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ARTICLE

Mucosal administration of anti-bacterial antibodies provide long-term cross-protection against *Pseudomonas aeruginosa* respiratory infection

Aubin Pitiot ^{1,2}, Marion Ferreira ^{1,2}, Christelle Parent ^{1,2}, Chloé Boisseau ^{1,2}, Mélanie Cortes ^{1,2}, Laura Bouvart ^{1,2}, Christophe Paget ^{1,2}, Nathalie Heuzé-Vourc'h ^{1,2,†} and Thomas Sécher ^{1,2,†,✉}

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Bacterial respiratory infections, either acute or chronic, are major threats to human health. Direct mucosal administration, through the airways, of therapeutic antibodies (Abs) offers a tremendous opportunity to benefit patients with respiratory infections. The mode of action of anti-infective Abs relies on pathogen neutralization and crystallizable fragment (Fc)-mediated recruitment of immune effectors to facilitate their elimination. Using a mouse model of acute pneumonia induced by *Pseudomonas aeruginosa*, we depicted the immunomodulatory mode of action of a neutralizing anti-bacterial Abs. Beyond the rapid and efficient containment of the primary infection, the Abs delivered through the airways harnessed genuine innate and adaptive immune responses to provide long-term protection, preventing secondary bacterial infection. *In vitro* antigen-presenting cells stimulation assay, as well as *in vivo* bacterial challenges and serum transfer experiments indicate an essential contribution of immune complexes with the Abs and pathogen in the induction of the sustained and protective anti-bacterial humoral response. Interestingly, the long-lasting response protected partially against secondary infections with heterologous *P. aeruginosa* strains. Overall, our findings suggest that Abs delivered mucosally promotes bacteria neutralization and provides protection against secondary infection. This opens novel perspectives for the development of anti-infective Abs delivered to the lung mucosa, to treat respiratory infections.

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INTRODUCTION

Since the advent of monoclonal antibody (Ab) technology, the use of Abs has proven to be successful for the treatment or prevention of a variety of disorders. Abs have radically changed the management and treatment of fatal diseases. The success of Abs is based on its high specificity, predictable toxicity, and unique pharmacological profiles, giving them advantages over small-molecule drugs. Beyond cancer and inflammatory diseases, Abs are now considered as potential game changers for the management of infectious diseases. Four anti-infective Abs are already approved, targeting either bacterial or viral pathogens, and several other entities already reached clinical trials and may enrich the therapeutic armamentarium against infectious diseases^{1,2}. Overall, anti-infective Ab therapy offers prophylactic or therapeutic advances against infections when vaccines and/or conventional drugs are neither available nor efficacious, by overriding an immune system that is unresponsive to immunization or countering pathogen resistance.

Anti-infective Abs have multifaceted mechanisms of action. Few of them are designed to inhibit pathogen toxins, while most Abs recognize cell surface antigens through their antigen-binding (Fab) domain, thereby preventing the attachment,

entry, or production of virulence factors into the host cell. Although anti-infective Abs are primarily neutralizing, the killing of the pathogen may also be facilitated by effector functions, including Ab-dependent cellular phagocytosis/cytotoxicity and complement-dependent cytotoxicity, that depend on the interaction between the Fc domain and Fcγ receptors (FcγR) or enzyme cascade of the complement.

Accumulating evidence highlights a novel function of Abs, which can engage and promote endogenous innate and adaptive immune systems to induce long-lasting protection³. This has been widely exemplified at the preclinical and clinical level, with antitumor- and anti-viral Abs. For instance, patients treated with anti-Mucin 1 or anti-human epidermal growth factor receptor 2 Abs generate anti-Mucin 1 and human epidermal growth factor receptor 2-specific T-cell responses^{4,5}. Similarly, a single course of treatment with anti-cluster of differentiation (CD)20 Abs result in long-lasting tumor surveillance in patients with lymphoma⁶, in whom an anti-idiotypic T-cell response against lymphoma antigens was detected^{7,8}. Ab-mediated immune-stimulating response has also been illustrated during viral infections^{9,10}. A notable common characteristic, in both disease contexts, is the importance of the Fc domain, which connects

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specific recognition of tumor or viral antigens with immune cells mediating Ab-dependent cellular cytotoxicity or phagocytosis through the engagement of FcγR. Immune complexes (IC), formed between therapeutic Abs and tumor or viral particles infected, will promote antigen-presenting cells (APC) stimulation, maturation, and presentation of pathogenic antigens to T cells, eventually leading to long-term immune memory response^{3,11}. The inhibition of regulatory T-cell expansion was mandatory for the induction of these protective responses^{12,13}. Interestingly, while Ab-mediated long-term antitumor protection was solely associated with cellular responses¹⁴, anti-viral Abs prompted both host cellular and humoral immunity^{15,16}, essential to long-term containment of viremia. Although the mechanisms underlying Ab-mediated long-lasting response are not identical across disease contexts, taken as a whole, those findings bring a new concept in Ab immunotherapy.

Here, we hypothesized that Ab-mediated long-term protection may apply to neutralizing anti-bacterial Abs and benefit the treatment of respiratory infections, in particular those due to pathogens associated with persistent chronic or recurrent acute respiratory infections, like the ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), which are often multi-drug resistant and a major threat for human health.

In respiratory medicine, drugs can be delivered parenterally or locally, using the airways as a direct portal to the lungs. Although intravenous (IV) injection is the conventional route for anti-infective Abs, it is not optimal for the delivery of full-length Abs to the airways, where pathogens mainly infect, propagate, and disseminate. Indeed, the transport of Abs across the lung epithelial layer is relatively inefficient in either direction^{17–19}. In contrast, we and others have shown that airway administration of Abs conferred greater protection than parenteral administration^{20–23}. Airway delivery favors a rapid onset of action, resulting in the efficient neutralization of pathogens^{22,23} and the pharmacokinetics profile is favorable: Abs accumulate at higher concentrations in the airways than after IV infusions and pass slowly and in low amounts from the airways into the circulation^{19,22}. In addition, the respiratory mucosa, which is directly accessible through the airways, provides a specific immune environment, favorable for establishing long-term protection. Indeed, comparative studies have shown that mucosal routes provide superior efficacy in the induction of mucosal immune responses as compared to systemic administration²⁴. In particular, higher protection was obtained, at local or even remote sites, against airway pathogens when immunization was applied by the mucosal route^{25,26}. Here, we speculated that mucosal delivery of Abs through the airways is well-suited to achieve both direct control and long-term containment of bacterial respiratory pathogens.

Using an acute model of respiratory infection, due to *Pseudomonas aeruginosa*, a bacterium with a frequent multi-resistance phenotype and high rate of recurrence, we found that mucosal delivery of neutralizing Abs provided both an optimal defense against primary infection and frontline immunity for the remote protection against subsequent infections. Ab-mediated long-lasting protection was dependent on the size of the inoculum, the dose of Abs, and the presence of the cognate antigen during the primary infection. Remarkably, the immune response was sufficient to promote partial heterologous protection against *P. aeruginosa* strains from different ser-

otypes; based on compositional differences in the O units of lipopolysaccharide. The long-lasting response of mucosal Abs in bacterial respiratory infection relies, at least partly, on the establishment of a sustained and protective humoral immune response, mostly associated with immunoglobulin (Ig)G3.

RESULTS

Prophylactic and therapeutic mucosal administration of anti-bacterial antibodies, through the airways, mediates long-term protection against *P. aeruginosa*

As previously demonstrated, mAb166, a murine IgG2b recognizing *pcrV* (an antigen belonging to the type 3 secretion system) protects mice from a lethal pulmonary infection with *P. aeruginosa*²² after airway delivery. Here, we investigated whether the rescued animals were protected from a subsequent lung infection.

Mice were treated with 100 μg of inhaled mAb166 or IgG2b control Ab (MPC11) either prior to or after being inoculated intrapulmonary with a lethal dose of *P. aeruginosa* PA103 strain (Figs. 1A and 1C) to mimic both a prophylactic and therapeutic Ab intervention. No protection was observed in mice treated with MPC11 after the primary infection (Supplementary Fig. 1). After 30 days, the surviving mice were re-infected with the same lethal dose of PA103 (as for the primary infection), without additional mAb166 treatment, and at a time mAb166 was no longer detectable in the serum and broncho-alveolar lavage fluid (BALF) (Supplementary Fig. 2 and 22). It is noteworthy that the prophylactic intervention afforded 80%–90% protection (Fig. 1B) after the primary infection, while the therapeutic one rescued 60%–80% of the animals (Fig. 1D). After the secondary infection, a significant proportion of animals that previously survived the primary infection resisted the lethal dose infection (Figs. 1B and 1D). This beneficial effect of the immunotherapy was associated with improved control of the bacterial load in both lung and BAL after the primary and secondary infection (Fig. 1K).

In comparison to systemic administration (IV) (Fig. 1E), which is the conventional delivery route for anti-infective Abs, the overall response to repeated bacterial infections was significantly better if the mAb166 was delivered through the airways upon the primary infection (Fig. 1F). It is noteworthy that the benefit of mucosal delivery is mainly attributable to better control of primary infection, since the animals exhibited similar survival rates after the secondary infection, whatever the Ab route of administration. Overall, this highlights the superiority of the airways for the delivery of anti-infective Abs to contain pathogens after primary and subsequent infections.

