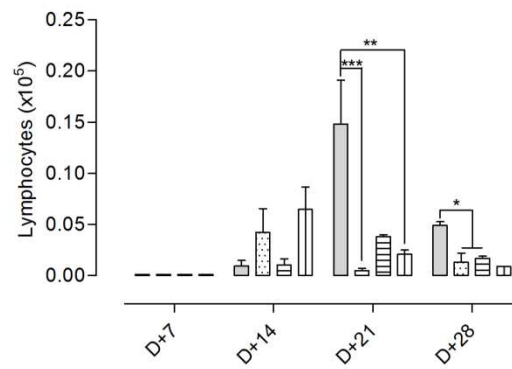
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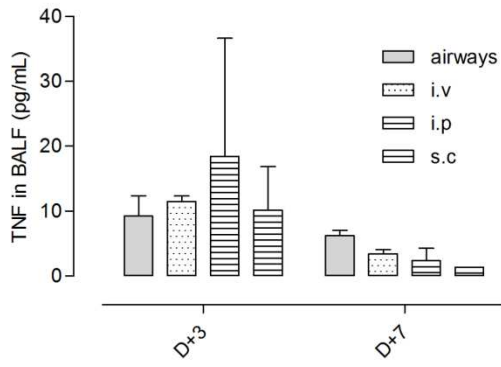


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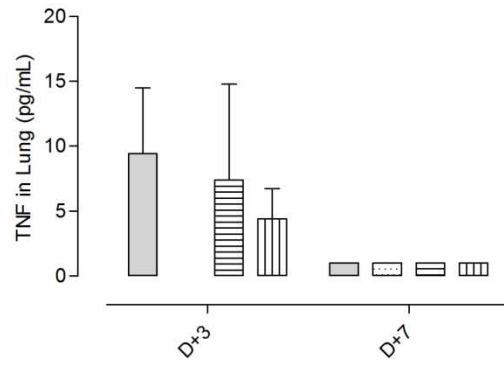
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574 **Supplementary Figure 4**

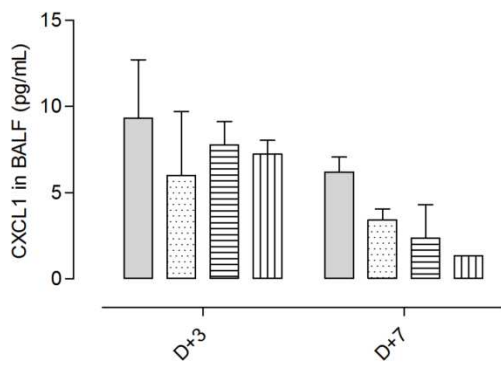
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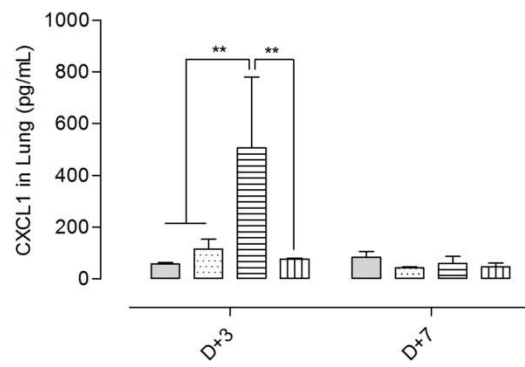
B.



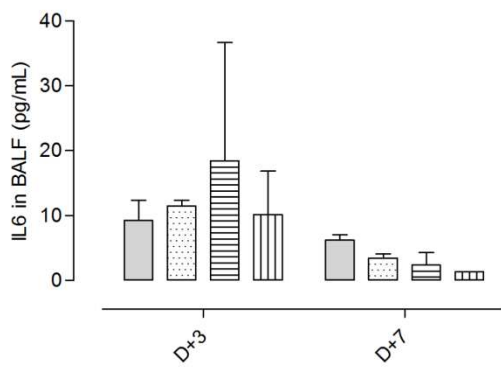
C.



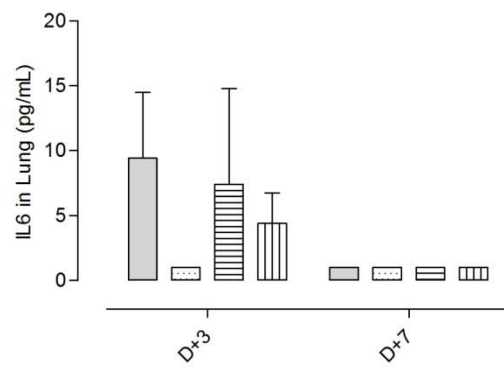
D.



E.



F.



575

576

577

**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
$k_{10}$ ( $\text{day}^{-1}$ )	0,46	14	
$\beta_{k10(\text{Weight})}$	-1,07		0,0040
$k_{13}$ ( $\text{day}^{-1}$ )	0,06	78	
$k_{31}$ ( $\text{day}^{-1}$ )	0,62	78	
$F_{AW}$ (-)	0,08	42	
$V_B$ (mL)	5,61	22	
$k_{a(\text{SC})}$ ( $\text{day}^{-1}$ )	0,54	81	
$\beta_{ka(\text{SC})(\text{Weight})}$	-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),  $\beta_{ka(\text{SC})}$  (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.

