

Neutralizing human monoclonal antibodies that target the PcrV component of the Type III Secretion System of *Pseudomonas aeruginosa* act through distinct mechanisms

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eLife Assessment

This **useful** work identifies new monoclonal antibodies produced by cystic fibrosis patients against *Pseudomonas aeruginosa* type three secretion system. The evidence supporting authors' claim is **solid**. Nonetheless, the manuscript may benefit from a more in depth description of what the authors learned from their structure-based analyses of antibodies targeting PcrV.

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Abstract

Pseudomonas aeruginosa is a major human opportunistic pathogen associated with a high incidence of multi-drug resistance. The antibody-based blockade of *P. aeruginosa* virulence factors represents a promising alternative strategy to mitigate its infectivity. In this study, we employed single B cell sorting from cystic fibrosis patients to isolate human monoclonal antibodies (mAbs) targeting proteins from the *P. aeruginosa* Type 3 Secretion System (T3SS) and characterized a panel of mAbs directed at PscF and PcrV. Among those, two mAbs, P5B3 and P3D6, that bind to the injectisome tip protein PcrV, exhibited T3SS blocking activity. We solved the crystal structure of the P3D6 Fab-PcrV complex, which revealed that the Ab binds to the C-terminal region of PcrV. In addition, we compared the T3SS-blocking activity of three PcrV-targeting mAbs, including two from previous independent studies, using two distinct assays to evaluate pore formation and toxin injection. We conducted a mechanistic and structural analysis of their modes of action through modeling based on the known structure of a functional homolog, SipD from *Salmonella typhimurium*. The analysis suggests that anti-

PcrV mAbs may act through different mechanisms, ranging from preventing PcrV oligomerization to disrupting PcrV's scaffolding function, thereby inhibiting the assembly and function of the translocon pore. Our findings provide additional evidence that T3SS-targeting Abs, some capable of inhibiting virulence, are elicited in *P. aeruginosa*-infected patients. The results offer deeper insights into PcrV recognition by mAbs and their associated mechanisms of action, helping to identify which Abs are more likely to be therapeutically useful based on their mode of action and potency. This paves the way for the development of effective alternatives to traditional antibiotics in the fight against this resilient pathogen.

Introduction

The emergence of antimicrobial resistance is a major threat to human health. Among the microorganisms whose resistance rates have increased the most dramatically are ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp*) for which novel antibacterial treatments are urgently needed. However, an antibiotic discovery hiatus that occurred during the last few decades severely heightened the resistance threat (Murray et al., 2022), underlining the importance of exploring alternative strategies such as host-targeting, bacteriophage, anti-virulence, and Ab-based therapies (De Melo et al., 2024; Kaufmann et al., 2018; Morrison, 2015).

Therapeutic mAbs have been successfully developed to fight viral infections ranging from Ebola to SARS-CoV-2 (Crowe, 2022; Levin et al., 2022; Mulangu et al., 2019). To date, however, only three therapeutic Abs have been marketed against bacteria, all of which target toxins. Other types of bacterial virulence factors could also serve as potential high importance targets for mAbs. Recent examples include the development of mAbs that target lipopolysaccharides, O-antigen, and outer membrane transporter proteins, notably in *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* (Pennini et al., 2017; Rollenske et al., 2018; Watson et al., 2021). The advantages of targeting virulence factors through mAbs include notably a high specificity and the decreased likelihood of the emergence of resistance among bacteria (La Guidara et al., 2024). Additionally, the employment of mAb engineering platforms offers the potential for improved efficacy through modifications such as half-life extension and alterations of Fc effector functions (Morrison, 2015; Vacca et al., 2022). Finally, strategies such as the use of mAb cocktails targeting different specificities and the combination with traditional antibiotics further expand the range of Ab-based treatment options (Duan et al., 2021; Morrison, 2015; Tabor et al., 2018).

Pseudomonas aeruginosa is a major nosocomial pathogen and the leading cause of acute pneumonia and chronic lung infections, particularly in ventilator-assisted and cystic fibrosis (CF) patients. Infections with *P. aeruginosa* ultimately lead to loss of lung function and death in CF patients. Worldwide, *P. aeruginosa* is responsible for more than 300,000 deaths associated or attributed to resistance each year. The natural resistance of *P. aeruginosa* to a broad range of antibiotics, its ability to grow as biofilms, as well as its widespread presence in hospital settings (Horcajada et al., 2019; Murray et al., 2022), have called for urgent efforts towards the development of new therapeutic agents. Aggressive acute infections by *P. aeruginosa* are highly dependent on its Type III Secretion System (T3SS), a needle-like, multicomponent secretion machinery located on the cell surface and that transports effectors from the bacterial cytoplasm directly into the host cell cytosol (Goure et al., 2004; Hauser, 2009; Quinaud et al., 2007, 2005). It is of note that in other human pathogens such as *Yersinia pestis*, *Salmonella typhi*, *Shigella dysenteriae* and *Escherichia coli* the T3SS also plays a key role in virulence, participating in the causation of diseases such as plague, typhoid fever, and bacillary dysentery, respectively (Coburn et al., 2007; Diepold and Wagner, 2014; Hu et al., 2017; Serapio-Palacios and Finlay, 2020).

A key component of the T3SS is the injectisome, membrane-embedded protein rings extended by a hollow needle, composed of the PscF protein that protrudes outwards from the bacterial surface. Injectisome-dependent toxin delivery, which occurs upon contact with the eukaryotic target cell, also requires formation of the ‘translocon’, a complex of three proteins that are exported through the interior of the polymerized needle, assemble at its tip and form a pore in the eukaryotic cell membrane, an essential step for effector injection (Mueller et al., 2008 [\[1\]](#)). The translocon is composed of two hydrophobic proteins (PopB and PopD in *P. aeruginosa*), as well as a hydrophilic partner – PcrV, or the V antigen-in *P. aeruginosa* (Mattei et al., 2011 [\[2\]](#)). PopB and PopD have been shown to act as *bona fide* pore-forming toxins (Schoehn et al., 2003 [\[3\]](#); Faudry et al., 2006 [\[4\]](#); Montagner et al., 2011 [\[5\]](#)) that, upon membrane disruption, can trigger the manipulation of host processes including histone dephosphorylation and mitochondrial network disruption (Dortet et al., 2018 [\[6\]](#)). PcrV, on the other hand, oligomerizes at the tip of the T3SS needle and aids PopB and PopD in their membrane disruption process (Gébus et al., 2009 [\[7\]](#); Goure et al., 2005 [\[8\]](#); Guo and Galán, 2021 [\[9\]](#); Mattei et al., 2011 [\[2\]](#)). Crystal structures of monomeric homologs of PcrV (LcrV, SipD, BipD) have shown that they fold into an elongated coiled-coil buttressed by an α -helical hairpin at the N-terminus and an α/β carboxy-terminal region (Derewenda, 2011 [\[10\]](#); Erskine et al., 2006 [\[11\]](#); Lunelli et al., 2011 [\[12\]](#)). Notably, the cryo-EM structure of a needle filament complex composed of PrgI (needle protein) with SipD (tip protein) at its extremity confirmed that the latter forms a pentamer where the first and fifth subunits are separated by a gap, thus generating a heterogeneous assembly (Guo and Galán, 2021 [\[9\]](#)). This arrangement could be similar in numerous T3SS systems (Habenstein et al., 2019 [\[13\]](#)).

Given the importance of the T3SS for *P. aeruginosa* infection, components such as PcrV and PscF have been explored as targets for the development of therapeutic Abs and inhibitory small molecules, respectively (Berube et al., 2017 [\[14\]](#); Bowlin et al., 2014 [\[15\]](#)). Animal models have shown that blocking the T3SS, particularly the function of PcrV, can successfully diminish tissue damage due to *P. aeruginosa* infection (Frank et al., 2002 [\[16\]](#); Imamura et al., 2007 [\[17\]](#)). Moreover, in ventilated patients, pegylated Fabs that target PcrV (KB001-A) were shown to successfully reduce the incidence of pneumonia, which is consistent with the role of T3SS in the acute phase of infection (Jain et al., 2018 [\[18\]](#); Roy-Burman et al., 2001 [\[19\]](#)). However, this treatment did not benefit chronically colonized CF patients in terms of antibiotic needs (François et al., 2012 [\[20\]](#); Jain et al., 2018 [\[18\]](#); Yaeger et al., 2021 [\[21\]](#)). In addition, the bispecific MEDI3902 mAb targeting both PcrV and the Psl exopolysaccharide successfully protected against *P. aeruginosa* infection in animal models but was discontinued in phase II clinical trials (Chastre et al., 2022 [\[22\]](#); DiGiandomenico et al., 2014 [\[23\]](#)). Nevertheless, PcrV remains an attractive target, motivating the search for potentially more effective Abs (Simonis et al., 2023 [\[24\]](#)).

Here, we sorted specific single memory B cells from peripheral blood mononuclear cells (PBMCs) of cystic fibrosis patients to identify mAbs against PcrV and PscF with potential T3SS-inhibiting activity. Two anti-PcrV mAbs (P5B3 and P3D6) showed inhibition of the injection of the T3SS effector ExoS into epithelial cells, with mAb P5B3 displaying blocking activity against five major PcrV variants representing more than 80% of clinical isolates sequenced to date. We obtained the crystal structure of a P3D6 Fab-PcrV complex and further compared the mechanisms of actions of different anti-PcrV mAbs targeting various epitopes, including one mAb from a recent publication (Simonis et al., 2023 [\[24\]](#)). These structure-based analyses of the mechanisms of action of the different mAbs provide valuable insights for the development of improved antipseudomonal treatments and preventive approaches.

Results

Selection of donors exhibiting T3SS-inhibiting circulating IgG responses

Our approach was based on single cell sorting of recombinant PcrV and PscF specific memory B cells from human donor peripheral blood mononuclear cells (PBMCs). To identify donors with anti-PcrV and –PscF mAbs with T3SS-inhibitory activity, we first evaluated in ELISA the reactivity of sera from a cohort of CF patients that were chronically colonized with *P. aeruginosa* against recombinant PcrV and PscF before testing them in functional assays (**Figure 1A**). Among the 34 sera tested, donors 16 and 25 exhibited the strongest reactivity for both proteins (**Figure 1B**).

To assess the capacity of Protein A-purified serum IgGs (predominantly IgG1, IgG2 and IgG4) to block T3SS effector translocation, we used a previously developed cellular model that is based on the T3SS-dependent translocation of the ExoS effector fused to β -lactamase, ExoS-Bla (Verove et al., 2012). Briefly, epithelial cells were exposed to *P. aeruginosa* CHA Δ ExoS expressing the ExoS-Bla reporter in the presence of patients' polyclonal purified IgGs. ExoS-Bla translocation was measured by monitoring fluorescence of the β -lactamase FRET-competent substrate CCF2-AM, and expressed as normalized reporter injection. Polyclonal IgGs from donors 16 and 25 showed a potent ExoS-Bla translocation blocking activity with an almost complete inhibition of injection at 160 μ g/mL (**Figure 1C**). To investigate whether the observed activity was driven by anti-PcrV and/or anti-PscF specific IgGs, we absorbed specific Abs on beads coated with recombinant PcrV or PscF to obtain polyclonal IgG samples depleted of the corresponding specific IgGs (**Figure 1D**, top). The T3SS-blocking activity of depleted polyclonal IgGs was then evaluated using the same method as above (**Figure 1D**, bottom). The results showed a decrease in inhibitory activity when anti-PcrV Abs were depleted from donor 25's IgGs and when anti-PscF Abs were depleted from donor 16's IgGs, suggesting the presence of inhibitory Abs against the respective proteins. Additionally, the findings demonstrated that our recombinant antigen baits could effectively bind T3SS-inhibitory Abs and could therefore be used to isolate memory B cells producing the corresponding IgGs.

Isolation of PcrV and PscF mAbs using a single cell direct sorting approach

To isolate mAbs specific to PcrV and PscF, PBMCs were purified from whole blood from the two selected donors. Next, using single cell sorting, IgG positive memory B cells were isolated based on their ability to recognize either PscF or PcrV (**Figure 2A**) and seeded at the frequency of one cell per well. Variable heavy and light chain gene sequences were retrieved from isolated B cells leading to the production of a total of 66 recombinant mAbs (53 and 13 putative anti-PscF and anti-PcrV, respectively). The specific binding capacities of 10 anti-PscF and 4 anti-PcrV mAbs were confirmed by ELISA against the corresponding recombinant proteins. EC₅₀ values calculated from ELISA data showed variable apparent affinities ranging from ~50 μ g/mL to 0.02 μ g/mL (**Figure 2B**). Isolated mAbs originated from a variety of variable gene germline families, as determined using the international immunogenetics information system (IMGT) database alignments, and did not present any notable features in term of mutation rates or HCDR3 length (Supplementary Table 1), with no particular enrichment noted.

The ability of ELISA-confirmed anti-PscF and anti-PcrV mAbs to block T3SS-mediated activity at a concentration of 100 μ g/mL was subsequently evaluated using the ExoS-Bla reporter system. No significant reduction in ExoS-Bla injection was observed for any of the anti-PscF mAbs tested. However, two out of four anti-PcrV mAbs, P5B3 and P3D6, significantly reduced ExoS-Bla injection, with P3D6 displaying significantly stronger efficacy (**Figure 2B**).