To investigate the specificity of this long-lasting response, we first assessed the capacity of standard anti-*P. aeruginosa* antibiotic therapy (amikacin) to protect animals from multiple infections. None of the animals that were rescued from the primary infection after airway administration of amikacin (Fig. 1G) survived a secondary infection without additional treatment (Fig. 1H). Secondly, we infected mice with an isogenic PA103 mutant deprived of *pcrV* expression, PA103Δ*pcrV* (Fig. 1I). This attenuated *P. aeruginosa* strain caused morbidity (Supplementary Fig. 3B) but no mortality in animals (Supplementary Fig. 3C). Interestingly, the animals primarily infected with PA103Δ*pcrV* and treated with mAb166, were not able to survive a second infection with the wild-type (WT) PA103 (Fig. 1J). Based on these results, we hypothesized that the formation of IC may promote adaptive immunity protecting against secondary infection. Thus, we incubated *in vitro* PA103 WT and mAb166 or

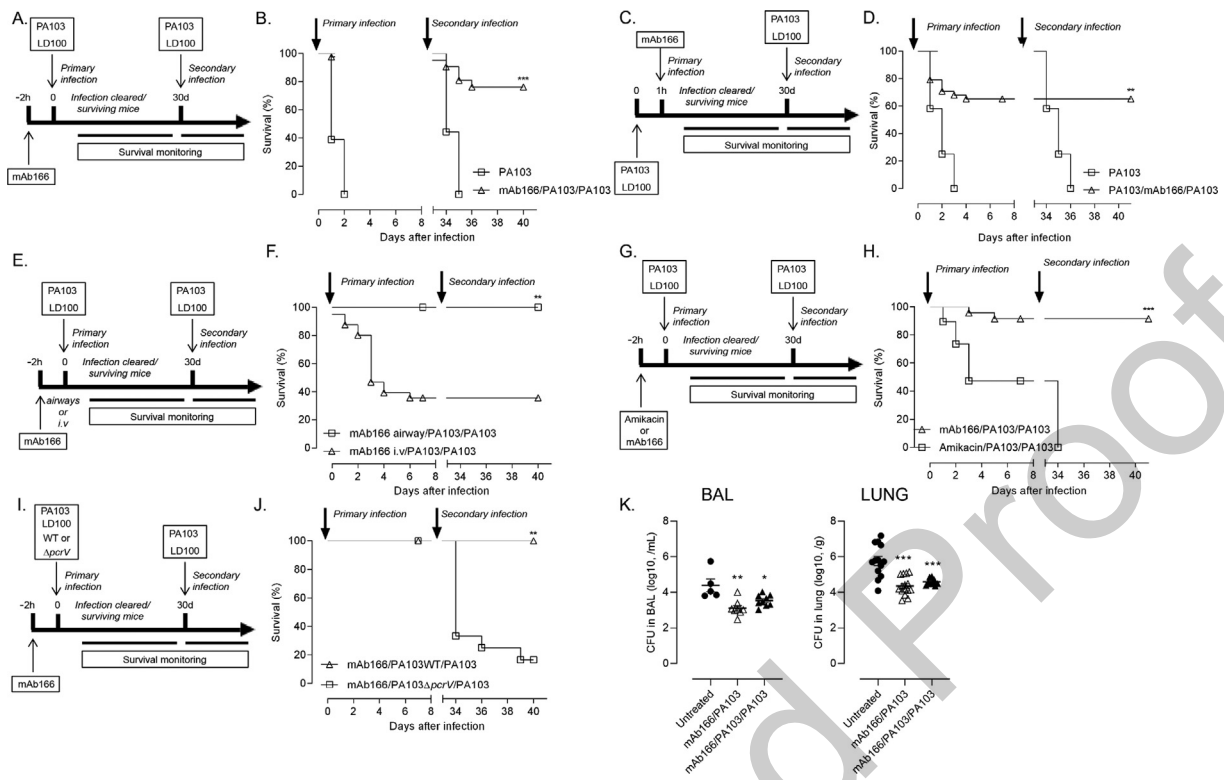


Fig. 1 Prophylactic and therapeutic mucosal administration of anti-bacterial antibodies (Ab), through the airways, mediates long-term protection against *Pseudomonas aeruginosa*. (A) C57/BL6J (B6) mice were treated or not with 100 μ g of mAb166 via the pulmonary (airway) route 2 hours before being infected by the orotracheal instillation of 40 μ L of *P. aeruginosa* strain PA103 (5×10^5 CFU / primary infection). Surviving animals were challenged, 30 days later (secondary infection) by the orotracheal instillation of 40 μ L of *P. aeruginosa* strain PA103 (5×10^5 CFU) without additional treatment. (B) Survival was monitored over the entire period. The results correspond to six pooled, independent experiments ($n = 27$ –43 mice per group), ***: $p < 0.001$ with a log-rank test. (C) B6 mice were infected by the orotracheal instillation of 40 μ L of *P. aeruginosa* strain PA103 (3×10^5 CFU = LD100 / primary infection) and 1 hour later were treated or not with 100 μ g of mAb166 via the pulmonary (airway) route. Surviving animals were challenged, 30 days later (secondary infection) by the orotracheal instillation of 40 μ L of *P. aeruginosa* strain PA103 (3×10^5 CFU) without additional treatment. (D) Survival was monitored over the entire period. The results correspond to six pooled, independent experiments ($n = 36$ –72 mice per group), **: $p < 0.01$ with a log-rank test. (E) B6 mice were treated with 100 μ g of mAb166 via the pulmonary (airway) or intravenous (IV) 2 hours before being infected as described in (A). (F) survival was monitored over the entire period. The results correspond to two pooled, independent experiments ($n = 8$ –27 mice per group), **: $p < 0.01$ with a log-rank test. (G) B6 mice were treated with 100 μ g of mAb166 or 25 mg/kg of Amikacin and were infected as described in (A). (H) Survival was monitored over the entire period. The results correspond to two pooled, independent experiments ($n = 19$ –24 mice per group), ***: $p < 0.001$ with a log-rank test. (I) B6 mice were treated with 100 μ g of mAb166 and infected with PA103 wild-type or with PA103 Δ pcrV as described in (A). (J) Survival was monitored over the entire period. The results correspond to two pooled, independent experiments ($n = 15$ –24 mice per group) **: $p < 0.01$ with a log-rank test. (K) The bacterial load (CFU) in the BAL and the lungs was measured 24 hours after the primary infection or after the secondary infection. The data are quoted as the mean values \pm standard error of the mean. The results correspond to four pooled, independent experiments ($n = 5$ –15 mice per group), *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ with a t test comparing with untreated groups. BAL = broncho-alveolar lavage; CFU = colony-forming unit; d = days; h = hours; mAb = monoclonal antibodies.

MPC11 and administered the IC or mixture, respectively, to animals. As shown in [Supplementary Fig. 4](#), animals infected with PA103 WT alone or co-incubated with MPC11 died quickly after primary infection while those treated with PA103 WT complexed with mAb166 were fully protected. Interestingly, the animals that were inoculated with IC and survived the primary infection, were not protected from the secondary infection. This suggests that long-term response cannot be induced with immunization by IC but requires an infectious trigger and subsequent therapeutic Ab treatment.

Overall, these results indicate that the induction of long-term protection against *P. aeruginosa* by Abs delivered through the airways depends on the Ab binding to its target antigen, as previously shown with antitumor Ab¹¹.

Antibody-mediated long-term protection against *P. aeruginosa* depends on a subtle balance between bacteria inoculum and antibody dose

To further characterize long-term protection induced by prior mucosal Ab treatment, we treated animals with a suboptimal quantity of mAb166 (50 μ g) ([Fig. 2A](#)), leading to 60% protection after the primary infection ([Fig. 2B](#)). Interestingly, this was not sufficient to protect all the rescued animals from a second infection ([Fig. 2B](#)). This was confirmed *in vitro*, using bone marrow-derived macrophages (BMDM) APC. BMDM were activated in a mAb166 and Fc γ R dose-dependent manner, as compared to BMDM treated with MPC11 ([Supplementary Fig. 5A](#)), suggesting that the amount of IC generated after infection was important to generate long-term protection²⁷. To investigate whether long-

term protection was only explained by the reduction of bacterial growth, we infected mice with a non-lethal dose of PA103 [5.10^4 colony-forming unit (CFU)] (Fig. 2C), which caused morbidity (Supplementary Fig. 6B) but not mortality (Supplementary Fig. 6C). We observed that these animals were not protected from a second infection with a lethal dose of PA103 (Fig. 2B). Interestingly, we observed *in vitro* a dose-dependent activation of APC regarding the quantity of bacteria used to generate IC (Supplementary Fig. 5B), suggesting that an adequate inflammatory microenvironment is necessary to promote long-term protection. Finally, we demonstrated that APC activation by IC was dependent on their ligation to FcγR as anti-CD16/CD32 Ab treatment blocked expression of interleukin (IL)-6 after 24 hours of stimulation.

Taken as a whole, our results suggest that an optimal amount of IC was critical to induce long-term protection.

