A NEW FORMAT OF MONOCLONAL ANTI-PCRV ANTIBODIES FOR
THE THERAPEUTIC HOST CLEARANCE OF PSEUDOMONAS
AERUGINOSA

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A New Format of Monoclonal Anti-PcrV Antibodies for the Therapeutic Host

Clearance of Pseudomonas aeruginosa

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ABSTRACT OF THE THESIS

A new format of monoclonal anti-PcrV antibodies for the therapeutic host clearance of *Pseudomonas aeruginosa* by Roxana Flores

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*Pseudomonas aeruginosa* is an opportunistic pathogen that can lead to mortality in immunocompromised patients and it is the second most common cause of nosocomial pneumonia from mechanical ventilation. The main bacterial virulence factor associated with higher morbidity and mortality is the type three secretion system (T3SS) which is used to deliver exotoxins into host cells. One critical structure of the T3SS is PcrV and has been a target of murine derived monoclonal antibodies (MAbs). Recent insight into structural differences of camelid antibodies has led to the generation of llama derived anti-PcrV monoclonal antibodies. In order to identify highly effective anti-PcrV Mabs, a pool of llama derived antibodies were characterized for their ability to block hemolysis and epithelial cell damage. Further investigation of antibodies that confer medium protection *in vitro* led to the development of a new format of MAbS which combine multiple epitopes. The tandem-biepitopic antibodies PcrV18-15 and PcrV18-20 were the most efficient disablers of the T3SS. To determine if the tandem-biepitopic antibodies confer better protection than the leading murine derived anti-PcrV antibody, V2L2MD, which has been previously shown to mediate significant *in vivo* protection. Pneumonia mouse models were performed and results show that the tandem-biepitopic antibodies have significant (P<0.0001) prophylactic protection compared to V2L2MD, thus demonstrating the potential for anti-PcrV immunotherapy improvement.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................ iv
LIST OF TABLES .................................................................................................. vii
LIST OF FIGURES .............................................................................................. viii
CHAPTER
  1 INTRODUCTION ............................................................................................... 1
    1.1 Significance of This Study ........................................................................... 1
    1.2 Pseudomonas aeruginosa Properties ....................................................... 2
    1.3 The Type Three Secretion System ............................................................. 2
    1.4 PcrV ........................................................................................................ 3
    1.5 Antibody Structure and Function ............................................................. 5
    1.6 Monoclonal Antibodies Targeting PcrV .................................................. 6
    1.7 Camelid Antibodies .................................................................................. 7
    1.8 Experimental Strategy ............................................................................. 8
  2 MATERIALS AND METHODS ......................................................................... 10
    2.1 Materials ................................................................................................ 10
    2.2 Bacteria Strains and Growth Conditions ................................................. 10
    2.3 Cell Lines ................................................................................................ 11
    2.4 Enzyme-linked Immunosorbent Assay ..................................................... 11
    2.5 Epitope Binning ...................................................................................... 12
    2.6 Hemolysis Assay .................................................................................... 12
    2.7 Cytotoxicity Assay ................................................................................ 13
    2.8 Fluoresce- Activated Cell Sorting (FACS) .............................................. 13
    2.9 Mouse Strains and Infection Models ....................................................... 14
    2.10 Data Analysis ....................................................................................... 14
  3 RESULTS ......................................................................................................... 15
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Anti-PcrV Antibodies Binding Characterization</td>
<td>15</td>
</tr>
<tr>
<td>3.2 Epitope Binning</td>
<td>18</td>
</tr>
<tr>
<td>3.3 New Format Antibody Characterization</td>
<td>20</td>
</tr>
<tr>
<td>3.4 \textit{in vivo} Prophylaxis and Treatment Effect of PcrV antibodies</td>
<td>23</td>
</tr>
<tr>
<td>3.5 Investigating anti-PcrV Antibodies Blocking Effect</td>
<td>25</td>
</tr>
<tr>
<td>4 CONCLUSION</td>
<td>27</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>29</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>30</td>
</tr>
</tbody>
</table>
LIST OF TABLES

PAGE

Table 3.1. Anti-PcrV antibody equilibrium dissociation constants ($K_D$)..........................16
Table 3.2. New format antibody structures........................................................................20
LIST OF FIGURES

Figure 1.1 Structure of P. aeruginosa T3SS. The Type 3 Secretion System consists of a basal body that spans the inner membrane (IM), peptidoglycan layer (PG) and the outer membrane (OM). Source: 26 ................................................................. 3