To map the epitopes of the isolated mAbs, we next performed competition ELISAs (Supplementary Table 2). Antibodies directed at PscF grouped into three clusters, with P1D8 and P5G10 mAbs competing only against themselves. Similarly, anti-PcrV mAbs also grouped into three clusters,

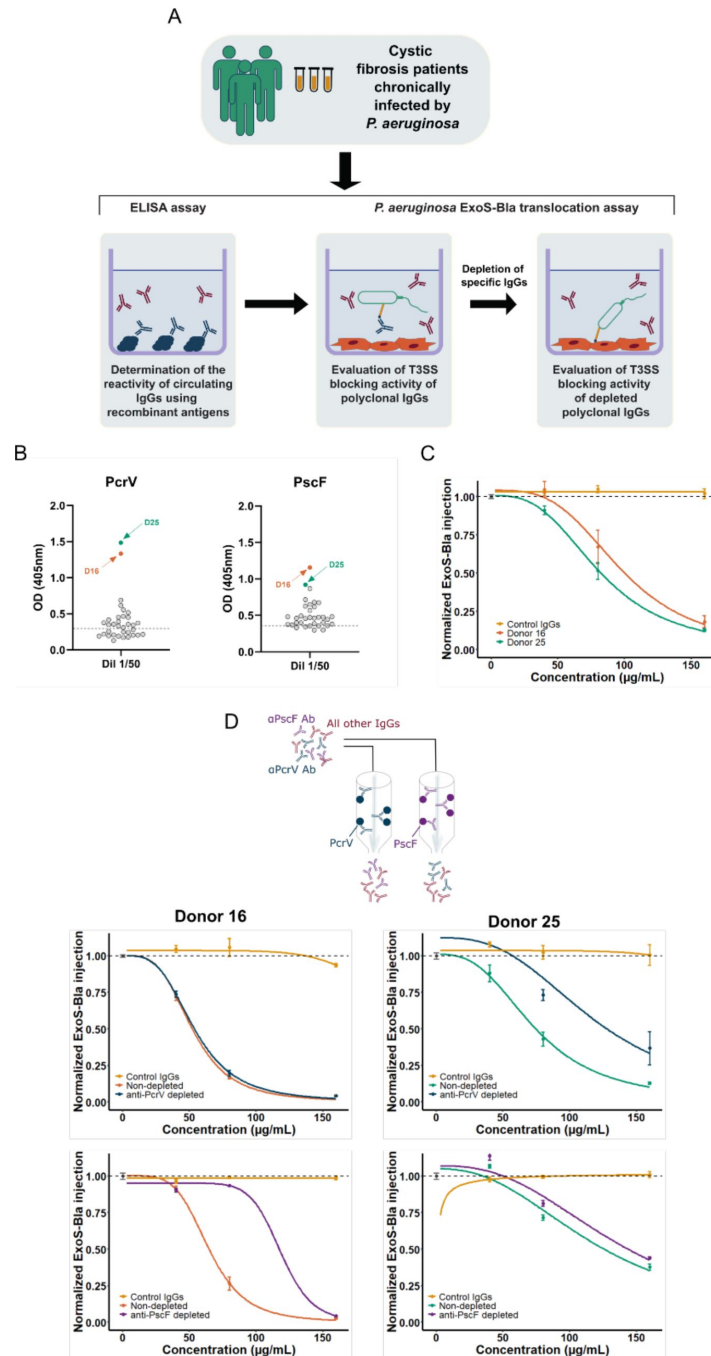


Figure 1.

Screening workflow and donor selection

(A) Schematic representation of the workflow from patient selection to evaluation of T3SS-blocking activity. (B) Patients sera (1/50 dilution) were tested in ELISA against recombinant PcrV and PscF. (C) ExoS-Bla translocation blocking activity of serum IgGs from donors 16 and 25. (D) (top) scheme of depletion experiment of specific Abs on either PscF- or PcrV-loaded columns. (bottom) blocking activity of depleted sera for both donors. The dots and bars represent the means and standard-deviations of experimental triplicates. The curves correspond to the modeled log-logistic dose-response curves. The dashed lines represent the mean of normalized ExoS-bla injection in the absence of Ab.

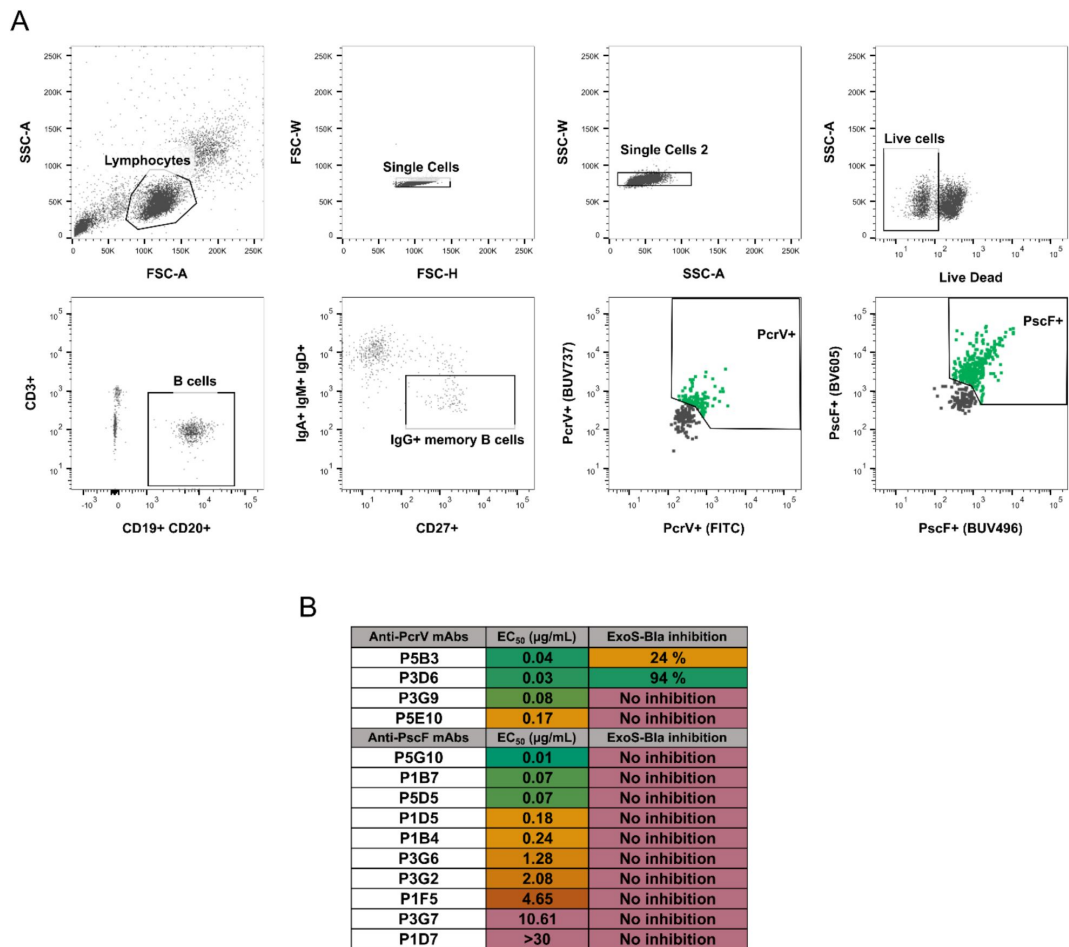


Figure 2.

Selection of B cells from donors 16 and 25.

(A) B cells sorting and isolation using PscF and PcrV baits. (B) Table summarizing the EC₅₀ values of selected Abs obtained by ELISA and the percentage of inhibition of ExoS-Bla injection into epithelial cells at 100 μg/mL. ExoS-Bla inhibitions were compared using ANOVA and “No inhibition” means an absence of significant difference with the control (adjusted p-values > 0.05). The P5B3 and P3D6 mAbs exhibited differences with the control (no Ab) with adjusted p-values < 0.001.

with the two anti-PcrV mAbs exhibiting T3SS inhibitory activity, P5B3 and P3D6, seemingly targeting overlapping epitopes. Precise affinities of both mAbs were measured using biolayer interferometry (BLI), revealing subnanomolar K_D values (Supplementary Table 3). Notably, P3D6 exhibited approximately 30-fold lower affinity compared to P5B3, despite demonstrating greater efficacy in the inhibitory assay.

P5B3 inhibits T3SS-dependent toxin injection by recognizing a highly conserved epitope of PcrV

Polymorphism in PcrV protein sequences was reported among *P. aeruginosa* clinical isolates and should be considered in the development of therapeutic human monoclonal Abs targeting PcrV (Figure 3A) (Tabor et al., 2018). To determine whether the blocking activity of mAbs P5B3 and P3D6 was impacted by the PcrV sequence, the reporter ExoS-Bla was introduced into a strain that lacked isogenic PcrV ($\Delta pcrV$) and synthesized the five most prevalent PcrV variants found in over 80% of clinical isolates (Tabor et al., 2018). Monoclonal Ab P5B3 showed statistically significant T3SS blocking activity towards all variants (Figure 3B) with estimated IC_{50} values ranging from 100 $\mu\text{g/mL}$ to 400 $\mu\text{g/mL}$ for the five variants (no statistically significant difference; Supplementary Figure 1). In contrast, mAb P3D6 had no effect on variants 2, 3, 4 and 5, but strongly inhibited variant 1 (Figure 3B) with an estimated IC_{50} of 3.7 $\mu\text{g/mL}$ (Supplementary Figure 1), indicating that the epitope recognized by P3D6 differs between PcrV variants.

Anti-PcrV mAbs block translocon pore assembly

It has been suggested that PcrV scaffolds the assembly of the PopB/PopD translocon within host membranes by interacting with the PopD component of the pore (Goure et al., 2004; Kundracik et al., 2022; Mattei et al., 2011). Furthermore, polyclonal Abs raised against PcrV have been shown to inhibit the assembly of the translocon in target membranes (Goure et al., 2005).

To investigate the mechanistic details of the inhibitory activity of mAbs P3D6 and P5B3, we used a *P. aeruginosa* strain deprived of all three T3SS effectors, ExoS, ExoT and ExoY. This strain, named PAO1A3Tox (Cisz et al., 2008) harbors PcrV variant 1 and provokes toxin-independent macrophage pyroptosis upon membrane insertion of the PopB/PopD translocation pore (Dacheux et al., 2001). Death of J774 macrophages was monitored during 4h post-infection by measuring an increase in propidium iodide fluorescence due to DNA binding to the nuclei of dead cells. Both mAbs significantly reduced the cytotoxicity induced by PAO1A3Tox by 28% and 73%, respectively (Figure 4A). Monoclonal Ab P3D6 exhibited a dose-response inhibition with an estimated IC_{50} of 11.8 $\mu\text{g/mL}$ (Figure 4B), while P5B3 did not exhibit a significant dose-response effect at concentrations below 100 $\mu\text{g/mL}$ (Figure 4C). Overall, these results indicate that the binding of both mAbs to PcrV reduces the formation of the translocation pore in target cell membranes, with P3D6 exhibiting more potent activity.

Crystal structure of PcrV* bound to Fab P3D6

In order to identify the PcrV epitopes recognized by the two mAbs, we generated a plasmid encoding a form of PcrV (PcrV*) amenable to crystallization (Tabor et al., 2018) as well as Fab fragments from both P3D6 and P5B3 mAbs. PcrV* was expressed in *E. coli* while both Fabs were expressed in HEK293F cells. Individual proteins were purified by affinity and size-exclusion chromatographies. PcrV* was incubated with either Fab fragment and samples were co-purified using size exclusion chromatography. Despite the fact that both PcrV*-Fab P3D6 and PcrV*-Fab P5B3 complexes co-eluted in gel filtration, only the PcrV*-Fab P3D6 complex subsequently generated diffracting crystals. Data were collected at the ESRF synchrotron in Grenoble and the structure was solved by molecular replacement using Phaser (McCoy et al., 2007). Iterative manual model building and model improvement led to the structure whose statistics for data collection and refinement are presented in Supplementary Table 4.