Long-term protection is associated with improved lung inflammatory response against *P. aeruginosa*

To gain first insight into the mechanisms accounting for long-term lung protection during *P. aeruginosa* infection, we quantified, in the airways and lung tissue, the recruitment of neutrophils—the prototypal leukocytes associated with the control of *P. aeruginosa* infections²⁸. After 4 hours, we observed that they were significantly more neutrophils in both BALF and lungs of the protected animals as compared to the susceptible controls (Figs. 3A and 3B). This was accompanied by a significant reduction of PA103 load (Figs. 3C and 3D). At 24 hours after secondary infection, a time at which there were fewer bacteria in the lungs of the mice, there were no longer differences in the number of airways neutrophils (Figs. 3A and 3B). These data suggest that long-term lung protection generated by mucosal-administered Abs remodels lung immune response to improve neutrophil recruitment, which in turn achieves better control of *P. aeruginosa*.

Although a neutrophilic-dependent immune response is necessary for bacterial clearance, a protracting and non-resolving inflammatory response is associated with poor host outcomes in *P. aeruginosa* acute lung infection model²⁹. Therefore, we investigated pro-inflammatory cytokines and chemokines in BALF upon *P. aeruginosa* secondary infection. Interestingly, the expression of the innate immunity mediators CXCL1, IL-6, CCL3, and to a lesser extent tumor necrosis factor-α (Figs. 3E–H) was significantly reduced in the BALF of mice with protective immunity within 4 hours after the challenge. Despite an increased neutrophil influx, dampening of pro-inflammatory pathways may mediate the early resolution of the acute inflammatory response. At a later time point, the levels of CXCL16 and CCL7, two chemokines associated with the recruitment and activation of cells associated with adaptive immunity, were significantly increased in mice protected from secondary infection (Figs. 3I and 3J).

To determine whether mAb166 treatment triggers a specific lung immune microenvironment after the primary infection, which may contribute to long-term protection, we analyzed immune cells in the lungs of mice, 28 days after the primary infection. Interestingly, the frequency and number of neutrophils were increased in the group, which will resist secondary infection (Supplementary Fig. 7B and 7C), while alveolar macrophages were not modified (data not shown). This was associated with an increased expression of CXCL1 (Supplementary Fig. 7D) while other pro-inflammatory cytokines (IL-6, tumor necrosis fac-

tor) were unchanged (data not shown). Additionally, we depleted CD4+ T cells, which are important players for proper maintenance and recall of memory responses against respiratory pathogens, 1 day before the secondary infection (Supplementary Fig. 7G). The marked reduction of CD4+ T cells in the lung compartment did not affect animal survival, anti-PA103 humoral response, and neutrophil recruitment (Supplementary Fig. 7H–L).

Overall, our data suggested that protection from *P. aeruginosa* secondary may be attributable to a better defense of the mucosal surface, which is promoted by improved recruitment of neutrophils.

Mucosal administration of mAb166 leads to the development of a sustained and protective humoral immune response against *P. aeruginosa*

The enhanced lung bacterial clearance after the secondary infection might be due to improved uptake/bactericidal features of recruited neutrophils. This may be due to better bacterial opsonization afforded by circulating anti-*P. aeruginosa* IgG. Indeed, humoral immunity is the main specific immune response to protect against extracellular pathogenic bacteria^{30,31}.

To delineate the immune mechanisms of long-term protection against bacterial pneumonia, we analyzed the primary as well as recall humoral response against *P. aeruginosa*. We observed a significant enrichment of anti-PA103 IgG in the serum of mice infected with a lethal dose of PA103 and treated with an optimal quantity of mAb166 as compared to the ones infected with a non-lethal dose (Fig. 4A), the $\Delta pcrV$ mutant or animals treated with a suboptimal quantity of mAb166 (Supplementary Fig. 8). Moreover, under protective conditions, we observed a rapid and robust induction of the humoral response after the secondary infection (Fig. 4B). Interestingly, comparing before and after the secondary infection, we showed a significant enrichment of anti-PA103 IgG in the serum only in protected mice as compared to susceptible controls suggesting the induction of a memory humoral response (Fig. 4C). We next addressed the frequencies of CD19+ B cells in the spleen, as a surrogate of systemic and local humoral responses, at 1 day after the secondary infection. The number of systemic B cells was enhanced in mice that developed long-term protection, as compared to sensitive animals (Figs. 4D and 4E), with a significant proportion of them with an MZ phenotype (CD21^h IgM^h, CD19⁺) (data not shown), which are critical for the Ab responses³². It is noteworthy that similar results were obtained in the prophylactic intervention model (Supplementary Fig. 8). This enrichment of lung B cells was not associated with the development of inducible bronchial-associated lymphoid tissue after either the primary or the secondary infection (Supplementary Fig. 9). We next investigated whether *P. aeruginosa* prior exposure established such humoral response within the airways and observed while IgG titers in BALF were present in a lesser extent as compared in serum (Fig. 4F), they were significantly enriched in protected mice (Fig. 4G). Interestingly, we observed similar BALF enrichment of IgG before the secondary infection, while IgA was not detectable (Supplementary Fig. 7E and 7F). This was associated with an enrichment of CD19+ B cells in the lung parenchyma (Figs. 4H and 4I) as well as an increased expression of BAFF—which is known as a crucial cytokine for B cell activation and maturation³³—24 hours after the secondary infection (Fig. 4J).

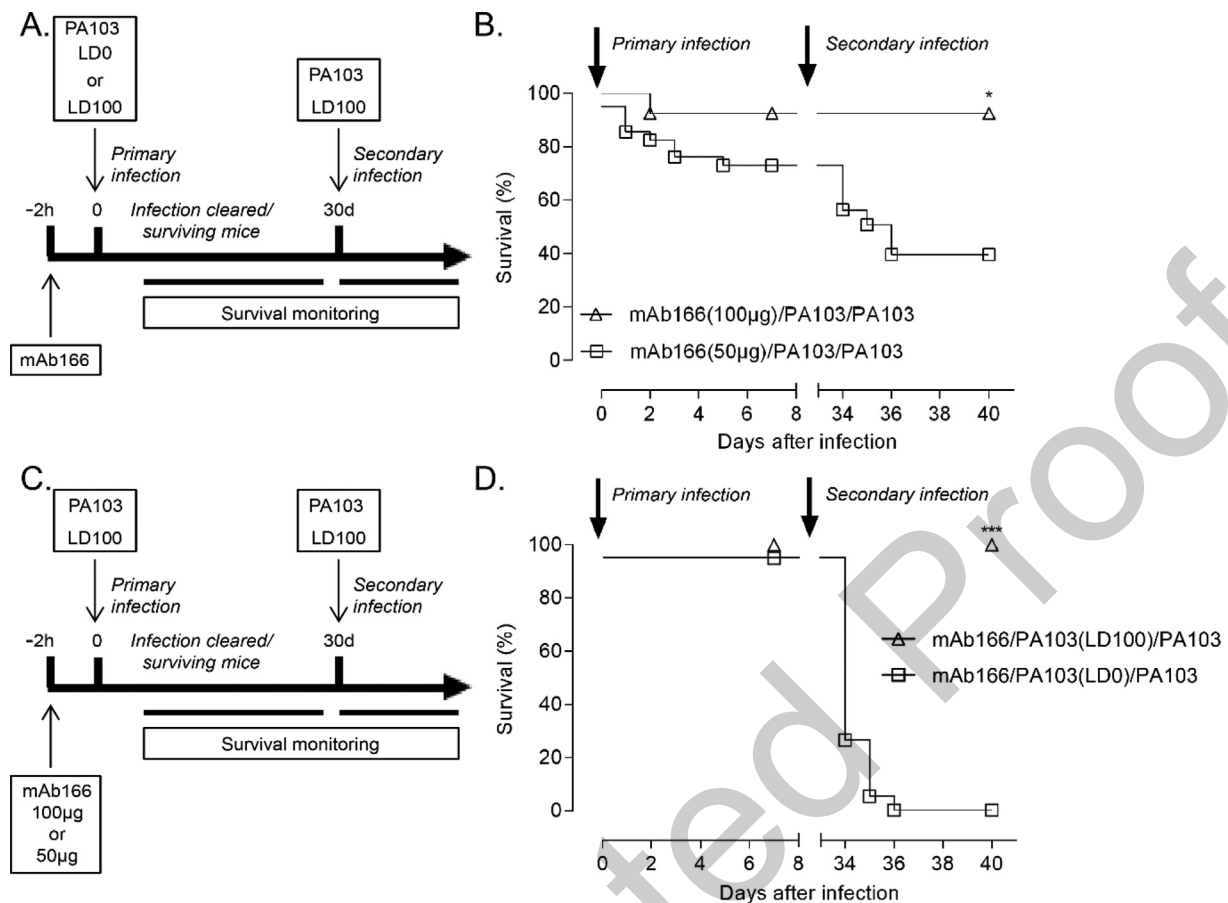


Fig. 2 Antibody-mediated long-term protection against *Pseudomonas aeruginosa* depends on a subtle balance between bacteria inoculum and antibody dose. (A) B6 mice were treated with 100 µg or 50 µg of mAb166 and infected as described in Fig. 1A. (B) Survival was monitored over the entire period. The results correspond to four pooled, independent experiments ($n = 27$ – 32 mice per group), *: $p < 0.05$ with a log-rank test. (C) B6 mice were treated with 100 µg of mAb166 and infected with a lethal dose (5×10^5 CFU / primary infection) or a non-lethal dose (5×10^4 CFU / primary infection) as described in Fig. 1A. (D) Survival was monitored over the entire period. The results correspond to five pooled, independent experiments ($n = 16$ – 30 mice per group), ***: $p < 0.001$ with a log-rank test. CFU = colony-forming unit; d = days; h = hours; mAb = monoclonal antibodies.