Figure 1.2. PcrV structure modeled from LcrV (PDB:1r6f chain A). The conserved helices and the protective epitope regions circled in white. Source: 25 ......................... 4

Figure 1.3. General structure of a conventional IgG antibody and its fragmented forms: Fab, Fv and scFv by protein digestion. Source: 56 ................................................................. 5

Figure 1.4. Camelid antibodies also known as heavy chain IgG consists of a variable heavy-chain fragment (VHH) and constant domains (CH2 and CH3). The full antibody weighs approximately 95 kDa while the VHH is around 15 kDa. Source: 56 ................................................................. 8

Figure 3.1. Binding of PcrV antibodies by ELISA to rPcrV coated plates (2 ug/mL). This is preliminary data of n=1 in duplicate. ................................................................. 15

Figure 3.2. PcrV antibodies ability to block hemolysis. The figure is representative of n=3 experiments ........................................................................................................... 16

Figure 3.3. PcrV antibodies ability to block lung epithelium cell (A549) damage. This is preliminary data (n=1) that supports hemolysis experiment ........................................... 17

Figure 3.4. Leading antibodies PcrV15 and PcrV20 demonstrated superior blocking ability as shown in their ability to block cytotoxicity in A549 cells. ......................... 17

Figure 3.5. General antibody hemolysis blocking levels. PcrV15 and PcrV20 are high binding, PcrV18 and PcrV17 demonstrated to be medium and low hemolysis blockers respectively ................................................................. 18

Figure 3.6. Epitope binning of high binding PcrV15 and PcrV20. PcrV15 was challenged against itself (red line) and PcrV20 (violet line) which proved to bind the same epitope. Step 1 represents washing of the sensor. Step 2 is the coating of the sensor with biotinylated rPcrV. Step 3 is the quenching of empty space on the sensor with biotin. Step 4 is another washing and removing of unbound protein. Step 5 is the addition of the first antibody: red line is of PcrV15 and violet line is of PcrV20. Step 6 is the addition of PcrV15 and step 5 antibody. Step 7 is the last wash to determine dissociation rate ....................... 19
Figure 3.7. PcrV15 and PcrV18 epitope binning challenge. Red line indicates PcrV15 against PcrV15 and green line represents PcrV18 against PcrV15. The respective steps are the same as previously described in Figure 3.6.  

Figure 3.8. Antibody combination of leading antibodies, PcrV15 and PcrV20 with PcrV18 showed synergistic effect for improved cytotoxicity inhibition.  

Figure 3.9. PcrV15 antibody format lysis inhibition comparison. PcrV15VHH are single VHHs, PcrV15 have the normal camelid structure shown in Figure 1.4, PcrV15-15 is the tandem tetravalent-monospecific. There is an increase in protection with increasing epitope binding as shown by the left curve shift.  

Figure 3.10. Hemolysis inhibition by PcrV15 and PcrV18 tandem biepitopic antibodies.  


Figure 3.12. Cytotoxicity tests of phagocytic cells. Anti-PcrV antibody are able to protect cells against Pa103 lysis (MOI 10). Antibodies tested reflect their availability at the time of the assay.  

Figure 3.13. There was 100% survival in the PcrV15, and 87.5% survival in V2L2 and PcrV20 group at the high concentration of 5 mg/kg or equimolar amount of 2.7 mg/kg. For the lower antibody concentration, there was 25%, 62.5%, 50% survival for the lower concentration PcrV15, PcrV20 and V2L2 groups respectively. There was a 25% for the control, most likely indicative not no infection. Groups are n=8 for except the controls which are n=4.  

Figure 3.14. In vivo mouse pneumonia model experiment, prophylaxis treatment. There was a final 87.5% survival for PcrV18-15 at n=8 per group. Both groups were significantly different compared to control.  

Figure 3.15. In vivo mouse pneumonia model experiment, prophylaxis treatment. There was a final 87.5% survival for PcrV18-15 at n=8 per group.  