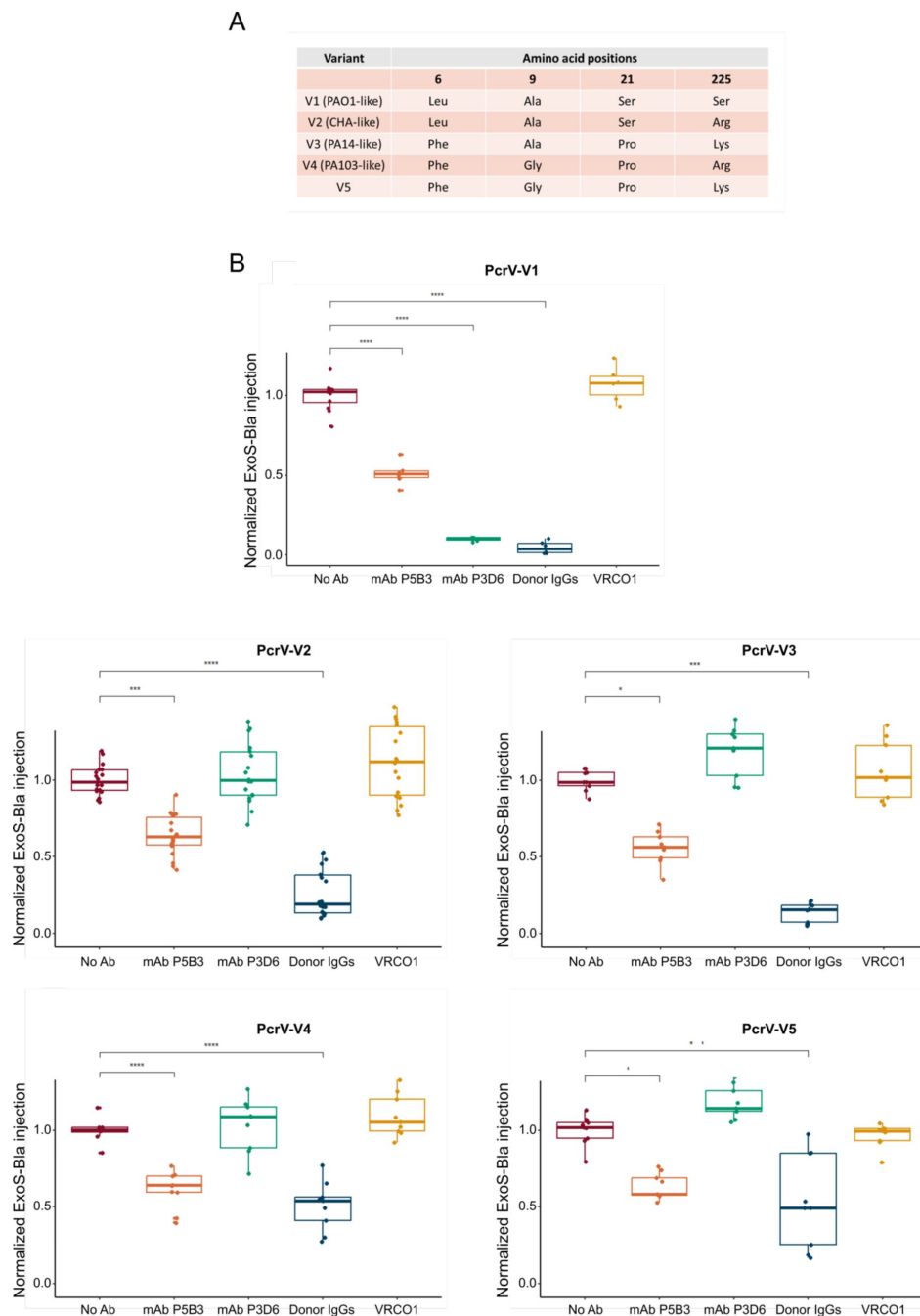


Figure 3.

mAbs P5B3 and P3D6 activity on PcrV variants.

(A) PcrV variability in clinical strains. The most variable position (225) can either be Ser, Arg or Lys. Representative strains are indicated when available (PAO1 for V1, CHA for V2, PA14 for V3 and PA103 for V4). (B) Inhibition of ExoS-Bla activity following infection of A549 epithelial cells with *P. aeruginosa* expressing the V1 variant. Normalized ExoS-Bla injection values in the presence of 100 $\mu\text{g/mL}$ Abs were compared to the control (no Ab) using ANOVA. Pairwise t-test significance is indicated by the symbols *, **, *** and **** for adjusted p-values below 0.05, 0.01, 0.001 and 0.0001, respectively. The absence of symbol corresponds to adjusted p-values > 0.05.

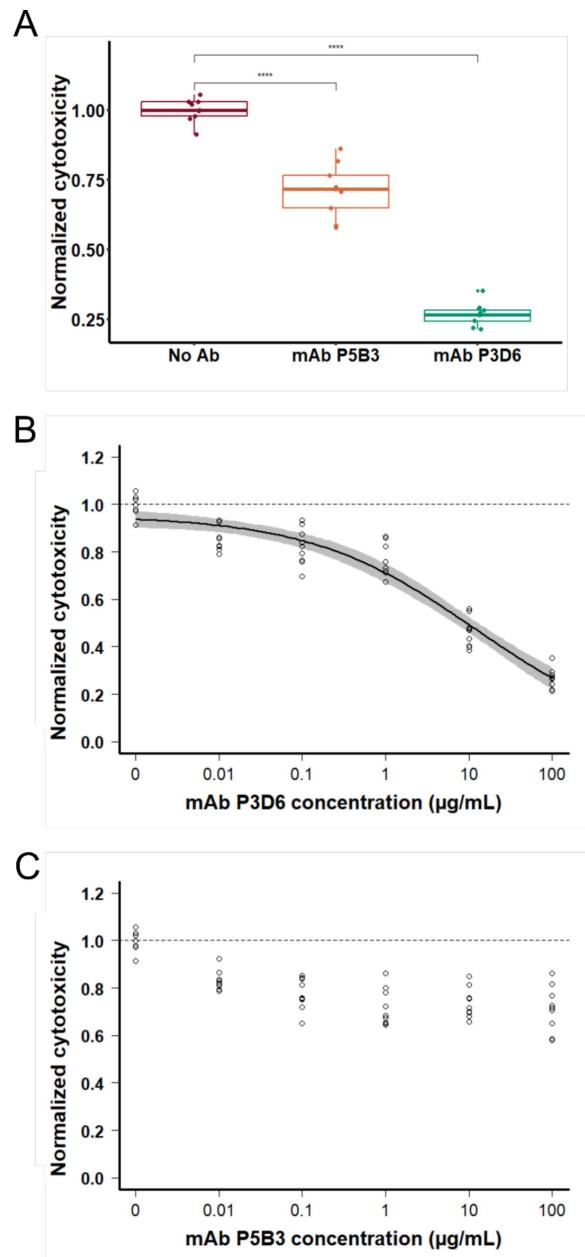


Figure 4.

mAb P3D6 efficiently inhibits the PopB/PopD translocation pore.

J774 macrophages were infected with *P. aeruginosa* strain (PAO1, V1) deprived of all three T3SS toxins. The cell death (cytotoxicity) resulting from insertion of the translocon pore was measured by propidium iodide incorporation and normalized to the wild-type strain without addition of mAbs. (A) Normalized cytotoxicity values in the presence of mAb at 100 $\mu\text{g/mL}$. Specific mAbs were compared to the control (no Ab) using ANOVA. Pairwise t-test significance is indicated by the symbol ****, meaning p-values below 0.0001. (B, C) Dose-response analysis with mAb concentrations ranging from 0.01 to 100 $\mu\text{g/mL}$. The circles, the black line and the grey area represent the experimental values, the log-logistic modeled dose-response curve and the 95% confidence interval, respectively. The dashed lines represent the mean of normalized cytotoxicity in the absence of Ab. The Ab concentration is presented in logarithmic scale. No black curve nor grey area is displayed for P5B3 because no dose-response could be modeled. In contrast, P3D6 exhibits an IC_{50} of 11.8 $\mu\text{g/mL}$.

PcrV* is composed of six helices interwoven by loop regions. α -helices 1, 4 and 6 are the major secondary structure elements in PcrV*, while helices 2, 3 and 5 are 1- or 2-turn helices. Most of the contacts formed between PcrV* and Fab P3D6 involve Helix 6 and the loop preceding it (**Figures 5A** [↗](#), **5B**) and implicate a binding platform made by both LC and HC from Fab P3D6 (Supplementary figure 3). From the PcrV side, the interaction region is highly polar, being formed by the side chains of Lys208, Gln217, Glu220, Lys222, Ser225, Asp 226, Tyr 228, Glu231, Asn234, Thr243, Asp246 and Arg247. The substitution of Ser225 in PcrV variant V1 by Lys or Arg in variants V2 to V5 is consistent with P3D6 being inefficient on strains harboring these four variants, since a residue with a bulky side chain in this position would invariably clash with the loop formed by residues 52-56 of the Fab.

In order to understand the protective role of mAb P3D6 in the context of the PcrV pentameric oligomer located at the tip of the PscF needle, we generated a model using the cryo-EM structure of the SipD pentamer ([Guo and Galán, 2021](#) [↗](#)) and aligned our co-crystal structure onto this model (**Figures 5C** [↗](#), **5D**). This analysis revealed that Fab P3D6 can successfully bind to one PcrV monomer (dark red and orange in **Figure 5** [↗](#), respectively), but would be unable to bind to a pre-formed PcrV pentamer due to the generation of clashes with neighboring subunits of the oligomeric form (**Figure 5** [↗](#); the structure of the Fab can be seen overlaid with that of the pentamer subunits).

Discussion

We generated a panel of anti-PscF and -PcrV human mAbs through specific memory B cell sorting from selected individuals. Although adsorption experiments with recombinant PscF suggested the presence of anti-PscF Abs with T3SS inhibitory activity in the donor from whom they were isolated, none of the isolated mAbs exhibited this activity. Competition mapping showed that the anti-PscF mAbs targeted three distinct regions of PscF, none of which seemingly involved in inhibitory activity. Further epitope mapping would be necessary to gain deeper insight; however, in the absence of a PscF structure, this remains challenging. Isolating a greater number of mAbs from a selected donor with strong anti-PscF inhibitory activity would certainly increase the likelihood of identifying one with T3SS-inhibiting properties.

Of the four anti-PcrV mAbs isolated, two exhibited T3SS inhibitory capacity. Their mechanism of action could potentially involve (i) prevention of effector secretion by acting as a cap for PcrV; (ii) prevention of effector translocation towards the host cell by disruption of the PcrV-PopB/PopD interaction; or (iii) prevention of oligomerization of PcrV itself ([Gébus et al., 2009](#) [↗](#); [Sawa et al., 2019](#) [↗](#)). Here, we measured the ability of the inhibiting anti-PcrV mAbs we isolated to block PopB/PopD pore formation and toxin injection, and carried out a mechanistic and structural analysis of their activity, in parallel with other mAbs targeting PcrV.

We set out to investigate and compare the mechanism of action of several antiPcrV mAbs: P3D6 mAb and P5B3 (this work), 30-B8 ([Simonis et al., 2023](#) [↗](#)), as well as a previously reported humanized, bivalent PcrV-Psl mAb ([DiGiandomenico et al., 2014](#) [↗](#)). In order to do so, we produced mAb 30-B8 and subsequently purchased mAb MEDI3902 from MedChem. The P3D6, P5B3, 30-B8 and MEDI3902 mAbs were notably compared by employing two assays capable of detecting T3SS inhibition, each with a different readout: injection of the ExoS-Bla reporter into epithelial cells and cytotoxicity measurements in macrophages as a read-out for translocon assembly. In addition, we performed structural analyses on three of the mAb-PcrV complexes, from the viewpoints of recognition of both monomeric and pentameric forms of PcrV.

Both MEDI3902 and 30-B8 mAbs potently inhibited the injection of ExoS-Bla into target cells, with IC₅₀ values of 117 ng/mL and 21.3 ng/mL, respectively (**Figure 6A** [↗](#)). Monoclonal Abs isolated in this study also inhibited toxin injection, although significantly less potently, with IC₅₀ values of

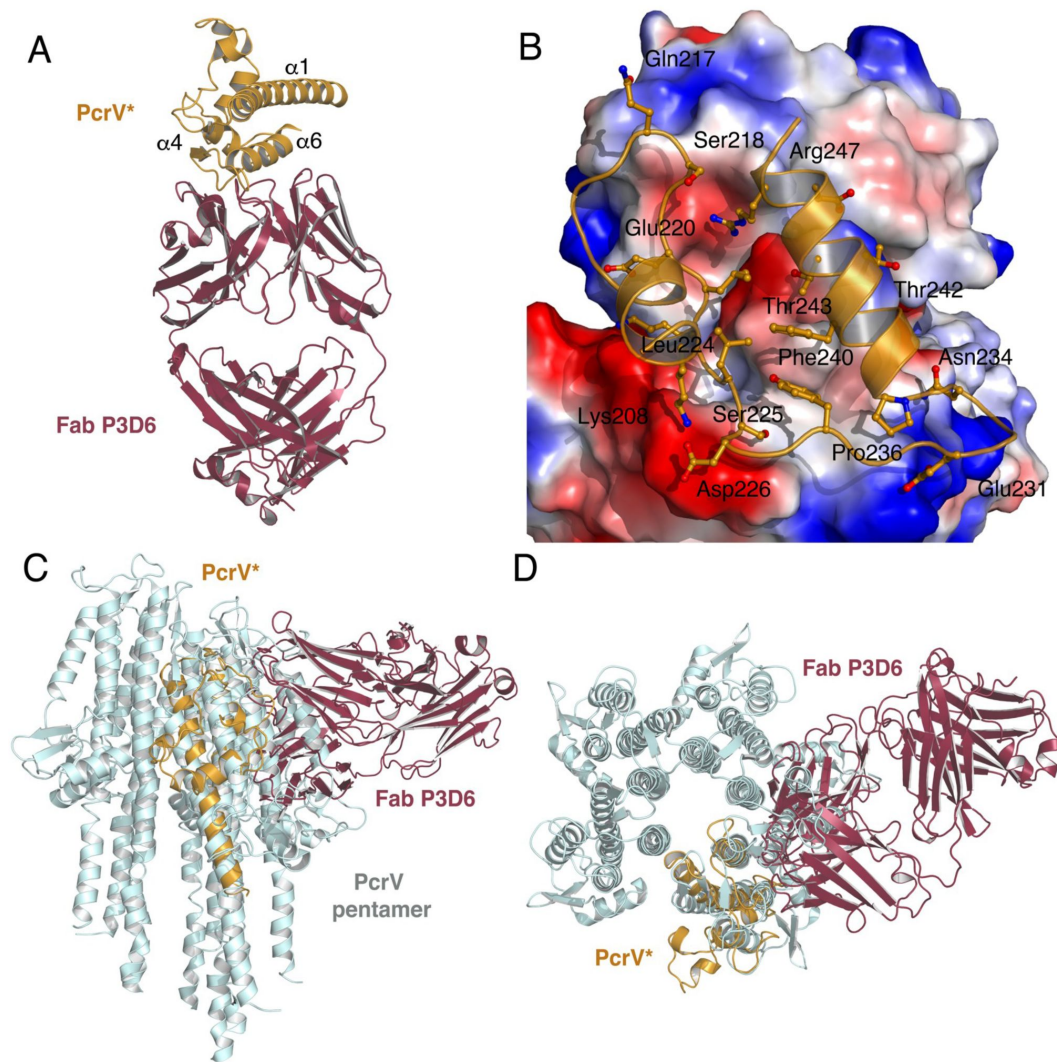


Figure 5.