We next assessed the relative amount of different anti-PA103 IgG subclasses present in animals exhibiting a different ability to limit the extent of the secondary infection. IgG1 and IgG3 were the most abundant isotypes found in the serum and BALF (data not shown) after *P. aeruginosa* infection. We observed a significant enrichment of IgG3 in contrast with IgG1 in the serum of the mice resistant to the secondary infection, as compared to the susceptible animals (Figs. 4K and 4L). Indeed, a formal correlation analysis of bacterial burden in the lung with IgG titers in the serum showed that the presence of IgG3 correlated with better bacterial control, as compared to IgG1 after PA103 secondary infection (Figs. 4M and 4N). Overall, our data support the idea that the mice infected and treated with mAb166, under optimal conditions, resist re-infection by PA103 due to an efficient anti-bacterial humoral immune response, which is recalled after the second bacterial infection.

To investigate whether this protracted humoral anti-PA103 response was protective on its own, we collected sera from animals that were either protected from a secondary infection or not and uninfected/mAb-treated control mice. First, we explored the impact of serum on PA103 growth. As shown in Fig. 5A, only serum from protected mice limited bacterial growth and the

inhibitory effect was independent of the activity of the complement (Fig. 5A). Interestingly, enrichment of IgG3 in the serum was significantly associated with the limitation of bacterial growth, as compared to IgG1 (Figs. 5B and 5C). To determine whether serum IgG would be sufficient to confer protection against *P. aeruginosa* infection, sera from infected/treated animals containing anti-PA103 IgG (Supplementary Fig. 10) were decomplexed and administered intraperitoneally to naïve mice (Fig. 5D) before and after PA103 infection. Animals, which were either not subjected to serum transfer (data not shown) or treated with control sera, developed severe pneumonia and died within a few days. In contrast, mice, which received serum from immune-(LD100-mAb166) animals survived better from PA103 infection as compared to those which received serum from non-protected-(LD0-mAb166) animals (Figs. 5E and 5F). This result suggests that the humoral anti-PA103 response developed in animals resistant to secondary infection contributes to the protective anti-bacterial response.

Long-lasting humoral response induced by anti-bacterial antibodies mediates cross-protection against *P. aeruginosa*
Although numerous *P. aeruginosa* antigens have been investigated as immunotherapy or vaccine candidates, their develop-

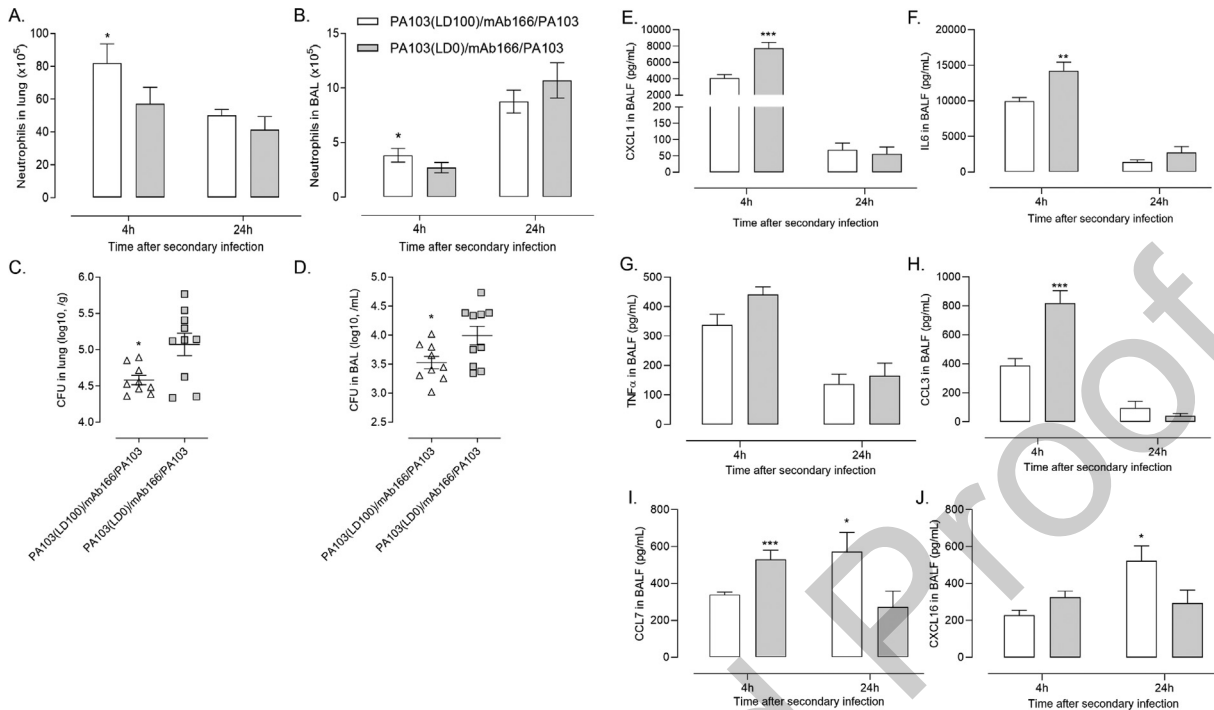


Fig. 3 Long-term protection is associated with improved lung inflammatory response against *Pseudomonas aeruginosa*. B6 mice were treated/infected as described in Fig. 1C. Neutrophils (CD45+ CD11b+ SiglecF- Ly6G+ cells) number in lung (A) and BAL (B) were determined 4 and 24 hours after the secondary infection. The data are quoted as the mean values \pm SEM. The results correspond to three pooled, independent experiments ($n = 13$ mice per group), *: $p < 0.05$ with t test. Bacterial load in lung (C) and BAL (D) were determined 4 hours after the secondary infection. The data are quoted as the mean values \pm SEM. The results correspond to two pooled, independent experiments ($n = 10$ mice per group), *: $p < 0.05$ with t test. At 4 and 24 hours after the secondary infection, BAL fluid was analyzed by Multiplex assay for the production of (E) CXCL1, (F) IL-6, (G) TNF- α , (H) CCL3, (I) CCL7, (J) CXCL16. The data are quoted as the mean values \pm SEM. The results correspond to three pooled, independent experiments ($n = 13$ mice per group), *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ with a t test. BAL = broncho-alveolar lavage; CFU = colony-forming unit; d = days; h = hours; IL = interleukin; mAb = monoclonal antibodies; SEM = standard error of the mean; TNF = tumor necrosis factor.

387 ment has been hampered by the inability to achieve broad pro-
 388 tection across different serotypes³⁴. Here, we investigated
 389 whether Ab-mediated long-term response in mice infected with
 390 PA103 (serotype O11) and treated with mAb166 may protect
 391 animals from a secondary infection by heterologous strains of
 392 *P. aeruginosa* (Fig. 6A). We used CLJ1 and PA14 strains, which
 393 belong to the serogroups O12 and O10, respectively (Supple-
 394 mentary Table 1). PA14 express *pcrV*, while not CLJ1, but none
 395 of them are sensitive to mAb166 immunotherapy (Supplemen-
 396 tary Fig. 11). Although the long-term effect associated with the
 397 infection with PA103- 3.10^5 colony-forming unit (CFU) and the
 398 treatment with mAb166-100 μ g was not sufficient to protect ani-
 399 mals against a lethal challenge with CLJ1 (Supplementary
 400 Fig. 12), it significantly protects individuals from a sublethal
 401 infection by CLJ1 or lethal infection by PA14 (Fig. 6B). Inter-
 402 estingly, this protection was associated with the production of
 403 anti-CLJ1 or anti-PA14 IgG, which, even lower as compared to
 404 the production of anti-PA103 IgG, was significantly superior to
 405 controls (Fig. 6C). These results suggest that prior lung infec-
 406 tion rescued by passive inhaled immunotherapy induces a humoral
 407 protective immunity against *P. aeruginosa* pneumonia induced
 408 by heterologous strains.