Figure 3.16. FACS analysis for antibody binding indicative of opzonization. Overlapping antibodies include V2L2, Control IgG, PcrV18, PcrV15, PcrV18-15, PcrV 15-15, and no antibody control.
CHAPTER 1

INTRODUCTION

1.1 SIGNIFICANCE OF THIS STUDY

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a bacterium responsible for a major proportion of morbidity and mortality in immunocompromised patients\(^1\)–\(^4\). It is an increasing cause for concern in the intensive care unit (ICU) especially in burn patients, those using mechanical ventilation\(^5\)–\(^7\) and patients with persistent lung infections such as cystic fibrosis (CF). Over the past decade, *P. aeruginosa* infections have increased\(^8\) in reported cases ranging from nosocomial pneumonia, urinary tract infections (UTI), surgical site infection and bacteremia\(^8\)–\(^11\). In burn victims, burn wound infections is the major cause of morbidity and mortality with pseudomonas being the major gram-negative bacteria isolated\(^1,6\). In the hospital setting, *P. aeruginosa* is one of the major causes of longer ICU stay, need for mechanical ventilation and accounts for 50% mortality rate in systemic infection\(^9,10,12–14\). Although the proportion of *Pseudomonas aeruginosa* infections have remained stable throughout the years, the rate of multidrug resistant (MDR) strains is alarmingly increasing\(^12,15,16\).

Due to inappropriate antimicrobial therapy, there is an increasing number of antibiotic resistant strains\(^12,17\) being clinically isolated. One approach to combat these resistant strains is the use of old antibiotics\(^18\), but clinical studies have not shown improved outcome compared to standard treatment. Novel antibacterial agents are underway in the form of antibodies that target virulence factors responsible for the bacteria’s pathogenesis\(^19\). *P. aeruginosa* is the most common MDR gram-negative pathogen\(^15\) isolated and it is important to develop new and more effective therapeutic ways that veer off antibiotics, improve survival, decreases hospital stay and lower overall treatment cost.
1.2 PSEUDOMONAS AERUGINOSA PROPERTIES

*Pseudomonas aeruginosa* is a rod shaped, aerobic Gram-negative bacteria with a single polar flagellum for motility and ranges in size from 0.5 to 0.8 um by 1.5 to 3.0 um\(^{20}\). The bacteria forms part of the normal human microflora and it is also found in soil and water\(^{20}\). It has minimal nutritional requirements which allows it to adapt to multiple environments\(^{19,21}\), it can even grow anaerobically and in temperatures ranging from 25°C to as high as 42°C\(^{20}\).

*P. aeruginosa* has a low outer membrane permeability, numerous membrane efflux pumps\(^{8,15}\) and plasmid-mediated antibiotic resistance factors\(^{10}\), which makes it a difficult bacteria to combat with traditional antibiotic therapy. It is an opportunistic pathogen that causes acute infections when the host immune system is impaired\(^{22}\) and it is important to understand its virulence factors for improved therapeutic targets.

1.3 THE TYPE THREE SECRETION SYSTEM

An important virulence factor associated with increased resistance, severe disease and mortality is the Type Three Secretion System (T3SS)\(^{4,19,23,24}\). The T3SS is a specialized apparatus for the injection of toxins into host cells, believed for the purposes of delaying or preventing local innate response to allow infection\(^{25}\). It is a needle-like projection made up of over 20 proteins consisting of a basal body within the outer and inner membrane, a protruding needle (secretion apparatus), a V-tip complex (PcrV) on the distal end of the needle and a translocon that assembles on the host membrane consisting of PopB and PopD proteins\(^{25}\). The system can be broken down to three functional levels: the secretion apparatus, the translocation apparatus and the chaperones and toxins involved which work to alter cell signaling, and cause cell death or interfere with host immune responses\(^{26}\) such as neutrophil function\(^{27–29}\).

The system is expressed under two main conditions: contact with eukaryotic host cells and under extracellular low calcium levels\(^{30,31}\). In order for the system to effectively deliver the toxins into the host cell cytosol, there needs to be close contact between the host cell and the bacteria\(^{27}\).
Figure 1.1 Structure of *P. aeruginosa* T3SS. The Type 3 Secretion System consists of a basal body that spans the inner membrane (IM), peptidoglycan layer (PG) and the outer membrane (OM). Source:26

The T3SS uses the basal body to transfer exotoxins from the bacterial cytosol to the injectosome made up of PsF proteins32,33. There are four known effector proteins (exotoxins): ExoU, ExoS, ExoT and ExoY8,34. Different strains express a combination of exotoxins, but the presence of genes for ExoU and ExoS appears to be mutually exclusive8. The main *P. aeruginosa* strains clinically isolated from acute invasive infections are ExoU positive35.