Structure of Fab P3D6 in complex with PcrV*.

(A) Crystal structure of Fab P3D6 in complex with PcrV*. Fab P3D6 is shown in brown, while PcrV* is in orange. Contacts are made between PcrV* and an interaction platform formed by both HC and LC of P3D6. (B) Closeup of the interaction between PcrV* and P3D6, with the latter being shown as an electrostatic surface where acidic regions are shown in red, and basic in blue. Side (C) and top (D) views of the modeled PcrV pentamer, in light blue) onto which the structure shown in (A) was overlaid.

3.65 $\mu\text{g/mL}$ for P3D6, and around 100 $\mu\text{g/mL}$ for P5B3. Monoclonal Ab MEDI3902 had previously been shown to bind to different PcrV variants (Tabor et al., 2018) and here we confirmed that mAb 30-B8 was similarly efficient at inhibiting toxin injection by strains carrying five different PcrV variants (Simonis et al., 2023) (Supplementary Figure 2). Monoclonal Ab P5B3 was also able to inhibit all variants, but only at high concentrations, while P3D6 was only active against variant V1. Therefore, P5B3 appears to recognize a highly conserved epitope whereas P3D6 seems to bind an overlapping epitope that includes the variable Ser225, as suggested by the ELISA mapping competition and structural data, but does so in a more effective manner.

In the macrophage cytotoxicity assay, translocon assembly was inhibited by mAbs 30-B8 (IC_{50} of 45.2 ng/mL) and P3D6 (IC_{50} of 11.8 $\mu\text{g/mL}$), while P5B3 and MEDI3902 did not exhibit significant dose-response inhibition. Together, these results suggest that the T3SS-inhibiting activity of these anti-PcrV Abs may occur through distinct mechanisms.

In order to understand the differences in potency and potentially in mechanisms of action at a structural level, we compared the Fab-PcrV interaction regions for the P3D6, 30-B8 and MEDI3902 Fabs. Monomeric PcrV is an elongated, dumbbell-shaped molecule, and the chimeric form used in this study displays the same characteristics (Figure 5A). Fab MEDI3902 binds to one of the extremities of monomeric PcrV, extending in the longitudinal axis of the molecule (Figure 6B, top). This mode of binding is distinct from that of 30-B8 and P3D6 Fabs, both of which recognize the C-terminal region of PcrV (Figure 6B, middle) (Simonis et al., 2023). Therefore, the binding region of 30-B8 and P3D6 does not, by itself, appear to explain the significant difference in potency between the two mAbs. Moreover, a difference in affinity does not account for the difference in potency either, as both mAbs bind to recombinant monomeric PcrV with comparable apparent affinities of around 30 ng/mL (our data and Simonis et al., 2023).

In order to perform this comparative analysis in the context of a PcrV oligomer, we employed the model of the PcrV pentamer generated as described above, which was based on the cryo-EM structure of SipD from *S. typhimurium* (Guo and Galán, 2021). According to this analysis, the only Fab that is able to bind to all PcrV protomers in the pentamer without generating clashes with either PcrV or other Fabs is 30-B8 (Figure 6C). In the case of MEDI3902, the Fab can bind the oligomer, but only with a 1:5 stoichiometry, possibility due to clashes between Fabs. Finally, P3D6 is unable to bind to a pre-formed pentamer, and can only recognize a PcrV protomer in its monomeric form.

This structural analysis suggests different potential mechanisms of action. For P3D6, the inability to bind to the pentamer points towards a mechanism involving inhibition of oligomerization. Indeed, the analysis shows that binding of P3D6 to a single PcrV monomer prevents the association of additional PcrV protomers through Fab-protomer direct clashes. A similar mechanism of action may be suggested for MEDI3902, as Fab-bound PcrV protomers cannot oligomerize, in this case due to clashes between the Fabs themselves (Fig. 6C). However, the fact that MEDI3902 does not appear to prevent translocon insertion suggests that such a mechanism is unlikely for this mAb as PcrV oligomerisation is required at the tip of the needle for translocon assembly. In contrast to P3D6, MEDI3902 can bind the oligomerized PcrV pentamer (with a stoichiometry of one) and its mechanism of action may thus be related to this ability. Our results suggest that when MEDI3902 is bound to the PcrV oligomer, the formation of the pore is not efficiently blocked while toxin injection is strongly inhibited (Fig. 6C). Therefore, the presence of one MEDI3902 Ab molecule at the tip of the needle does not appear to efficiently prevent either the secretion of the translocator proteins PopB/PopD through the needle, nor the interactions between PcrV and PopB/PopD, which have been described as required for pore formation (Kundracik et al., 2022). However, the MEDI3902 Ab molecule seems to interfere with further interactions between PcrV and the PopB/PopB complex, or with the sensing by PcrV of the host cell, both of which are needed for toxin injection (Lee et al., 2010).

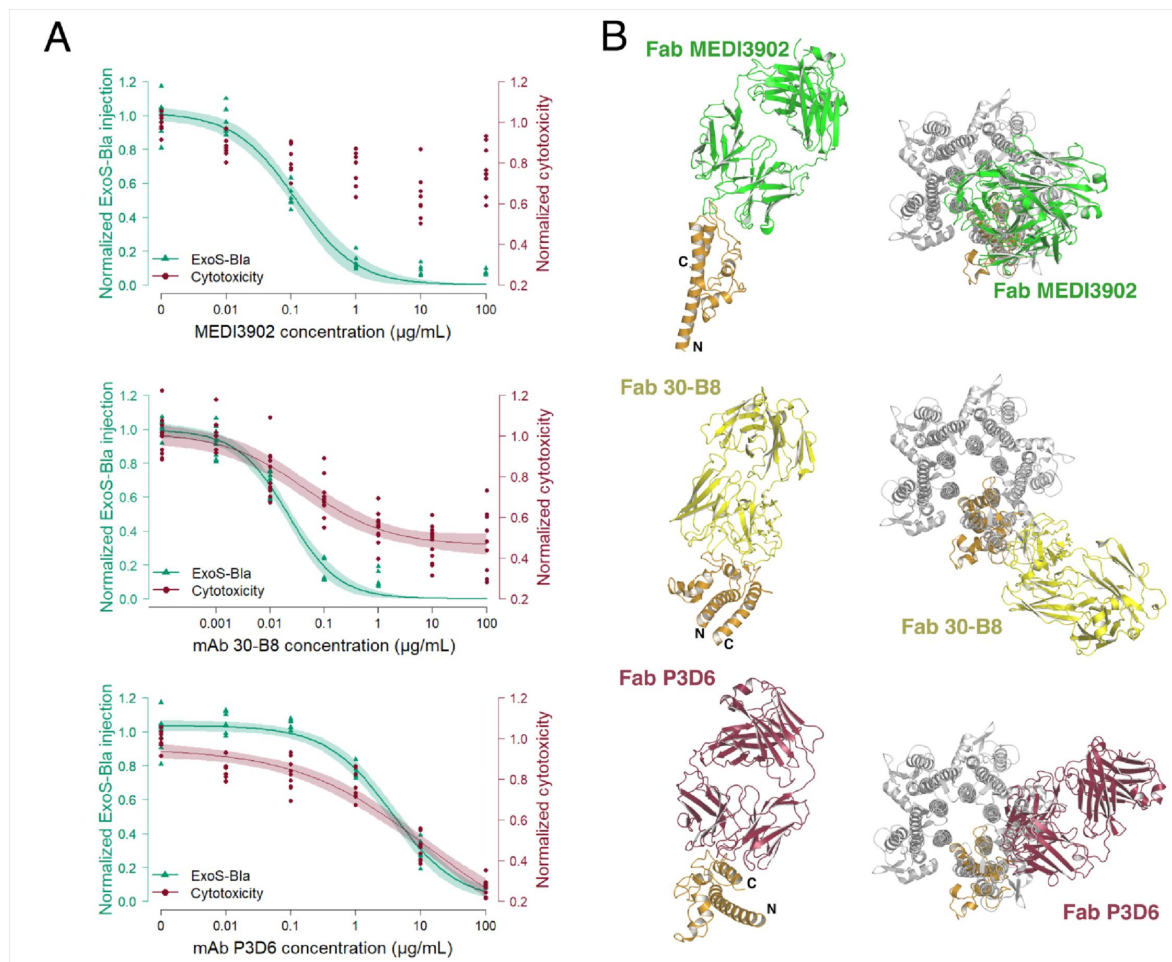


Figure 6.

Functional and structural comparisons between anti-PcrV mAbs.

(A) Dose-dependent inhibition of T3SS in two functional assays reflecting toxin injection (ExoS-Bla injection) and translocon assembly (J774 macrophage cytotoxicity) for three mAbs. The circles, the dark lines and the light-colored areas represent the experimental values, the log-logistic modeled dose-response curves and the 95% confidence intervals, respectively. The Ab concentration is presented in logarithmic scale. No red curve nor red area is displayed for MEDI3902 because no dose-response could be modeled. (B) Structures of PcrV-Fab complexes in the context of a PcrV monomer, as well as of a pentamer modeled based on the cryo-EM structure of SipD (Guo and Galán, 2021). The N- and C-termini of PcrV are indicated in all structures, which are referenced in the main text.

Lastly, 30-B8, which can bind the formed pentamer with a stoichiometry of five, appears to be the most effective at blocking both pore formation and toxin injection. The fact that the PcrV-bound 30-B8 Ab probably projects towards the cell membrane, associated with its ability to bind to the PcrV pentamer at full occupancy, may result in remarkable efficacy in blocking the interactions between PcrV and PopB/PopD.

In conclusion, here we show that patients with chronic infection with *P. aeruginosa* can elicit anti-PscF and anti-PcrV mAbs that recognize different regions within these proteins. Anti-PcrV Abs can act as T3SS inhibitors through different mechanisms, with some exhibiting significantly greater efficacy than others. The strategy employed here, involving the analysis of structural and functional data on antiT3SS mAbs should open new avenues towards deciphering the mechanism of T3SS toxin translocation and enable the isolation of more effective mAbs targeting a broad range of clinical strains.

Materials and methods

Clinical sample collection

The study was approved by the French ethics committee (ID-RCB 2020A00311-38) and was carried out according to the Declaration of Helsinki, Good Clinical Practice (GCP) guidelines and current French regulations. Written consent for participation was not required for this study. The first phase was a non-interventional study involving data and samples from human participants conducted according to Reference Methodology No. 004 issued by French authorities (Commission Nationale de l'Informatique et des Libertés). Screening and functional assays were performed on human sera previously collected at Grenoble Alpes University Hospital (France), from CF patients chronically infected with *P. aeruginosa*. Participants were all informed and did not object to this phase of the study. Inclusion criteria for the second phase of the study were: patients with positive screening during phase one, ≥ 18 years old, ≥ 32 Kg, with a programmed blood sampling at Grenoble Alpes University Hospital and not being opposed to the second phase of the project. Whole blood was then collected using BD Vacutainer® EDTA tubes (Becton Dickinson) and PBMCs were purified by density gradient centrifugation using Lymphoprep (Eurobio scientific) following manufacturing guidelines. Cells were then stored in liquid nitrogen until further use.

Bacterial strains, genetic manipulations and growth conditions

P. aeruginosa strains used in this study are listed in Supplementary Table 5. Strains were cultured in LB media at 37 °C. For infection experiments, bacteria were grown until the measured optical density at 600 nm (OD_{600nm}) of 1. Genes encoding the most common variants of PcrV were cloned into the *Pseudomonas* replicative vector derived from pUCP21 (West et al., 1994 [\[1\]](#)) containing the *PprcG* promoter that drives *pcrGVHpopDB* operon expression (West et al., 1994 [\[1\]](#)). The plasmids, kindly provided by Simona Barzu (Sanofi Pasteur, Lyon), were transformed into the *P. aeruginosa* strain CHA lacking *pcrV* (Goure et al., 2004 [\[2\]](#)).