DISCUSSION

409 Despite considerable advances in antimicrobial chemotherapy,
 410 treatment of *P. aeruginosa* infections has become challenging
 411 because of the increasing prevalence of intrinsic and acquired
 412 multi-drug resistance to antibiotics^{35–38}. Notably, *P. aeruginosa*
 413 has become the most common multi-drug resistant Gram-
 414 negative bacteria causing pneumonia in hospitalized patients,
 415 associated with premature mortality³⁹. In addition, managing
 416 antibiotic-resistance infections of *P. aeruginosa* is costly, adding
 417 pressure on overburdened healthcare systems⁴⁰. Besides acute
 418 pneumonia, *P. aeruginosa* can also cause persistent infections
 419 in patients with chronic pulmonary diseases, including cystic
 420 fibrosis (CF)⁴¹, chronic obstructive pulmonary disease⁴², or
 421 non-CF bronchiectasis⁴³ and is also associated with recurrent
 422 infections in patients in the intensive care unit⁴⁴. Anti-
 423 infectious Ab therapies have gained an important place in the
 424 therapeutic arsenal against infectious diseases. As illustrated in
 425 the treatment of *Clostridium difficile* or HIV infections, they offer
 426 novel perspectives for addressing antimicrobial resistance and
 427 chronic/recurrent *P. aeruginosa* infections. Our group and others
 428 have demonstrated that the airways constitute an attractive and
 429 feasible alternative route for Ab delivery into the lungs^{19,45–47}
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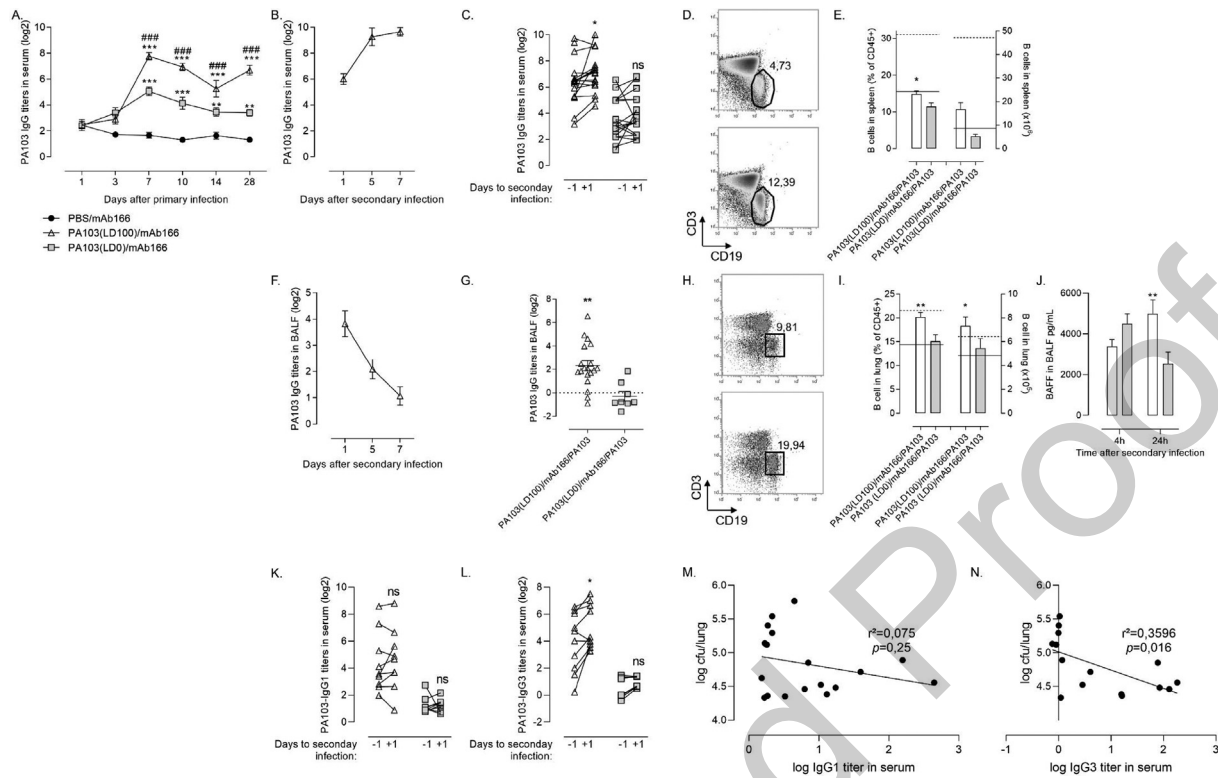


Fig. 4 Mucosal administration of mAb166 leads to the development of a sustained and protective humoral immune response against *Pseudomonas aeruginosa*. B6 mice were treated/infected as described in Fig. 1C. (A) The concentration of total anti-PA103 IgG in serum was determined by ELISA at days 1, 3, 7, 10, 14, 28 after the primary infection. The data are quoted as the mean values \pm SEM. The results correspond to seven pooled, independent experiments ($n = 10\text{--}33$ mice per time-points), ###: $p < 0.001$ with two-way analysis of variance followed by a Bonferroni post-test, comparing PA103(LD100)/mAb166 with PA103(5.10^4 CFU = LD0)/mAb166. **: $p < 0.01$; ***: $p < 0.001$ with two-way analysis of variance followed by a Bonferroni post-test, comparing with PBS controls. (B) The concentration of total anti-PA103 IgG in serum was determined by ELISA at days 1, 5, and 7 after the secondary infection. The data are quoted as the mean values \pm SEM. The results correspond to three pooled, independent experiments ($n = 10$ mice per time-points). (C) The concentration of total anti-PA103 IgG in serum was determined by ELISA at days -1, +1 after the secondary infection. The data are quoted as individual values. The results correspond to four pooled, independent experiments ($n = 17\text{--}19$ mice per group), *: $p < 0.05$; with a paired t test. 24 hours after the secondary infection, (D) representative dot plot depicting CD19+ B cells in the spleen, (E) frequency and total number of splenic CD19+ B cells (CD45+ CD3- CD19+ cells). The data are quoted as the paired mean values \pm SEM. The results correspond to two pooled, independent experiments ($n = 10$ mice per time-points), *: $p < 0.05$; with a t test. (F) The concentration of total anti-PA103 IgG in BALF was determined by ELISA at days 1, 5 and 7 after the secondary infection. The data are quoted as the mean values \pm SEM. The results correspond to six pooled, independent experiments ($n = 8\text{--}18$ mice per time-points). (G) the concentration of total anti-PA103 IgG in BALF was determined by ELISA at day 1 after the secondary infection. The data are quoted as the mean values \pm SEM. The results correspond to three pooled, independent experiments ($n = 8\text{--}19$ mice per time-points). **: $p < 0.01$; with a t test. 24 hours after the secondary infection, (H) representative dot plot depicting CD19+ B cells in the lung, (I) total number of lung CD19+ B cells; and (J) BAFF expression in the BALF. The data are quoted as the paired mean values \pm standard error of the mean. The results correspond to two pooled, independent experiments ($n = 10$ mice per time-points), *: $p < 0.05$; **: $p < 0.01$ with a t test. The concentration of anti-PA103 IgG1 (K) and IgG3 (L) in serum was determined by ELISA at days -1, +1 after the secondary infection. Correlation analysis of PA103 burden (depicted in Fig. 3C) in lung and IgG1 (M) or IgG3 (N) in the serum. The data are quoted as individual values. The results correspond to four pooled, independent experiments ($n = 17\text{--}19$ mice per group), *: $p < 0.05$; with a paired t test. Dotted lines indicated data from five uninfected/untreated mice; continuous lines indicated data from five infected/untreated mice. BALF = bronchoalveolar lavage fluid; CD = cluster of differentiation; CFU = colony-forming unit; ELISA = enzyme-linked immunosorbent assay; h = hours; Ig = immunoglobulin; mAb = monoclonal antibodies; PBS = phosphate-buffered saline; SEM = standard error of the mean.

431 enhancing their local concentration, limiting their passage into
432 the systemic circulation, and leading to a better antimicrobial
433 protective response as compared to other delivery
434 routes^{20,22,23,48}. After mucosal delivery, Ab-mediated anti-
435 infective response relies mostly on Fab-dependent neutraliza-
436 tion of pathogen and recruitment of immune mediators via
437 Fc γ R.

438 Using a robust and straightforward murine model of pneumo-
439 nia mimicking the initial phases of lung infection during *P.*
440 *aeruginosa* colonization of the airways, we demonstrated for
441 the first time a long-lasting protection associated with
442 mucosal-delivered Abs, protecting individuals from a secondary
443 infection. Recent clinical and preclinical studies have identified
444 similar functions for Abs, which act as immunomodulators that