578

579 **Figure Legends**

580

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

583 C57/BL6jRj (B6) mice received a 40  $\mu$ L orotracheal instillation of *P. aeruginosa* strain PA103 ( $5 \times 10^4$ ,  
584  $5 \times 10^5$  or  $5 \times 10^6$  cfu). Survival (A) was monitored for 7 days after infection. In another experiment, B6  
585 mice received a 40  $\mu$ L orotracheal instillation of *P. aeruginosa* ( $5 \times 10^5$  cfu) and were injected  
586 intranasally with dextran-FITC 30 min before analysis. The bacterial load (cfu) in the lung (B), the  
587 translocation of 4 kDA dextran-FITC (FD4) into blood (C), and the bacterial load in the blood (cfu) (D)  
588 were measured 0, 1, 3, 6 and 18 hours after the infection. The proportion of mice with *P. aeruginosa* in  
589 the blood (E) was evaluated. The data are quoted as the mean  $\pm$  SEM. \*, \*\* and \*\*\*:  $p < 0.05$ ,  $p < 0.01$   
590 and  $p < 0.001$ , respectively, in a one-way ANOVA with Newman-Keuls's correction for multiple  
591 comparisons. The results are representative of two independent experiments (n=5-10 mice for each).

592

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

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

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

606

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

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

614

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

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

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

625

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

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

635

636 Table 1: mAb166 compartmentalized PK model

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

644

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

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

652

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

655 B6 mice were treated (or not) with 100  $\mu$ g of mAb166 or an IgG2b isotype control via the pulmonary  
656 route 2 hours before being infected by orotracheal instillation of 40  $\mu$ L of *P. aeruginosa* strain PA103  
657 ( $5 \times 10^5$  cfu). Survival was monitored for 7 days after infection. \*:  $p < 0.05$  in a log-rank test. The results  
658 are representative of 1 experiment (n= 5-8 mice).

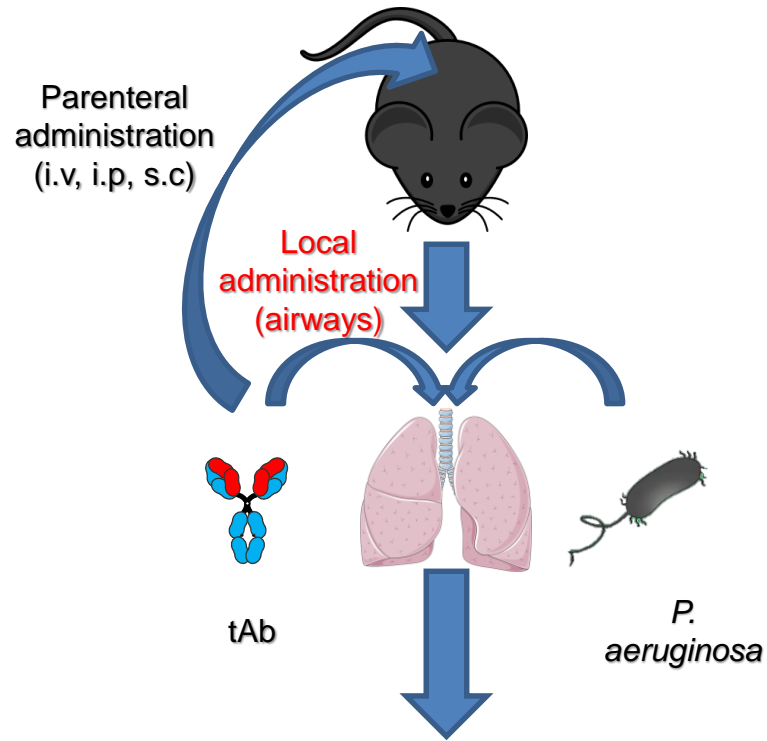
659 Supplementary Figure 3: Late leukocyte recruitment in the lungs of *P. aeruginosa*-infected mice.

660 B6 mice were treated (or not) with 100 µg of mAb166 via the pulmonary (airway), intravenous (i.v.),  
661 intraperitoneal (i.p.) or subcutaneous (s.c.) route 2 hours before being infected by the orotracheal  
662 instillation of 40 µL of *P. aeruginosa* strain PA103 ( $5 \times 10^5$  cfu). Total protein (A), the total cell count (B)  
663 and counts of neutrophils (C), macrophages (D) and lymphocytes (E) in the BALF were evaluated 7,  
664 14, 21 and 28 days after the infection. The data are quoted as the mean  $\pm$  SEM. \*, \*\* and \*\*\*:  $p < 0.05$ ,  
665  $p < 0.01$  and  $p < 0.001$ , respectively, in a one-way ANOVA with Newman-Keuls' correction for multiple  
666 comparisons. The results are representative of four independent experiments (n=5-10 for each).

667

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

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



**Local vs Parenteral**