Expression and purification of full length PcrV and PcrV*

Expression of full-length PcrV from strain PAO1, cloned into a pET15b vector, was performed in *E. coli* BL21(DE3) as previously described, with small modifications (Nanao et al., 2003 [\[3\]](#)). Expression was induced with 1mM of Isopropyl β -D-1thiogalactopyranoside (IPTG) at $OD_{600} = 0.8$ AU and cells were then grown overnight at 20°C with shaking at 250 rpm. Cells were harvested by centrifugation and lysed by passing through a French Press 3 times at 25 Kpsi in lysis Buffer (50 mM Tris pH 8, 200 mM NaCl, 20 mM Imidazole) supplemented with a protein inhibitor cocktail tablet (Roche). The supernatant was cleared by centrifugation at 18,000 rpm and subsequently loaded onto Ni-IDA resin (Macherey-Nagel). The resin was washed with lysis buffer and the sample was eluted with lysis buffer supplemented with 100 mM imidazole. Fractions containing the

sample were pooled and applied to a size exclusion chromatography column (Superdex 200 HiLoad 16/600) pre-equilibrated in SEC buffer (20 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA). A chimeric form of PcrV (PcrV*) consisting of amino acids 1-17 fused to 136-249 whose design was inspired by the construct described in Tabor et al. (2018) [\[18\]](#), was employed for crystallization purposes. The purification protocol was the same as above, the only difference being that 250 mM imidazole was employed to elute the sample from the Ni resin.

Expression and purification of PscF

Expression of PscF from strain PAO1 was performed in *E. coli* BL21(DE3) grown in Terrific Broth. Expression was induced with 1 mM IPTG at OD₆₀₀ = 0.6 AU and cells were then grown for an additional 3 hours at 37 °C with shaking at 250 rpm. Cells were harvested by centrifugation and lysed by passing through a French Press 3 times at 25 kpsi in lysis buffer (50 mM Tris pH 8, 200 mM NaCl, 20 mM Imidazole, 2 % glycerol) supplemented with a protein inhibitor cocktail tablet (Roche). The supernatant was cleared by centrifugation at 18,000 rpm and applied to a Ni-IDA resin (Macherey-Nagel). The resin was washed with lysis buffer and the protein was eluted in the same buffer supplemented with 250 mM imidazole. Fractions were then buffer exchanged in an Amicon Ultra 10 kDa cutoff concentrator against a buffer exempt of imidazole (50 mM Tris pH 8, 200 mM NaCl, 2 % glycerol).

ELISA assays

For direct ELISAs, 96-well ELISA plates (Fisher # 11530627 Nunc Maxisorp) were coated overnight at 4° C with the respective antigen diluted to 1µg/ml in PBS. Plates were then washed with PBS-Tween 0.01%, and blocked for 1 h at room temperature (RT) with 3% BSA in PBS. Next, sera or mAbs serially diluted in PBS-BSA 1 % were added and incubated for 1h at RT. Antibody binding was detected using alkaline phosphatase-coupled goat anti-human IgG (Jackson Immuno #109 056 098) and a para-nitrophenylphosphate substrate (Interchim #UP 664791). The enzymatic reaction was read at 405 nm using a TECAN Spark 10M plate reader. Polyclonal Abs raised in rabbits against PscF and PcrV (Goure et al., 2004 [\[19\]](#)) were used as positive controls.

For competition ELISAs, serial dilutions of competitor mAbs were transferred into antigen-coated wells. Following a 30-minute incubation, biotinylated mAbs were added to the wells at their EC₇₀ concentration (effective concentration for 70% binding). Binding of biotinylated mAbs was detected using alkaline phosphatase-conjugated streptavidin.

Sorting of specific memory B cells

Briefly, PBMCs were stained for 30 min at 4°C in the dark, using FACS-Buffer (PBS-1X 0.5 % BSA, 2mM EDTA) with Live Dead staining (Thermo L34957), Antihuman CD3-Vio-Blue (Miltenyi 130-114-519), Anti-human CD20 Pe-Vio707 (Miltenyi 130-111-345), Anti-human CD19 Pe-Vio707 (Miltenyi 130-113-649), Anti-human IgM PE (Miltenyi 130-093-075), Anti-human IgA PE (Miltenyi 130-113-476), Anti-human IgD PE (Miltenyi 130-110-643), Anti-human CD27 APC (Miltenyi 130-108-336), in the presence of recombinant biotinylated His-PcrV-Avitag coupled with streptavidin BUV737 (BD 612775) or streptavidin Vio-515 (Miltenyi 103-107-459), and recombinant biotinylated His-PscF-Avitag coupled with streptavidin BUV496 (BD 612961) or streptavidin BV605 (Biolegend 405229). After washing, the cells were resuspended in FACS-Buffer and PscF or PcrV positive B cells were sorted and clonally seeded in 96 plates containing lysis buffer using BD FACSAria Fusion cytometer (BD Biosciences).

Isolation and production of mAbs

Sequences coding for variable regions of both heavy and light (κ and λ) chains were isolated by reverse transcription on total mRNA followed by a multiplex nested PCR using a set of primers covering the diversity of V-region diversity. V-regions family was attributed after sequencing of amplicons and alignment in the IMGT database (<https://imgt.org> [\[20\]](#)). An additional round of PCR

using primers specific to the identified family was performed followed by the cloning of V-regions genes into corresponding vectors containing IgG1H, IgGκ and IgGλ constant regions. Regarding 30-B8, the sequences coding for the variable regions of heavy and light chains were synthesized by Eurofins according to the sequence published by Simonis and coworkers (Simonis et al., 2023 [\[1\]](#)). Cloning and production were performed as described above.

Monoclonal Abs were produced by transient transfection in HEK293F cells (ThermoFisher scientific) and purified by affinity chromatography using a Protein A Sepharose column (Sigma #ge17-1279-03). Elution was performed with 4.5 ml of glycine 0.1 M (pH 2.5), followed by neutralization with 500 µl of 1 M Tris (pH 9). Purified mAbs were then subjected to buffer exchange and concentration using AmiconUltra centrifugal filters (Merck #36100101).

P3D6 and P5B3 Fab production

Sequences coding for Fab fragments were obtained by inserting stop codons on genes corresponding to heavy chains of the mAbs by PCR using site directed mutagenesis (Quickchange II, Agilent) according to manufacturer's instructions. Mutated heavy and corresponding light chain genes were cloned into appropriate expression plasmids for eukaryotic cell expression and were co-transfected at a 2:1 ration into FreeStyle 293-F cells (ThermoFisher). Fabs were purified using KappaSelect affinity chromatography (Cytiva).

Cellular tests for T3SS activity

ExoS-Bla translocation

The T3SS-dependent toxin injection into epithelial A549 cells was measured using the reporter system based on Bla/CCF2 enzyme/substrate combination (Charpentier and Oswald, 2004 [\[2\]](#)) previously described for *P. aeruginosa* (Verove et al., 2012 [\[3\]](#)). *P. aeruginosa* strain CHAΔexoS carrying ExoS-Bla fusion on the chromosome was used to infect A549 cells at the multiplicity of infection (MOI) of 5. The level of injected ExoS-β-lactamase was measured using CCF2 substrate, as described previously (Verove et al., 2012 [\[3\]](#)). Inhibition of ExoS-Bla translocation was evaluated in the presence of serial dilutions of Protein-A purified serum IgGs and mAbs. Serum IgG purification was performed as described above for mAb purification. All values were normalized using noninfected cells and cells infected in the absence of Abs as references.

Pore formation / propidium iodide incorporation into macrophages

To assess the formation of a T3SS translocation pore, macrophages were infected with a *P. aeruginosa* strain PAO1A3Tox devoid of three exotoxins (Cisz et al., 2008 [\[4\]](#)). Two days before the experiment, J774 cells were seeded in a 96-well plate (Greiner, 655090) at a density of 100 000 cells per well in DMEM supplemented with 10% FCS. The day before the experiment, the strain was grown overnight in LB medium. The next day, bacteria were sub-cultured in fresh LB media until an OD_{600nm} of 1 and the macrophages were washed twice with PBS before addition of 65 µL of DMEM 10% FCS containing 2 µg/mL of propidium iodide. Antibodies diluted in DMEM, 10% FCS (25 µL) were then added followed by 10 µL of bacteria diluted in DMEM 10% FCS to give a MOI of 5. Propidium iodide fluorescence was recorded in a Fluoroskan fluorimeter every 10 minutes. The data of each fluorescence kinetics of the triplicates were processed in R Studio to calculate the Area Under the Curve, as described before (Ngo et al., 2019 [\[5\]](#)). This metrics was then normalized using noninfected cells and cells infected in the absence of Abs as references.

Data processing and analysis

Data from independent cell experiments were pooled and analyzed with R version 4.3.2 (R Core Team, 2023) by one-way ANOVA followed by paired t-test or Kuskal-Wallis followed by the Dunn test with Benjamini-Hochberg p-value adjustment. Dose-response fitting was performed using the

drc package (Ritz & Streb, 2016) based on a three-parameters log-logistic model and IC_{50} were compared using the function `comParm()`.

Bio-layer interferometry

BLI experiments were performed on an OctetRED96e from Satorius/FortéBio (former Pall/FortéBio) and were recorded with software provided by the manufacturer (Data Acquisition v11.1). All protein samples were diluted in analysis buffer (1X PBS pH 7.4, 0.02% Tween-20). 10 mM glycine pH 2.0 was used as regeneration buffer. Commercial SA or SAX (streptavidin) biosensors (Pall/FortéBio) were used to capture biotinylated PcrV. Kinetic analyses were performed in black 96-well plates (Nunc F96 MicroWell, Thermo Fisher Scientific) at 25°C with agitation at 1000 rpm. After incubation and equilibration of biosensors in analysis buffer, PcrV samples were applied at a concentration of 2.5 mg/mL by dipping biosensors until reaching a spectrum shift between 1.2 and 2 nm, followed by an additional equilibration step in analysis buffer. For association measurements, all analyte samples were diluted in analysis buffer at concentrations either between 3.12 and 200 nM for IgGs or between 50 and 3200 nM for Fab fragments. Association phases were monitored while dipping the functionalized biosensors in analyte solutions for 5 min after recording a baseline for 2 min, and the dissociation phases monitored in analysis buffer for 10 min. To assess and monitor unspecific binding of analytes, measurements were performed with biosensors treated with the same protocols but replacing ligand solutions with analysis buffer. All measurements were performed in duplicate using sample preparations. Kinetic data were processed with software provided by the manufacturer (Data analysis HT v11.1). Signals from zero-concentration samples were subtracted from the signals obtained for each functionalized biosensor and each analyte concentration. Resulting specific kinetics signals were then fitted using a global fit method and 1:2 bivalent analyte model for full Abs/IgG and 1:1 Langmuir model for Fab. Reported kinetics parameter values were obtained by averaging the values obtained with duplicated assays and reported errors as the standard deviation.

Crystallization of the PcrV*-Fab P3D6 complex

PcrV* and Fab P3D6 were mixed in a 1:2 ratio for 1 hr at room temperature prior to being subjected to size exclusion chromatography using a Superdex 200 10/300GL increase column in SEC buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA). Peaks harboring PcrV*:Fab complexes in SDS-PAGE were pooled, concentrated and used for crystallization trials using the ISBG HTX crystallization platform in Grenoble. Initial crystallization conditions (25 % PEG 1000, 1mM $ZnCl_2$, 100 mM sodium acetate pH 5.5) were optimized manually, and diffracting crystals were obtained using microseeding. All crystals were grown using the hanging drop vapor diffusion method at 20 °C. Single crystals were mounted in cryo-loops and flash cooled in liquid nitrogen. X-ray diffraction data were collected under a nitrogen stream at 100° K at the European Synchrotron Radiation Facility (ESRF, Grenoble, France).

Structure determination and refinement

The best diffraction data were collected to 2.56 Å on beamline ID30A-1 (ESRF) (Bowler, 2015). The diffracting crystal was in space group $P2_1$ and displayed one 1:1 PcrV:Fab complex per asymmetric unit. Statistics on data collection and refinement are summarized in supplementary table 5. X-ray diffraction images were indexed and scaled with XDS (Kabsch 2010). ADXV (Arvai, 2020) and XDSGUI (Brehm et al., 2023) were used to perform data quality and resolution cutoff check-ups (Karplus and Diederichs, 2015). The maximum possible resolution was determined using the STARANISO server (Tickle, 2007). The reduced X-ray diffraction data was imported into the CCP4 program suite (Agirre et al., 2023). The PcrV*-Fab P3D6 structure was solved by molecular replacement using PHASER (McCoy et al., 2007) and an AlphaFold2 ColabFold-generated model (Mirdita et al., 2022). The PcrV* and Fab model chains were placed sequentially. The structure was completed by cycles of manual model building with COOT (Emsley and Cowtan, 2004, p. 200). Water molecules were added to the residual electron density map as implemented in COOT.