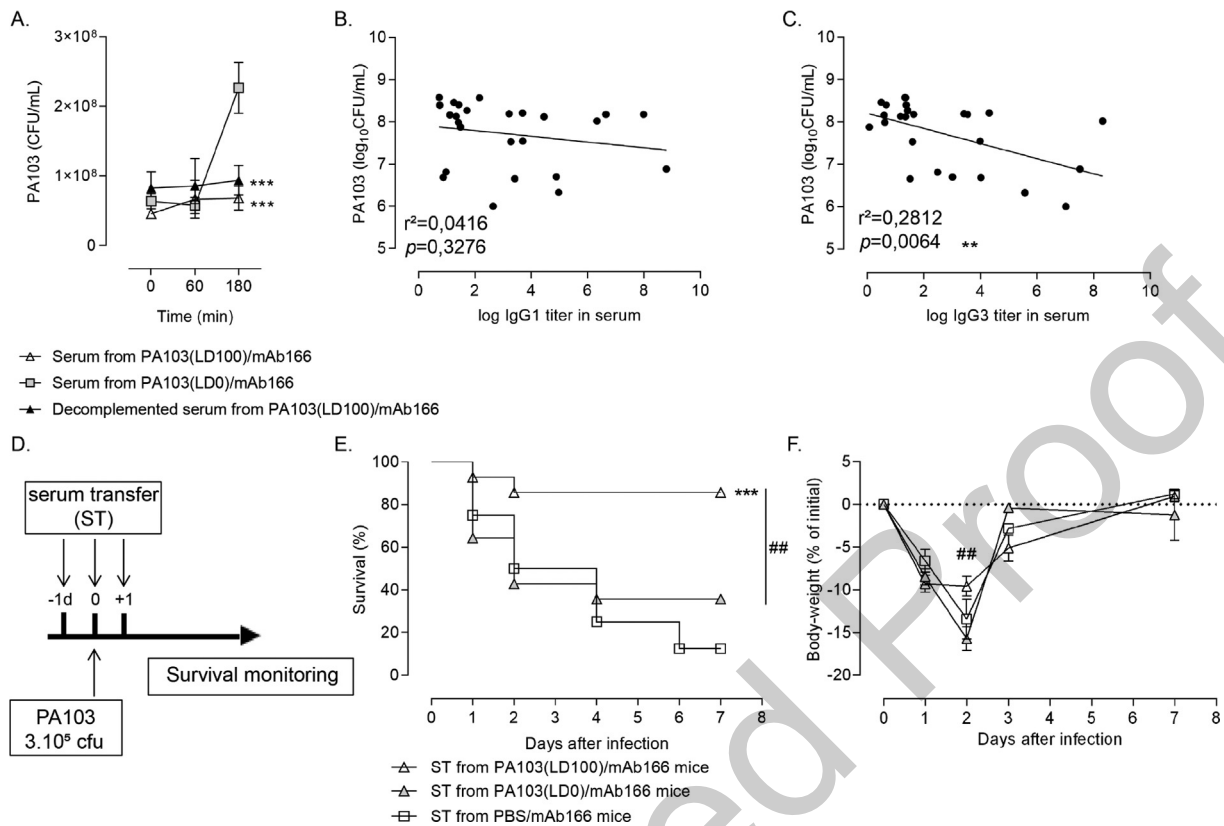


Fig. 5 Serum from challenged immune mice protects against *Pseudomonas aeruginosa* infection. (A) Bacterial growth assay using samples collected at D+28 after primary infection with *P. aeruginosa* (LD100 or LD0) and treated with mAb166 (100 μ g). Correlation analysis of PA103 burden (depicted in Fig. 5A) and IgG1 (B) or IgG3 (C) in the serum. The data are quoted as individual values. The results correspond to three pooled, independent experiments ($n = 9$ –17 mice per group), ****: $p < 0.001$; two-way analysis of variance followed by Bonferroni post-test. (D) Serum from B6 mice that have been previously infected with *P. aeruginosa* (3.10^5 CFU = LD100) and treated with mAb166 (100 μ g) were collected at D+28 after primary infection. Sera were injected intraperitoneally in naïve B6 mice at D-1, D0 and D+1 after an infection with *P. aeruginosa* (3.10^5 CFU); survival (E) and body weight (F) were monitored over 7 days. The results correspond to three pooled, independent experiments ($n = 15$ mice per group), ****: $p < 0.01$ with a log-rank test comparing with mAb166/PBS group; ##: $p < 0.01$ with a log-rank test comparing with mAb166/PA103(5.10^4) group. CFU = colony-forming unit; D = day; Ig = immunoglobulin; mAb = monoclonal antibodies.

could bridge innate, acquired, cellular, and humoral immune responses. However, this Ab-mediated long-term effect was for instance limited to cancer or viral infection and after a systemic administration of the therapeutic agent^{3,16}. Our results showed that this effect was not related to unspecific vaccination process (due to natural immunity), as the long-term immunity was dependent on the dose of the Abs and the size of bacteria inoculum while an antibiotic treatment was unable to promote protection against secondary infection. Similarly, we observed that this effect was restricted to the presence of the antigen as demonstrated by the absence of protection after a secondary infection when the mice have been primarily infected with a *P. aeruginosa* strain devoid of antigen expression. This indicates a critical role of IC between therapeutic Abs and bacteria expressing the cognate antigen. Our results support this hypothesis, showing that the amount of IC was correlated to the activation of APC in an Fc γ R-dependent manner. Evidence in the literature showed that receptor-mediated capture of extracellular antigen by APC considerably enhanced the efficiency of antigen uptake, processing, and presentation on major histocompatibility complex molecules to T cells^{49–52}, especially under inflammatory conditions⁵³. As suggested by the literature, the induction of

Ab-mediated long-term protection may be supported by the ability of APC to internalize IC and promote protective adaptive memory responses^{54,55}.

To translate the observations of Ab-mediated long-term protection into human benefits, it appears essential to identify the molecular and cellular mechanisms supporting these long-term protective responses. During viral infections, Ab-mediated long-term protection relies on the induction of endogenous humoral response^{9,10,15,56}, but not in cancer models¹⁴. Here, we provided evidence that anti-*P. aeruginosa* humoral response is a critical component of Ab-mediated long-term protection. In fact, historical data have established that impaired serum-killing of *P. aeruginosa* was associated with poor outcome in infected patients⁵⁷. Here, we first showed that anti-bacterial IgG and especially IgG3 are generated in a greater extent after primary infection in individuals that will subsequently survive the challenge, with a direct effect on the control of the bacterial growth and lung load. This subclass stimulated by a pro-T helper 1 inflammatory milieu⁵⁸ was already demonstrated to actively contribute to the protection against other respiratory bacteria⁵⁹. In particular, it was shown that IgG3 had superior binding to capsular polysaccharide, better ability to control bacterial growth,

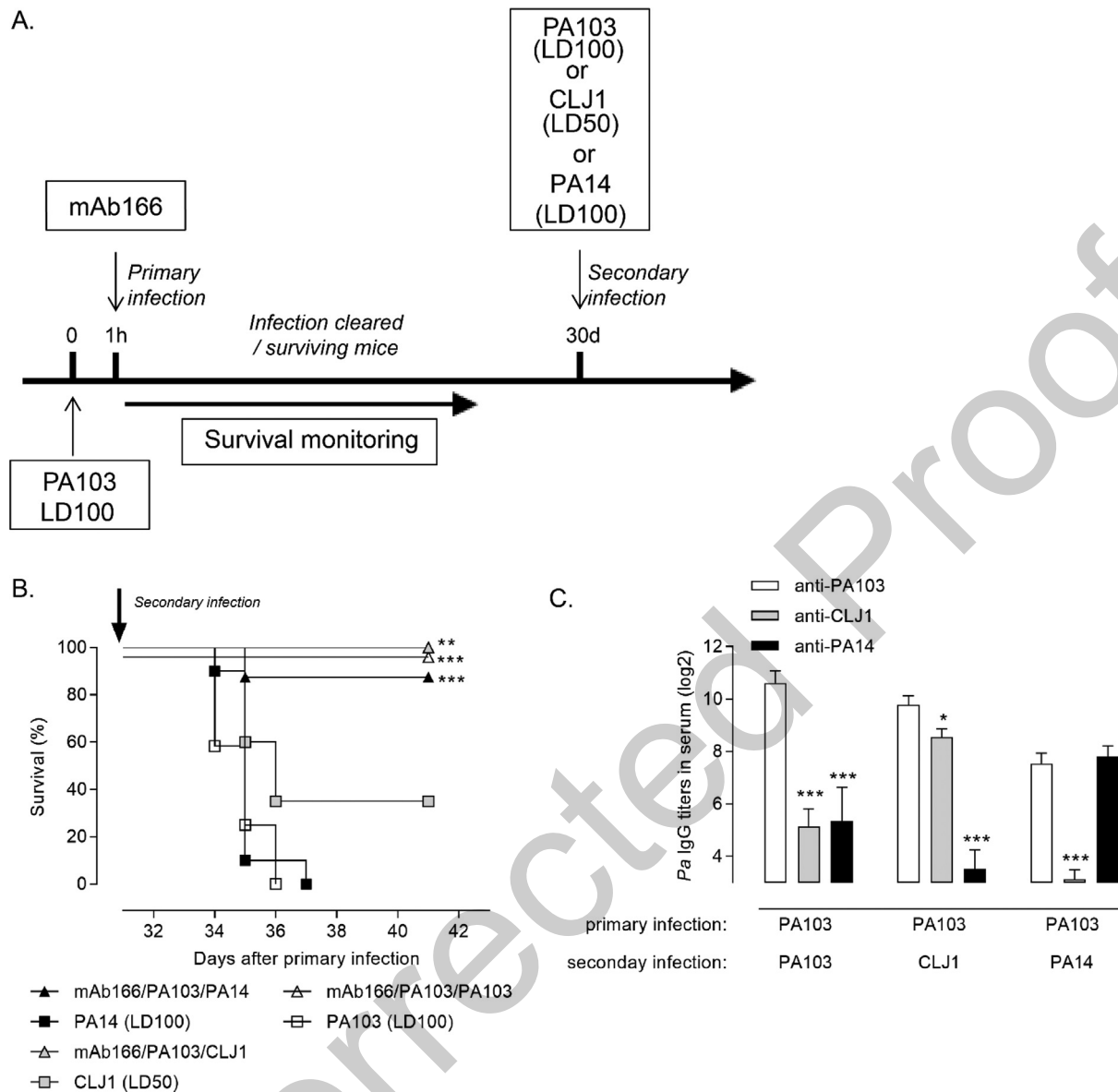


Fig. 6 Long-lasting humoral response induced by anti-bacterial Ab mediates cross-protection against *Pseudomonas aeruginosa*. (A) B6 mice were treated/infected as described in Fig. 1C. At the challenge, mice were infected with either homologous strain PA103 wild-type (LD100), or heterologous strains CLJ1 (LD50) or PA14 (LD100). (B) Survival was monitored over the entire period. The results correspond to three pooled, independent experiments ($n = 10-36$ mice per group), **: $p < 0.01$; ***: $p < 0.001$ with a log-rank test comparing with respective untreated groups. (C) Total anti-PA103 (white bars), anti-CLJ1 (black bars) or anti-PA14 (gray bars) IgG in serum was determined by enzyme-linked immunosorbent assay at D+7 after secondary infection. The data are quoted as the mean values \pm standard error of the mean. The results correspond to three pooled, independent experiments ($n = 5-10$ mice per group). D = day; Ig = immunoglobulin; mAb = monoclonal antibodies.

489 and also promoted neutrophil-mediated killing against Gram-
 490 negative lung pathogen as compared to IgG1⁶⁰. Secondly, trans-
 491 ferred anti-bacterial IgG3 enriched serum provided protection
 492 against *P. aeruginosa* infection. This protection due to opsonic
 493 IgG3 was not mediated through the complemented pathway,
 494 confirming the limited ability of mouse complement to mediate
 495 bacterial killing⁶¹⁻⁶³. Finally, both lung and systemic B cells are
 496 substantiated in the context of long-term protection. It is note-
 497 worthy that we observed lung plasma cells scattered within
 498 the lung parenchyma of the protected animals, but not isolated
 499 lymphoid follicles. This differs from an influenza lung infection,

which elicits tertiary lymphoid structures and is associated with
 memory responses^{64,65} while being similar to what was
 observed in pneumococcus-recovered lungs⁶⁶. Taken together,
 these results indicate that *P. aeruginosa*-specific memory
 humoral response induced by mucosal delivery of Abs mediates
 protection against repeated pulmonary infection. This is in
 accordance with previous evidence indicating an essential con-
 tribution of the humoral and B cell response to anti-viral Abs
 long-term response^{67,68}. Interestingly, in the context of cancer,
 the long-term antitumor protection induced by Ab therapy
 was independent of endogenous Ab response¹⁴, suggesting dis-

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tinct long-term protective immune responses depending on the inflammatory condition. Although, a complete characterization of the relative contribution of each B cell's subpopulations (notably long-lived plasma cells, marginal zone B cells, and B1 B cells) to anti-*P. aeruginosa* immunity remains to be addressed, the pre-opsionization and serum transfer experiments indicate that preformed plasma Abs may be involved in the long-term protection mediated by mAb166.

The long-term protection provided by inhaled Abs after resolution and challenge by *P. aeruginosa* appears complex and may involve multiple overlapping pathways which may remodel immune cells^{69,70}. In fact, CD4+ T cells are critical to coordinate innate and adaptive immune responses against *P. aeruginosa*^{31,71}, including B cell maturation and class-switching, providing bacterial clearance^{72,73}. Alveolar macrophages are first-line phagocytes preventing *P. aeruginosa* to invade airways⁷⁴. Although we cannot exclude the possibility that other immune cell populations are involved in long-term protection, our cell depletion and flow-cytometric analysis suggested that CD4+ T cells and alveolar macrophages might not contribute to long-term protection against pneumonia. Our results are in line with findings showing that CD4 depletion does not alter the production of anti-*P. aeruginosa* IgG3 after whole cell vaccination⁷⁵ or that alveolar macrophages do not play an important role in defense of the lung against *P. aeruginosa*^{76,77}. A shared downstream mechanism of anti-*P. aeruginosa* immunity involved improved neutrophil recruitment. Here, we showed that mucosal Ab treatment hastened neutrophil influx both before and after secondary infection in animals protected against *P. aeruginosa* pneumonia. Additional experiments will be necessary to provide a complete picture of the cellular and molecular partners accounting for the induction of long-term anti-*P. aeruginosa* humoral response, mediated by Ab treatment^{31,78}.

P. aeruginosa is a highly versatile pathogen intrinsically associated with chronic obstructive pulmonary disease or patients with CF, acting as a colonizer that contributes to the deterioration of lung function and fatal outcomes^{79,80}, or being also a causative agent of nosocomial pneumonia with significantly higher mortality as compared to other pathogens⁸¹ and even in readmitted patients⁴⁴. In this context, understanding immune mechanisms of long-term protection against *P. aeruginosa* is critical to developing effective Ab-based therapies that are broadly protective against bacterial pneumonia. For instance, whether Ab-mediated long-lasting effects are solely directed at the Ab-targeted antigen or provide epitope spreading remains unclear and may depend on the pathological context³. Historically, anti-*P. aeruginosa* vaccination has been notably hampered by the inability to provide broad protection across different serotypes⁸²⁻⁸⁴. In this study, animals primarily infected with PA103 strain (serotype O11) and treated with a monoclonal Abs targeting the type 3 secretion system were partly protected against secondary infections by heterologous *P. aeruginosa* strains (serotype O10 or O12). Interestingly, at the recall, the humoral response showed enrichment of IgG specific to the heterologous strains including the opsonic IgG3 subtypes (data not shown), suggesting that monoclonal Ab treatment has overcome the immunodominance of lipopolysaccharide antigen eliciting a broad anti-*P. aeruginosa* Ab response.

A limitation of our study relies on the complete understanding of the immune mechanisms accounting for this cross-protection, which may rely on the generation of a network of adaptive memory cells that can cross-react with antigens from

heterologous bacteria^{85,86} or through the induction of trained innate immunity which has been shown to mediate persistent broad-spectrum protection against lung pathogens⁸⁷⁻⁸⁹.

In conclusion, mucosal delivery of Abs offers the best protection against *P. aeruginosa*, a high-priority classified pathogen for which there is currently no approved vaccine or Ab. It will allow rapid pathogen neutralization limiting its replication while providing an optimal host immune response to eliminate the pathogen and induce long-lasting protection against secondary infections. This novel modality associated with anti-bacterial Abs may be of importance for the treatment of pathogen-causing recurrent acute infections as well as chronic respiratory infections, especially in the context of antibiotic resistance.

METHODS

Mice and antibodies

Adult male C57BL/6j (B6) mice (6-8 weeks old) were obtained from Janvier (Le Genest Saint-Isle, France). All mice were housed under specific-pathogen-free conditions at the PST "Animaleries" animal facility (Université de Tours, France) and had access to food and water *ad libitum*. All experiments complied with the French government's ethical and animal experiment regulations (APAFIS#7608-2016111515206630).

mAb166 was generated from PTA-9180 hybridoma (LGC Standards, France) and supplied as a sterile, non-pyrogenic, phosphate-buffered saline (PBS) solution under good manufacturing practice (BioXcell, USA). MPC11 (clone MPC11, murine IgG2b), depleting anti-CD4 Ab (clone GK1.5), and its isotype control (clone LTF2, rat IgG2b) were obtained from BioXcell.

P. aeruginosa primary infection

Several *P. aeruginosa* strains were used in the study: PA103 (ATCC 33348) and PA103 Δ pcrV, kindly provided by Pr. Teiji Sawa (Kyoto University, Japan), CLJ1 kindly provided by Dr. Ina Attree (CEA Grenoble, France) and PA14, kindly provided by Dr. Eric Morello (Tours University, France). The uniformity of the colonies was checked by plating on *Pseudomonas* isolation agar (PIA) plates. PA103 has been transformed by quadriparental mating by a mini-Tn7T transposon encoding allowing a constitutive expression of the *LuxCDABE* operon. Bacteria were prepared as previously described²². Mice were anesthetized with isoflurane 4% and an operating otoscope fit with intubation specula was introduced to both maintain tongue retraction and visualize the glottis. A fiber optic wire threaded through a 20G catheter and connected to torch stylet (Harvard Apparatus, France) was inserted into the mouse trachea. Correct intubation was confirmed using lung inflation bulb test and 40 μ L of the bacterial solution was applied using an ultrafine pipette tip. Inoculum size for infections were confirmed by counting CFU on PIA plates and were as follow: 5.10^4 CFU (LD0 dose), or 5.10^5 CFU (LD100, experiments with prophylactic intervention), or 3.10^5 CFU (LD100, experiments with therapeutic setting) for PA103; 5.10^5 CFU (non-lethal dose) for PA103 Δ pcrV. Mortality and body weight of animals were monitored daily. In all experiments, moribund animals or animals with a weight loss of more than 20% were sacrificed for ethical reasons and considered as dead animals due to the infection.

Antibody, antibiotic administration

mAb166 was either administered 2 hours before (prophylactic intervention) or 1 hour after the infection (therapeutic intervention), at the dose of 100 μ g or 50 μ g (suboptimal quantity) in 50

632 μL of PBS using a Microsprayer aerosolizer (Penn-Century, US)
633 introduced orotracheally, as described in the previous section.
634 Amikacin sulfate (Sigma, France), 25 mg/kg was administered
635 through the airways using the same protocol. For CD4+ T-cell
636 depletion, mice were treated at D+28 with 200 $\mu\text{g}/\text{mice}$ of
637 anti-CD4 Abs delivered by both intraperitoneal and intranasal
638 administration⁶⁶.