Crystallographic macromolecular refinement was performed with REFMAC (Murshudov et al., 2011 [↗](#)). Cycles of model building and refinement were performed until R_{work} and R_{free} converged. The TLS definition was determined and validated using the TLSMD (Painter and Merritt, 2006 [↗](#)) and PARVATI (Zucker et al., 2010 [↗](#)) servers. The stereochemical quality of the refined models was verified with MOLPROBITY (Chen et al., 2010 [↗](#)), PROCHECK (Laskowski et al., 1993 [↗](#)) and PDB-REDO (Joosten et al., 2014 [↗](#)). Secondary structure assignment was performed by DSSP (Kabsch and Sander, 1983 [↗](#)) and STRIDE (Heinig and Frishman, 2004 [↗](#)). Figures displaying protein structures were generated with PYMOL (<http://www.pymol.org> [↗](#)).

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files, with the exception of the final refined model coordinates and structure factors corresponding to the PcrV*-Fab P3D6 complex. Those were deposited in the Protein Data Bank (PDB, <https://www.rcsb.org> [↗](#)), ID code: 9FM0.

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Additional information

Author contributions

JMD, EF, CCM, FC, LSDD, AA, IB, VTC and FC performed experiments and analyzed the data; EF, JMD, VTC, CCM and LSDD prepared the figures; DF and YC provided the tools and materials; IA, AD and PP designed the study, analyzed the data, and wrote the manuscript. All authors participated in discussions and editing of the final version of the manuscript.

All data reported in this paper including antibody sequences will be shared by the lead contact upon request.

Supplementary data

Anti-PcrV mAbs						
Heavy chain	V Gene and allele	J Gene and allele	V region identity (%)	J region identity (%)	CDR3 length	Donor #
P3G9Hc	IGHV4-61*08	IGHJ6*02	92.44	91.94	18	16
P3D6Hc	IGHV3-23*01	IGHJ4*02	92.70	89.58	14	25
P5B3Hc	IGHV3-74*01	IGHJ3*02	96.88	94.00	11	25
P5E10Hc	IGHV3-21*01	IGHJ6*02	88.42	74.20	12	25
Light chain	V Gene and allele	J Gene and allele	V region identity (%)	J region identity (%)	CDR3 length	Donor #
P3G9kc	IGKV3-20*01	IGKJ3*01	94.68	100.00	10 9	16
P3D6kc	IGKV1-8*01	IGKJ4*01	96.41	97.37	11	25
P5B3kc	IGLV3-25*03	IGLJ2*01	96.10	100.00	9	25
P5E10kc	IGKV4-1*01	IGKJ4*01	95.00	100.00		25

Anti-PscF mAbs						
Heavy chain	V Gene and allele	J Gene and allele	V region identity (%)	J region identity (%)	CDR3 length	Donor #
P1B7Hc	IGHV3-23*01	IGHJ4*02	97.22	91.67	13	25
P1D5Hc	IGHV4-61*02	IGHJ6*02	83.85	80.65	19	25
P1B4Hc	IGHV3-23*01	IGHJ6*02	95.14	83.87	17	25
P1F5Hc	IGHV3-23*01	IGHJ6*02	77.86	83.87	19	25
P1D7Hc	IGHV3-23*01	IGHJ4*02	96.87	93.75	15	25
P3G2Hc	IGHV4-61*02	IGHJ4*02	91.40	93.75	16	25
P3G6Hc	IGHV3-23*01	IGHJ6*02	98.61	85.48	17	25
P3G7Hc	IGHV3-72*01	IGHJ3*01	86.39	88.00	18	25
P5D5Hc	IGHV3-23*01	IGHJ4*02	96.52	93.75	13	25
P5G10Hc	IGHV4-34*01	IGHJ6*02	91.22	77.42	16	25
Light chain	V Gene and allele	J Gene and allele	V region identity (%)	J region identity (%)	CDR3 length	Donor #
P1B7kc	IGKV1-16*02	IGKJ2*01	97.13	97.14	9	25
P1D5kc	IGKV2-28*01	IGKJ3*01	94.22	100.00	9	25
P1B4kc	IGKV3-20*01	IGKJ1*01	95.04	97.14	9	25
P1F5kc	IGKV2-28*01	IGKJ2*01	95.58	89.47	17	25
P1D7kc	IGLV3-21*02	IGLJ2*01	98.56	97.37	11	25
P3G2kc	IGLV2-8*01	IGLJ2*01	94.79	88.57	10 9	25
P3G6kc	IGKV3-20*01	IGKJ2*01	99.60	89.74	11 9	25
P3G7kc	IGLV1-51*01	IGLJ2*01	88.00	91.67	11	25
P5D5kc	IGKV1-16*01	IGKJ2*01	98.20	94.29		25
P5G10kc	IGLV1-47*01	IGLJ2*01	94.73	91.67		25

Supplementary Table 1.

Sequence conservation of V and J regions of selected mAbs compared to germline.

Percentage (%) of identity was obtained by aligning variable region sequences on IMGT database (<https://www.imgt.org>).

		Detection mAbs								IC50 ($\mu\text{g/mL}$)
		P1D8*	P1B7*	P5D5*	P3G6*	P1D6*	P1B4*	P1D5*	P5G10*	
Competitor mAbs	P1D8	0,01	ND	ND	ND	ND	ND	ND	ND	ND
	P1B7	ND	0,19	0,16	0,16	0,60	0,20	0,52	ND	9-20
	P5D5	ND	0,39	0,68	0,46	0,33	0,26	1,39	ND	4-8
	P3G6	ND	12,66	2,99	1,77	8,37	5,18	7,99	ND	<3
	P5G10	ND	ND	ND	ND	ND	ND	ND	0,46	
	P1D6	ND	15,02	0,89	1,17	1,62	1,83	5,30	ND	
	P1B4	ND	2,83	0,61	2,06	7,67	1,97	5,00	ND	
	P1D5	ND	14,21	0,86	0,70	3,85	0,23	0,69	ND	

		Detection mAbs				IC50 ($\mu\text{g/mL}$)
		P3G9*	P3D6*	P5B3*	P5E10*	
Competitor mAbs	P3G9	0,98	ND	ND	ND	ND
	P3D6	ND	0,8	0,28	ND	9-20
	P5B3	ND	2,42	0,41	ND	4-8
	P5E10	ND	ND	ND	0,51	<3

Supplementary Table 2.

Competition between A) anti-PscF mAbs and B) anti-PscF mAbs.

The indicated IC₅₀ values correspond to the concentration of competitor mAbs necessary to obtain half of the signal generated by the biotinylated mAbs without competitor. ND corresponds to a non-detectable competition.

IgG/His-PcrV-avitag	K_D (10^{-12} M)	K_{on} (10^2 /Ms)	k_{on2} (1/Ms)	K_{off} (10^{-7} /s)	k_{off2} (10^{-3} /s)	R^2
P5B3	23.9 ± n.d	362 ± 38.2	1.2 ± 0.2	9.3 ± n.d	321.15 ± 49.7	0.9998
P3D6	736.8 ± 19	618 ± 1.5	0.4 ± 0.2	463.5 ± 12,7	12.3 ± 5.9	0.9997

Supplementary Table 3.

Affinities of anti-PcrV mAbs for PcrV.

The reported values correspond to the average of the measurements obtained from two independent experiments (n=2). Standard Deviations were calculated by the BLI analysis software.

DATA COLLECTION

X-ray source (Beamline)	ESRF (ID30A-1)
Detector	Pilatus 2M
Wavelength (Å)	0.965459
Scan-range (°)	180
Oscillation (°)	0.2
Space group	P2 ₁
<i>a</i> (Å)	87.58
<i>b</i> (Å)	87.58
<i>c</i> (Å)	105.22
<i>a</i> (°)	90.00
<i>b</i> (°)	104.83
<i>g</i> (°)	90.00
Mosaicity (°)	0.129
Overall resolution (Å)	49.21-2.56
No. observed/unique reflections	176390/52157
High resolution shell (Å)	2.71-2.56
Completeness (%) (last shell)	99.5(99.2)
<i>R</i> _{sym} (%) (last shell)	21.7 (152.9)
<i>I</i> / <i>σ</i> (<i>I</i>) (last shell)	5.02 (0.72)
<i>CC</i> _{1/2}	97.7 (31.2)
Wilson plot B-factor (Å ²)	45.86

MOLECULAR REPLACEMENT

Phaser LLG	1032.93
<i>R</i> _{work} / <i>R</i> _{free} (%)	35.68/40.33

REFINEMENT

Initial <i>R</i> _{work} / <i>R</i> _{free} (%)	35.68/40.33
Final <i>R</i> _{work} / <i>R</i> _{free} (%)	19.69/23.64
Final+TLS <i>R</i> _{work} / <i>R</i> _{free} (%)	20.97/23.70

STEREOCHEMICAL QUALITY

RMS deviation, bond lengths (Å)	0.006
RMS deviation, bond angles (°)	1.266
Mean B-factor (Å ²)	46.74
No. of atoms	9052
No. of protein atoms	8614
No. of water molecules	392
Residues in most favored/allowed region of Ramachandran plot (%)	99.80

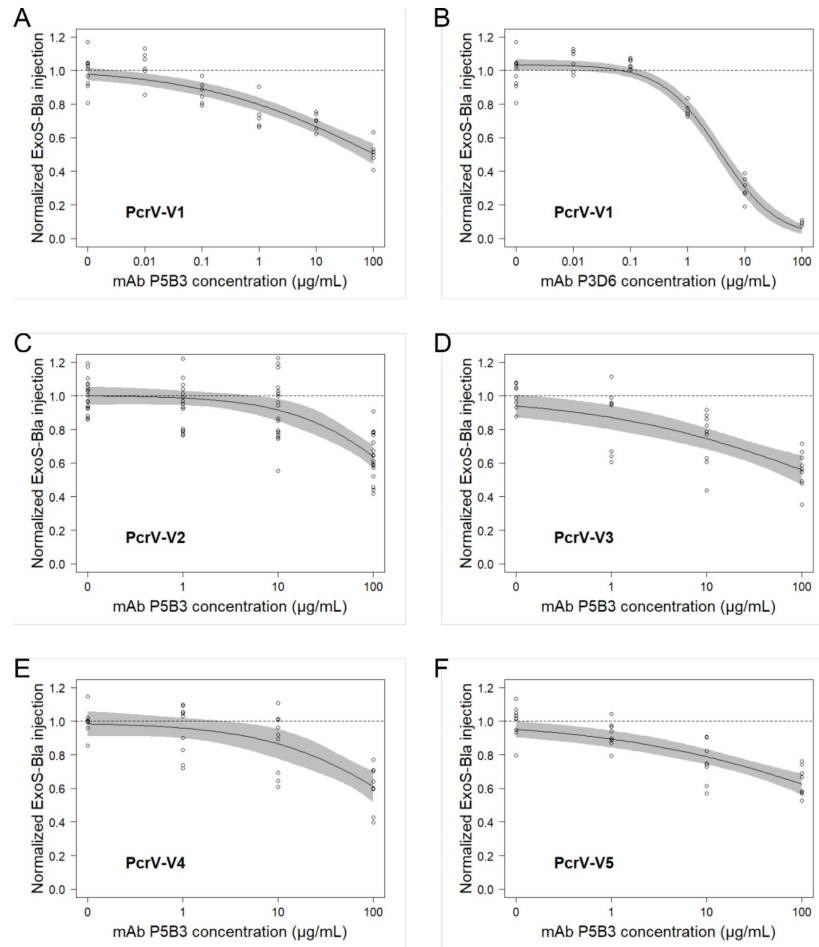
Supplementary Table 4.**Data collection, phasing and structure refinement statistics**

<i>P. aeruginosa</i> strains	Characteristics	Reference or source
CHAD _{ExoS::exoS-bla}	b-lactamase (bla) reporter for ExoS translocation	Verove et al., 2012
CHAD _{pcrV}	Strain lacking <i>pcrV</i>	Gouré et al., 2004
PAO1D3Tox	Strain lacking toxins ExoS, ExoT and ExoY	Cisz et al., 2008
Plasmids	Characteristics	Reference or source
pIApG- <i>pcrV</i> -V1 (PAO1)	Replicative plasmid for PcrV expression, *Leu ⁶ Ala ⁹ Ser ²¹ Ser ²²⁵	Lab collection; Simona Barzu, Lyon
pIApG- <i>pcrV</i> -V2 (CHA)	Leu ⁶ Ala ⁹ Ser ²¹ Arg ²²⁵	Lab collection; Simona Barzu, Lyon
pIApG- <i>pcrV</i> -V3 (PA14)	Phe ⁶ Ala ⁹ Pro ²¹ Lys ²²⁵	Lab collection; Simona Barzu, Lyon
pIApG- <i>pcrV</i> -V4 (PA103)	Phe ⁶ Gly ⁹ Pro ²¹ Arg ²²⁵	Lab collection; Simona Barzu, Lyon
pIApG- <i>pcrV</i> -V5	Phe ⁶ Gly ⁹ Pro ²¹ Lys ²²⁵	Lab collection; Simona Barzu, Lyon
pET15b-His-PcrV	Replicative plasmid for inducible His-PcrV expression	Nanao et al., 2003
pET22b-PscF-His	Replicative plasmid for inducible PscF-His expression	Quinaud et al, 2005
pESPRIT-His-PcrV-avitag	Replicative plasmid for inducible His-PcrV-avitag expression	This study
pESPRIT-His-PscF-avitag	Replicative plasmid for inducible expression His-PscF-avitag	This study

*variable residues within PcrV

Supplementary Table 5.

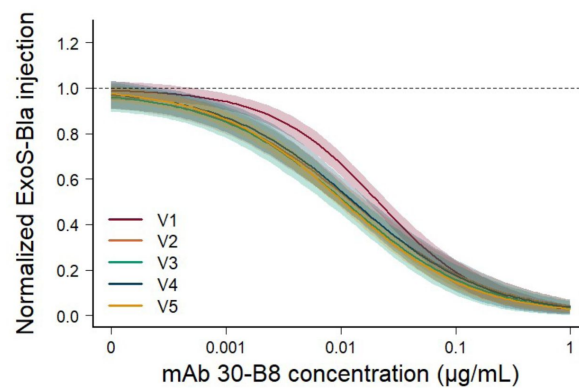
Bacterial strains and plasmids



Supplementary Figure 1.

Dose-dependent inhibition by mAbs P5B3 and P3D6 of ExoS-Bla injection from strains expressing five PcrV variants.

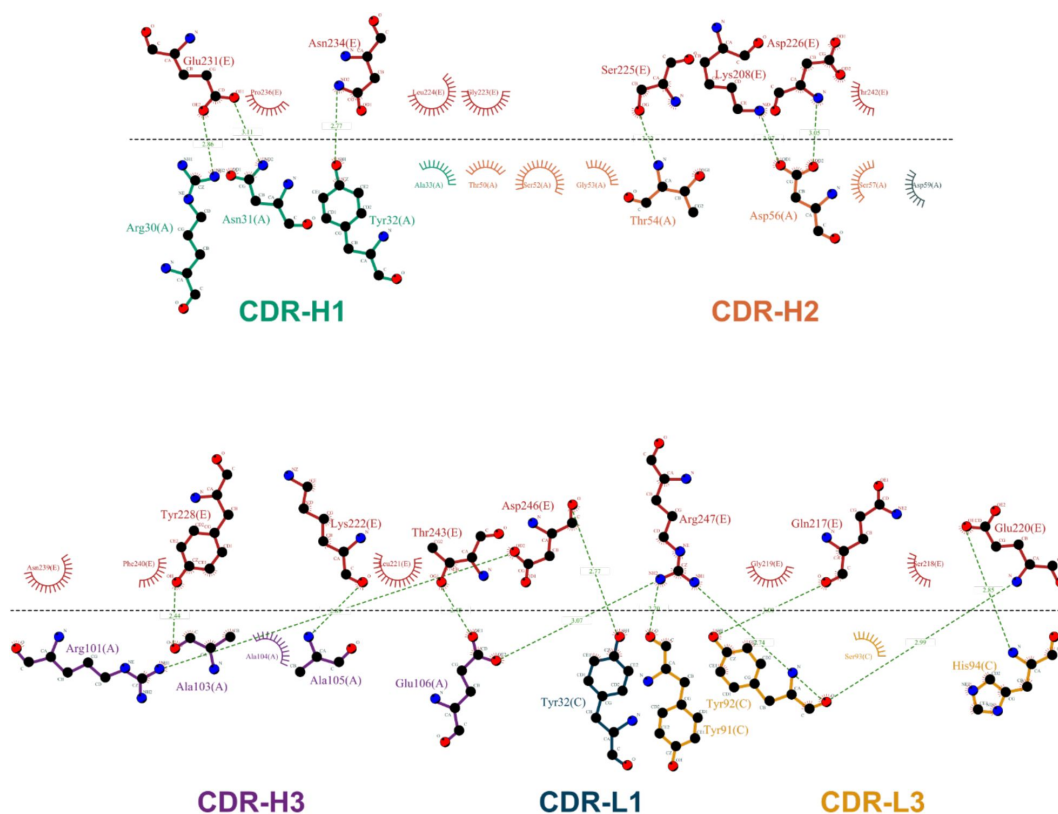
Inhibition of ExoS-Bla activity following infection of A495 epithelial cells with *P. aeruginosa* expressing the V1 (A and B), V2 (C), V3 (D), V4 (E) or V5 (F) variants, with mAbs concentration ranging from 0.01 to 100 µg/mL. The circles, the black line and the grey area represent the experimental values, the log-logistic modeled dose-response curve and the 95% confidence interval, respectively. The dashed lines represent the mean of normalized ExoS-Bla injection in the absence of antibody. The Ab concentration is presented in logarithmic scale. Monoclonal Ab P5B3 displays dose-response inhibition with the strains expressing the V1, V2, V3, V4 and V5 variants with respective IC₅₀s of 96, 206, 192, 212 and 426 µg/mL (no statistically significant difference). P3D6 (B) displays a dose-response inhibition with the strain expressing the V1 variant, with an IC₅₀ of 3.7 µg/mL, significantly different from the one of P5B3 against the same variant (p-value = 0.015).



Supplementary Figure 2.

Dose-dependent inhibition by mAbs 30-B8 of ExoS-Bla injection from strains expressing five PcrV variants.

Inhibition of ExoS-Bla activity following infection of A495 epithelial cells with *P. aeruginosa* expressing the V1, V2, V3, V4 or V5 variants, with mAbs concentration ranging from 0.001 to 1 μg/mL. Monoclonal Ab 30-B8 displays dose-response inhibition with V1, V2, V3, V4 and V5 variants with respective IC₅₀ of 21.3, 12.8, 11.2, 13.0, 10.5 ng/mL. The dark lines and the light-colored areas represent the log-logistic modeled dose-response curve and the 95% confidence interval, respectively. The dashed lines represent the mean of normalized ExoS-Bla injection in the absence of antibody. The Ab concentration is presented in logarithmic scale.



Supplementary Figure 3.

Interactions between PcrV* and Fab P3D6.

Chain codes correspond to: Fab HC (chain A), LC (chain C) and PcrV* (chain E). CDR H1, H2 and H3 of the heavy chain, as well as CDR L1 and L3 of the light chain, contribute to PcrV* binding. Interaction diagram generated with LigPlot+ v.2.9.9 (Lakowski & Swindells 2011, <https://www.ebi.ac.uk/thornton-srv/software/LigPlus/>). Hydrogen bonds are represented by green dashed lines and residues involved in hydrophobic contacts are indicated by spoked arcs.

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Reviewer #1 (Public review):

Summary:

Desveaux et al. describe human mAbs targeting protein from the *Pseudomonas aeruginosa* T3SS, discovered by employing single cell B cell sorting from cystic fibrosis patients. The mAbs were directed at the proteins PscF and PcrV. They particularly focused on two mAbs binding the T3SS with the potential of blocking activity. The supplemented biochemical analysis was crystal structures of P3D6 Fab complex. They also compared the blocking activity with mAbs that were described in previous studies, using an assay that evaluated the toxin injection. They conducted mechanistic structure analysis and found that these mAbs might act through different mechanisms by preventing PcrV oligomerization and disrupting PcrVs scaffolding function.

The antibiotic resistance crisis requires the development of new solutions to treat infections cause by MDR bacteria. The development of antibacterial mAbs holds great potential. In that context, this report is important as it paves the way for the development of additional mAbs targeting various pathogens that harbor the T3SS. In this report the authors present a comparative study of their discovered mAbs vs. a commercial mAb currently in clinical testing resulting in valuate data with applicative implications. The authors investigated the mechanism of action of the mAbs using advanced methods and assays for characterization of antibody and antigen interaction, underlining the effort to determine the discovered mAbs suitability for downstream application.

<https://doi.org/10.7554/eLife.105195.2.sa2>

Reviewer #2 (Public review):

Summary:

Desveaux et al. performed Elisa and translocation assays to identify among 34 cystic fibrosis patients which ones produced antibodies against *P. aeruginosa* type three secretion system (T3SS). Authors were especially interested in antibodies against PcrV and PcsF, two key components of the T3SS. The authors leveraged their binding assays and flow cytometry to isolate individual B cells from the two most promising sera, and then obtained monoclonal antibodies for the proteins of interest. Among the tested monoclonal antibodies, P3D6 and P5B3 emerged as the best candidates due to their inhibitory effect on the ExoS-Bla translocation marker (with 24% and 94% inhibition, respectively). The authors then showed that P5B3 binds to the five most common variants of PcrV, while P3D6 seems to recognize only one variant. Furthermore, the authors showed that P3D6 inhibits translocon formation, measured as cell death of J774 macrophages. To get insights into the P3D6-PcrV interaction, the authors defined the crystal structure of the P3D6-PcrV complex. Finally, the authors compared their new antibodies with two previous ones (i.e., MEDI3902 and 30-B8).

Strengths:

- Article is well written.
- Authors used complementary assays to evaluate protective effect of candidate monoclonal antibodies.
- Authors offered crystal structure with insights into the P3D6 antibody-T3SS interaction (e.g., interactions with monomer vs pentamers).
- Authors put their results in context by comparing their antibodies with respect to previous ones.

Weaknesses:

- Results shown in Fig. 6 should be initially described in the Results section and not in the Discussion section.
- The authors should describe, in the Discussion (and also in L146-147), in more detail the gained insights into how anti-PcrV antibodies work. This is especially important given previous reports of more potent antibodies (e.g., Simonis et al.) that significantly reduces the novelty of their work. Hence, authors could explicitly highlight how their study differentiate from previous work, and what unique insights were gained (in the current version is not completely obvious).

<https://doi.org/10.7554/eLife.105195.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public review):*Summary:*

Desveaux et al. describe human mAbs targeting protein from the Pseudomonas aeruginosa T3SS, discovered by employing single cell B cell sorting from cystic fibrosis patients. The mAbs were directed at the proteins PscF and PcrV. They particularly focused on two mAbs binding the T3SS with the potential of blocking activity. The supplemented biochemical analysis was crystal structures of P3D6 Fab complex. They also compared the blocking activity with mAbs that were described in previous studies, using an assay that evaluated the toxin injection. They conducted mechanistic structure analysis and found that these mAbs might act through different mechanisms by preventing PcrV oligomerization and disrupting PcrVs scaffolding function.

Strengths:

The antibiotic resistance crisis requires the development of new solutions to treat infections caused by MDR bacteria. The development of antibacterial mAbs holds great potential. In that context, this report is important as it paves the way for the development of additional mAbs targeting various pathogens that harbor the T3SS. In this report, the authors present a comparative study of their discovered mAbs vs. a commercial mAb currently in clinical testing resulting in valuable data with applicative implications. The authors investigated the mechanism of action of the mAbs using advanced methods and assays for the characterization of antibody and antigen

interaction, underlining the effort to determine the discovered mAbs suitability for downstream application.

Weaknesses:

Although the information presented in this manuscript is important, previous reports regarding other T3SS structures complexed with antibodies, reduce the novelty of this report. Nevertheless, we provide several comments that may help to improve the report. The structural analysis of the presented mAbs is incomplete and unfortunately, the authors did not address any developability assessment. With such vital information missing, it is unclear if the proposed antibodies are suited for diagnostic or therapeutic usage. This vastly reduces the importance of the possibly great potential of the authors' findings. Moreover, the structural information does not include the interacting regions on the mAb which may impede the optimization of the mAb if it is required to improve its affinity.

As described in the manuscript (Fig. 6), our mAbs are markedly less effective in every in vitro T3SS inhibition assay than the mAbs recently described by Simonis et al. They are therefore very unlikely to outperform these mAbs in in vivo animal models of *P. aeruginosa* infection. Considering the high cost of animal experiments and ethical concerns-and in accordance with the Reduction principal of the 3Rs guidelines-we chose not to pursue in vivo experiments. Instead, we focused on leveraging the new isolated mAbs to investigate the mechanisms of action and structural features of anti-PcrV mAbs.

Following the reviewer's suggestion, we have now added mAb interaction features into the structural data presented in the manuscript. However, based on the efficiency data, the structural analysis and the mechanistic insights presented, we do not consider further therapeutic use and optimization of our mAbs to be warranted.

Reviewer #2 (Public review):

Summary:

*Desveaux et al. performed Elisa and translocation assays to identify among 34 cystic fibrosis patients which ones produced antibodies against *P. aeruginosa* type three secretion system (T3SS). The authors were especially interested in antibodies against PcrV and PcsF, two key components of the T3SS. The authors leveraged their binding assays and flow cytometry to isolate individual B cells from the two most promising sera, and then obtained monoclonal antibodies for the proteins of interest. Among the tested monoclonal antibodies, P3D6 and P5B3 emerged as the best candidates due to their inhibitory effect on the ExoS-Bla translocation marker (with 24% and 94% inhibition, respectively). The authors then showed that P5B3 binds to the five most common variants of PcrV, while P3D6 seems to recognize only one variant. Furthermore, the authors showed that P3D6 inhibits translocon formation, measured as cell death of J774 macrophages. To get insights into the P3D6PcrV interaction, the authors defined the crystal structure of the P3D6-PcrV complex. Finally, the authors compared their new antibodies with two previous ones (i.e., MEDI3902 and 30-B8).*

Strengths:

- (1) The article is well written.*
- (2) The authors used complementary assays to evaluate the protective effect of candidate monoclonal antibodies.*
- (3) The authors offered crystal structure with insights into the P3D6 antibody-T3SS interaction (e.g., interactions with monomer vs pentamers).*

(4) The authors put their results in context by comparing their antibodies with respect to previous ones.