639 *P. aeruginosa* secondary infection

640 Mice recovering from the primary infection were re-infected oro-
641 tracheally as described above with PA103– 5.10^5 CFU (experi-
642 ments with prophylactic intervention) or 3.10^5 CFU
643 (experiments with therapeutic intervention); CLJ1– 1.10^6 CFU
644 (LD50) or PA14– 1.10^6 CFU (LD100), 30 days after the primary
645 infection.

646 Blood sampling and adoptive serum transfer experiments

647 Blood samples were collected every 3 days after the primary
648 infection and at day 1, 5, and 7 after the secondary infection
649 to assess endogenous anti-*P. aeruginosa* IgG concentrations. At
650 day 28 after the primary infection, mice were sacrificed and sera
651 were collected, pooled, heat-inactivated (56°C) for 30 minutes,
652 and diluted 1:6 in PBS. For serum transfer experiments, age-
653 matched mice were administered intraperitoneally 100 μL of
654 1/6-fold diluted serum in 1X PBS, on days -1, 0, and +1 post-
655 infection with an LD100 of PA103 (3.10^5 CFU).

656 Broncho-alveolar lavage, organ sampling, bacterial load 657 assay

658 BALF was collected after *P. aeruginosa* infection by introducing a
659 catheter into the trachea under deep pentobarbital anesthesia
660 and washing sequentially the lung with 1 x 0.5 mL and 2 x 1
661 mL of 1X PBS at room temperature. The lavage fluid was cen-
662 trifuged at 400 g for 10 minutes at 4°C and the supernatant of
663 the first lavage was stored at -20°C for analysis. The cell pellet
664 was resuspended in PBS, counted in a hemocytometer chamber
665 and used for subsequent analysis.

666 Spleen and lungs were perfused with 10 mL of PBS 1X and
667 harvested in GentleMACS C tubes (Miltenyi Biotec, Germany)
668 containing 2 mL of RPMI medium (Invitrogen, France) for flow
669 cytometry or GentleMACS M tubes (Miltenyi Biotec, Germany)
670 containing 2 mL of 1X PBS for microbiology assay.

671 Bacterial load in lung homogenates or BAL (before centrifugation)
672 was determined by plating tenfold serial dilutions on PIA-agar.
673 Plates were incubated at 37°C in a 5% CO₂ atmosphere,
674 and the CFU were counted after 24 hours.

675 The effect of serum on bacterial growth was performed in
676 triplicate as previously described⁹⁰. Briefly, PA103 were grown
677 overnight in 10 mL of LB at 37°C and resuspended in PBS to a
678 final concentration of 5.10^7 CFU/mL. 5 μL was then mixed with
679 45 μL of undiluted mouse serum at 37°C with shaking (180 rpm).
680 Bacterial growth was determined after 60 and 180 minutes by
681 plating tenfold serial dilutions on PIA-agar. Plates were incu-
682 bated at 37°C in a 5% CO₂ atmosphere, and the CFU were
683 counted after 24 hours.

684 Histology

685 At sacrifice, lungs were inflated with 2 mL of 4% paraformaldehyde
686 (PFA, Shandon) before being fixed in 4% PFA for 24 hours,
687 Lungs were then dehydrated in ethanol and embedded in parafin.
688 Serial sections (3 mm) were stained with hematoxylin and
689 eosin and all lung sections were analyzed by an independent

clinical pathologist. Pictures were sampled using an automated
upright microscope (Leica) with 10X and 20X objectives.

692 Preparation of pulmonary and spleen immune cells

693 Lung and spleen homogenates were prepared using a Gentle-
694 MACS tissue homogenizer (Miltenyi Biotec, Germany). Lung
695 pieces were then digested in a medium containing 125 $\mu\text{g}/\text{mL}$
696 of Liberase (Roche, France) and 100 $\mu\text{g}/\text{mL}$ of DNase I (Sigma,
697 France) for 30 minutes at 37°C under gentle agitation. After
698 washes, contaminating erythrocytes were lysed using Hybri-
699 Max lysis buffer (Sigma, France) according to manufacturer's
700 instructions. Samples were sequentially filtered over 100 μm
701 and 40 μm nylon mesh. After final wash, cell pellets were resus-
702 pended in PBS containing 2% FCS, 2mM EDTA, and 1X murine
703 Fc-block (Becton Dickinson, France)—described elsewhere as
704 flow cytometry staining (FACS) buffer.

705 Flow cytometry

706 Cells were incubated in FACS buffer for 20 minutes at 4°C. Then,
707 cells were stained in FACS buffer for 20 minutes at 4°C with
708 appropriate dilutions of the following Abs: CD45-APC-Cy7 (30-
709 F11), CD3 PerCP-Cy5.5 (17A2), CD19 V450 (1D3), IgM PE (RMM-
710 1), CD21 APC (7E9), CD11b PercP (M1/70), and Ly6G FITC (1A8)
711 from Biolegend and Siglec-F BV421 (E50-2440) from BD. Dead
712 cells were stained with the LIVE/DEAD Fixable Aqua Dead Cell
713 Staining kit (ThermoFisherScientific) and acquired on a MACS
714 Quant (Miltenyi Biotec) cytometer. Analyses were performed
715 using Venturi-One software (Applied Cytometry; UK).

716 Preparation of IC and primary BMDM

717 IC we prepared by mixing mAb166 (at the indicated concentra-
718 tion) and PA103 (with the indicated inoculums) for 1 hour at 4°C
719 under rotation. As control, we used PA103 co-incubated with
720 MPC11, in the same conditions. Murine bone-marrow cells were
721 isolated from femurs and differentiated into macrophages after
722 culturing 10^6 cells/mL for 7 days in DMEM (Sigma) supple-
723 mented with 10% FCS, 1% penicillin/streptomycin mixture
724 (Gibco) and 50 ng/mL of M-CSF (Gibco). Mature BMDM were pla-
725 ted in a 96-well plate at 1.10^5 cells/well in complete medium for
726 24 hours before being stimulated 6 hours with IC or MPC11-
727 PA103 mixture in the absence or presence of 1X Fcblock (Biole-
728 gend). Cell-free supernatant was harvested and assessed for IL-6.

729 Cytokines, mAb166, and IgG measurements

730 Pa-specific IgG, IgG1, and IgG3 titers, in serum and BALF levels,
731 were measured by sandwich enzyme-linked immunosorbent
732 assay. Briefly, high-binding Immulon 96-well plates (Thermo Fis-
733 cher Scientific, France) were coated with 0.5 $\mu\text{g}/\text{mL}$ of PA103,
734 PA14, or CLJ1 lysates, prepared from overnight cultures that
735 were then sonicated, and diluted in bicarbonate buffer. The
736 plates were then washed and blocked with 1% BSA-PBS. Serum
737 or BALF samples were incubated for 2 hours. A biotin-
738 conjugated goat anti-rat IgG Ab (Biolegend, France) was added
739 for 2 hours. After a washing step, peroxidase-conjugated strep-
740 tavidin (R&D) was added for 20 minutes. Between each step,
741 plates were thoroughly washed in 0.05% Tween20-PBS. Tetram-
742 ethylbenzidine was used as a substrate, and the absorbance was
743 measured at 450 nm using a microplate reader (Tecan, Switzer-
744 land). The titer was calculated by binary logarithm regression as
745 the reciprocal dilution of the sample, where the extinction was
746 2-fold the background extinction. Similar procedure was used
747 to quantify mAb166 in serum and BALF using recombinant *pcrV*

748 (0.5 µg/mL) as coating antigen. Before assay, Ab-antigen dissociation was performed, using acetic acid, as previously described²². Cytokines concentrations in BALF were assessed using a Bio-plex magnetic bead assay (Luminex, Biotechne, France) and analyzed using a Bio-plex 200 workstation (Biorad, France).

754 Statistical analysis

755 Statistical evaluation of differences between the experimental groups was determined by using one-way analysis of variance followed by a Newman–Keuls post-test (which allows comparison of all pairs of groups). Log-rank test was used for survival analysis. Student's t test was used for comparison between two groups and paired t test was used when comparing the same individual before and after challenge. All tests were performed with GraphPad Prism, Version 6 for Windows (GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com). All data are presented as mean ± standard error of the mean. A *p* value < 0.05 was considered significant.

766 AUTHOR CONTRIBUTIONS

767 TS and NHV conceived the study. All authors substantially contributed to the acquisition, analysis, or interpretation of data. CP provided expertise in the analysis of the immune response. AP, TS, and NHV contributed to manuscript drafting, revising, and critically reviewing. All authors approved the final version of this manuscript to be published.

773 DECLARATIONS OF COMPETING INTEREST

774 AP, MF, CP, CB, MC, LB, NA, CP, and TS, have nothing to declare. NHV is co-founder and scientific expert for Cynbiose Respiratory. In the past 2 years, she received consultancy fees from Eli Lilly, Argencx, Novartis, and research support from Sanofi and Aerogen Ltd.

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790 APPENDIX A. SUPPLEMENTARY DATA

791 Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mucimm.2023.03.005>.

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Uncorrected Proof