Weaknesses:

The authors used a similar workflow to the one previously reported in Simonis et al. 2023 (antibodies from cystic fibrosis patients that included B cell isolation, antibody-PcrV interaction modeling, etc.) but the authors do not clearly explain how their work and findings differentiate from previous work.

We employed a similar mAb isolation pipeline to that used by Simonis et al., beginning with the screening of a cohort of cystic fibrosis patients chronically infected with *P. aeruginosa*. As in Simonis et al., we isolated specific B cells using a recombinant PcrV bait, followed by single-cell PCR amplification of immunoglobulin genes. The main differences in methodology between the two studies are as follows: i) the use of individuals from different cohorts, and therefore having different Ab repertoires; ii) the nature of the screening assays, although in both cases the screening was focused on the inhibition of T3SS function; iii) the PcrV labeling strategy, with Simonis et al. employing direct labeling, whereas we used a biotinylated tag combined with streptavidin;

The number of specific mAbs obtained and produced was higher in Simonis et al. (47 versus 9 in our study). They sorted B cells from three individuals compared to two in our work and possibly started with a larger amount of PBMCs per donor, which may account for the higher number of specific B cells and mAbs isolated. Considering that the strategies were overall very similar, the greater number of mAbs isolated in Simonis et al. likely explains, to a large extent, why they identified mAbs targeting different epitopes compared to ours, including highly potent mAbs that we did not recover.

Our modeling study, unlike that of Simonis et al., which relied on an AlphaFold prediction of the multimeric structure of *P. aeruginosa* PcrV, was based on the experimentally determined structure of the homologous *Salmonella* SipD pentamer, as described in the manuscript. Furthermore, we compared our mAb P3D6 not only with 30-B8 from Simonis et al., but also with MEDI3902. Finally, in contrast to the approach of Simonis et al., we used functional assays to investigate the differences in mechanisms of action among these mAbs, which target three distinct epitopes.

(2) Although new antibodies against P. aeruginosa T3SS expand the potential space of antibodybased therapies, it is unclear if P3D6 or P5B3 are better than previous antibodies. In fact, in the discussion section authors suggested that the 30-B8 antibody seems to be the most effective of the tested antibodies.

As explained above and shown in the Results section (Figure 6), the 30-B8 mAb is markedly more effective at inhibiting T3SS activity in both in vitro assays used.

(3) The authors should explain better which of the two antibodies they have discovered would be better suited for follow-up studies. It is confusing that the authors focused the last sections of the manuscript on P3D6 despite P3D6 having a much lower ExoS-Bla inhibition effect than P5B3 and the limitation in the PcrV variant that P3D6 seems to recognize. A better description of this comparison and the criteria to select among candidate antibodies would help readers identify the main messages of the paper.

The P3D6 mAb shows stronger inhibitory activity than P5B3 in the two assays used, as shown in Supplementary Figure 1. An error in the table in Figure 2B was corrected and this table now reflects the results presented in Supplementary Figure 1.

The final sections of the manuscript focus on P3D6, which is more potent than P5B3, and for which we successfully determined a co-crystal structure with PcrV*. All parallel attempts to obtain a structure of P5B3 in complex with PcrV* failed. The P3D6-PcrV* structure was used to analyze epitope recognition and mechanisms of action in comparison to previously described mAbs. As previously mentioned, we do not consider further studies aimed at therapeutic development and optimization of our mAbs to be justified given the current data. Therefore, we believe that the main message of the paper is adequately captured in the title.

(4) This work could strongly benefit from two additional experiments:

(a) In vivo experiments: experiments in animal models could offer a more comprehensive picture of the potential of the identified monoclonal antibodies. Additionally, this could help to answer a naïve question: why do the patients that have the antibodies still have chronic P. aeruginosa infections?

As explained above, the mAbs we isolated are significantly less potent than those described by Simonis et al., and are therefore unlikely to outperform the best anti-PcrV candidates in vivo. In light of the data, and considering ethical concerns related to animal use in research and budgetary constraints, we decided not to proceed with in vivo experiments.

There are a number of reasons that may explain why patients with anti-PcrV Abs blocking the T3SS can still be chronically infected with Pa. First these Abs may be at limiting concentration, particularly in sites where Pa replicates, and thus unable to clear infection. In addition, it has been described that the T3SS is downregulated in chronic infection in cystic fibrosis patients. This suggests that a therapeutic intervention with T3SS inhibiting Abs may be more efficient if done early in cystic fibrosis patients to prevent colonization when Pa possesses an active T3SS. Finally, T3SS is not the only virulence mechanism employed by P. aeruginosa during infection. Indeed, multiple protein adhesins and polysaccharides are important factors facilitating the formation of bacterial biofilms that are crucial for establishing chronic persistent infection. In this regard, a combination of Abs targeting different factors on the P. aeruginosa surface may be needed to treat chronic infections.

(b) Multi-antibody T3SS assays (i.e., a combination of two or more monoclonal antibodies evaluated with the same assays used for characterization of single ones). This could explore the synergistic effects of combinatorial therapies that could address some of the limitations of individual antibodies.

Given the high potency of the Simonis mAbs and the mechanisms of action highlighted by our analysis, it is unlikely that our mAbs would synergize with those described by Simonis. Additionally, since our two mAbs cross-compete for binding, synergy between them is also improbable.

Reviewer #1 (Recommendations for the authors):

Line 166: How was the serum-IgG purified? (e.g., protein A, protein G).

Protein A purification was used, as now mentioned in the manuscript. Purified Igs were thus predominantly IgG1, IgG2 and IgG4, as indicated.

(2) Line 196: When mentioning affinities, it is preferable to present in molar units.

To facilitate comparisons, Ab concentrations were presented in µg/mL as in Simonis et al.

(3) Line 206: The author states that P3D6 displays significantly reduced ExoS-Bla injection (Figure 2B), but according to the presented table, ExoS-Bla inhibition was higher for P5B3. Additionally, when using "significantly", what was the statistical test that was used to evaluate the significance? Please clarify.

We thank the reviewer for pointing out this inconsistency. Indeed, the names of P3D6 and P5B3 were exchanged when building the table related to Figure 2B. The corrected version of this figure is now presented in the new version of the manuscript. An ANOVA was performed to evaluate the significance of the observed difference (adjusted p-values < 0.001) and it is now mentioned in the figure caption.

(4) Line 215: "P3B3" typo.

This was corrected.

(5) Figure 3B: Could the author explain the higher level of ExoS-Bla injection when using VRCO1 antibody compared to no antibody.

A slightly higher level of the median is observed in the case of three variants out of five. However, this difference is not statistically significant (p-value > 0.05).

(6) Supplement Figure 1: the presented grey area is not clear (is it the 95%CI?) and how was the IC50 calculated? With what model was it projected? Are the values for IC50 beyond the 100µg/mL mark a projection? It seems that projecting such greater values (such as the IC50 of over 400µg/mL for variant 5) is prone to high error probability.

The grey area represents the 95% confidence interval (95% CI) and it is now mentioned in the figure caption. The IC50 and 95% CI were both inferred by the dose-response drc R package based on a three-parameters log-logistic model and it is now explained in the Materials & Methods section. The p-values for IC50 beyond the 100µg/mL were below 0.05 but we agree that such extrapolation should be considered with precaution (see below our response to comment number 7).

(7) Line 227: The author describes that P5B3 has similar IC50 values towards variants 1-4, but the IC50 towards variant 5 is substantially higher with 400µg/mL, albeit the only difference between variant 4 and 5 is the switch position 225 Arg -> Lys which are very similar in their properties. Please provide an explanation.

As explained in our response to comment number 6, we agree that the comparison of IC50 that are estimated to be close or higher than the highest experimental concentration is somehow speculative. Indeed, we performed further statistical analysis that showed no significant difference between the IC50 toward the five PcrV variants of mAb P5B3. In contrast, the difference between the IC50 of mAbs P5B3 and P3D6 toward variant 1 is statistically significant. This is now explained in the manuscript.

(8) Line 233: Pore assembly: It is not clear how the data was normalized. The authors mention the methods normalization against the wildtype strain in the absence of antibodies, but did not elaborate clearly if the mutant strain has the same base cytotoxicity as the wild type. It would be helpful to show the level of cytotoxicity of the wild type compared to the mutant in the absence of antibodies to understand the baseline of cytotoxicity of both strains.

In these experiments we did not use the wild-type strain. As explained, the only strain that allows the measurement of pore formation by translocators PopB/PopD is the one lacking all effectors. All the experiments were done with this strain, and all the measurements were normalized accordingly.

(9) Figure 4: The explanation is redundant as it is clearly stated in the results. It would be better for the caption to describe the figure and leave interpretation to the results section. Overall, this comment is relevant to all figure captions, as it will reduce redundancy. My suggestion is to keep the figure caption as a road map to understand what is shown in the figure. For example, the Figure 4 caption should include that the concentration is presented in logarithmic scale, what is the dashed line, what is the grey area (what interval does it represent?), what each circle represents, and what is the regression model used?

Figure captions have been improved as suggested.

(10) Line 432: The authors apparently misquoted the original article describing the chimeric form PcrV by describing the fusion of amino acids 1-17 and 136-249. I quote the original article by Tabor et al. "[...] we generated a truncated PcrV fragment (PcrVfrag) comprising PcrV amino acids 1-17 fused to amino acids 149-236 [...]". Additionally, how does the absence of amino acid 21 in the variant affect the conclusion?*

Our construct was inspired by the one described in Tabor et al. but was not identical. We have therefore replaced "was constructed based on a construct by Tabor et al." for "whose design was inspired by the construct described in Tabor et al."

Amino acid 21 is only absent in the construct used for crystallization experiments; all other experiments looking at Ab activity were performed with bacteria bearing full-length PcrV. The difference in P3D6 activity between variants V1 and V2 appears to be explained by the nature of the residue at position 225, according to the structural data, as explained now in more detail in the manuscript. Accordingly, the difference in efficiency of P3D6 against the V1 and V2 variants is explained by the residue at position 225, as both variants have the same residue at position 21. However, while the nature of the residue at position 225 appears to explain the absence of efficiency of the Ab for the variants studied, an impact of residue 21 could not be totally ruled out in putative variants with a Ser at 225 but different amino acids at 21.

(11) Line 569: Missing word - ESRF stands for European Synchrotron Radiation Facility.

This has been corrected.

(12) Line 268-269 (Figure 5A): The description of the alpha helices in relation to the figure is incomplete. Helices 2,3 and 5 are not indicated.

Indeed, since the structure is well-known and in the interest of visibility and simplicity, we only included the most relevant secondary structure features.

(13) Line 271-272: It would be good to elaborate on the exact binding platform between LC and HC of the Fab and the residues on the PcrV side. For example, the author could apply the structure to PDBePISA (EMBL-EBI) which will provide details about the interface between the PcrV and the antibody. It is very interesting to learn what regions of the antibody are in charge of the binding, such as: is the H-CDR3 the major contributor of the binding or are other CDRs more involved? Additionally, in line 275 they state that the

substitution of Ser 225 with Arg or Lys is consistent with the P3D6 insufficient binding. What contributed to this result on the antibodies side?

In order to address this question, we are now providing a LigPlot figure (supplementary Figure 3) in which specific interactions between PcrV* and the Fab are shown.

(14) Line 291: It is unclear from what data the authors concluded that anti-PscF targets 3 distinct regions of PscF.

The data are shown in Supplementary Table 2, as mentioned in the manuscript. We have now modified the order of the anti-PcrV mAbs in the table to better illustrate the three identified epitope clusters (Sup table 2). Similarly, the anti-PscF mAbs appear to group into three clusters as P3G9 and P5E10 only compete with themselves, while mabs P3D6 and P5B3 compete with themselves and each other.

(15) Line 315: It is preferable to introduce results in the results section instead of the discussion.

While preparing the manuscript, we initially included these results as a separate paragraph in the Results section, but ultimately chose the current format to improve flow and avoid redundancy.

(16) Supplement Figure 2: What was the regression model used to evaluate IC50, and what is presented in the graph? What is the dashed line (see comment for Figure 4 above)?

The regression is based on a three-parameters log-logistic model and the light-colors area correspond to the 95% IC. The dashed lines visually represents 100% of ExoS-Bla injection. These information are now mentioned in the figure caption.

(17) Figure 6B: It would be better to show an additional rotation of the PcrV bound by Fab 30-B8 that corresponds to the same as the one represented with Fab MEDI3092. This would clear up the differences in binding regions. Same for Fab P3D6.

Figure 6 already depicts two orientations. Despite the fact that we agree that additional orientations could be of interest, we believe that this would add unnecessary complexity to the figure, and would prefer to maintain the figure as is, if possible.

(18) Line 356-358: The author proposes an experiment to support the suggested mechanism of P3D6, it would follow up with a bio-chemical analysis showing the prevention of PcrV oligomerization in its presence.

We understand the reviewers' comment regarding the potential use of biochemical approaches to test our hypothesis. However, this not currently feasible as we have been unable to achieve in vitro oligomerization of PcrV alone, possibly due to the absence of other T3SS components, such as the polymerized PscF needle.

(19) Line 456: Missing details about how the ELISA was conducted including temperature, how the antigen was absorbed, plate type, etc.

Experimental details have been added.

(20) Line 460: Missing substrate used for alkaline phosphatase.

The nature of the substrate was added to the methods.

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