ExoU is a potent phospholipase that causes rapid cell lysis including epithelial cells and macrophages36–38. ExoS and ExoT are capable of activating Rho GTPases and ADP-ribosyltransferase to disrupt cellular signals, block phagocytosis, and damage the epithelial barrier for dissemination which can lead to septic shock4,21,28,34,39,40. The effector proteins are activated by host cellular co-factors4 and therefore preventing the bacteria-host cell interaction will render an avirulent pathogen. Even though a functional T3SS is necessary for the secretion of toxins, research suggests a mechanism of causing cell damage without toxins27.

1.4 PcrV

Most T3SS positive clinical isolates have a needle V-tip protein known as PcrV, which is essential for the translocation of effector proteins into eukaryotic cells. PcrV is thought to possess various functions including: sensing the microenvironment, signaling the bacteria, regulation of secretion and translocation at the tip complex and transcriptional level25. PcrV is essential for the assembly of a functional translocation pore consisting of
PopB/PopD complex by aiding in the insertion and or stability of PopD into the host membrane\textsuperscript{41}. Studies have shown that PcrV is required for ExoU-dependent cytotoxicity\textsuperscript{23} and the transfer of ExoS into epithelial cells\textsuperscript{42}, but the exact mechanism is unclear.

Bacteria strains with a PcrV deletion are avirulent in mouse models\textsuperscript{4,23,43} and are ineffective at translocating effectors into the host cell cytosol \textit{in vitro}; translocation and cytotoxicity is restored upon complementing with a PcrV containing plasmid\textsuperscript{23,25,41}. PcrV positive strains are associated with higher mortality even in the absence of toxins\textsuperscript{3,10}. PcrV mutants lacking toxins can cause sepsis and distal organ injury unlike mutants that express toxins and lack PcrV\textsuperscript{4}. PcrV has clearly been shown to be a protein critical for host cell cytotoxicity\textsuperscript{4,34}.

Figure 1.2. PcrV structure modeled from LcrV (PDB:1r6f chain A). The conserved helices and the protective epitope regions circled in white. Source: \textsuperscript{25}

PcrV is a 295 amino acid protein consisting of conserved coiled-coil structure of two \(\alpha\)-helices with the protective-epitope region circled in white as shown in Figure 1.2\textsuperscript{44,45}. Previous studies indicate that PcrV may form a pentamer at the tip of the injectosome\textsuperscript{46–49}.

PcrV is fairly conserved among different strains of \textit{P.aeruginosa} worldwide\textsuperscript{8,50} and it has high homology to the protective antigen of \textit{Yersinia}, LcrV\textsuperscript{51–54}, which made it a target of interest for immune therapy. Antibodies targeting PcrV have been shown to reduce mortality and reduce inflammation in pneumonia animal models and maintain host cell phagocytic capability\textsuperscript{23,55}. 
Antibodies are recognition proteins that bind antigens and belong to the family of immunoglobulins, globular proteins. They are produced by B-lymphocytes in response to an antigen. There are five different classes of antibodies found in vertebrates (IgG, IgM, IgA, IgD and IgE) that differ in their structural and functional properties and range from 150 to 1000 kDa.

IgG (160 kDa) is the most abundant in the blood, it can enter tissue spaces and coat antigens and it is the main type for monoclonal antibody produced as a conventional antibody. The structure of IgG consist of two identical light (L) chains (23 kDa each) and two identical heavy (H) chains (50 kDa each) which pair with one another. Each chain has a constant (C) region, at the C-terminal domain, that is similar across other antibody types (even across other species) and a variable (V) region at the N-terminal part of the antibody which was shown to have great variability. The diversity of the V region accounts for antibody specificity. Each V region has three areas of even greater variability called the hypervariable regions or complementary-determining regions (CDRs: CDR1, CDR2, CDR3) of about 10 amino acids in length. While the V region recognizes the antigen, domains in the C region of the heavy chain can elicit effector functions such as: protection, placental transfer and cytophilic properties.

Antibodies can disable foreign organisms by neutralizing toxins or viruses, but cannot directly destroy them. They can however bind its antigen and activate host immune defense systems such as the complement system to cause bacterial lysis (complement-dependent
cytolysis). Antibodies can opsonize (coat) organisms for increased phagocytosis by macrophages and neutrophils. The activation of the immune cells are Fc (fragment constant) mediated as it is recognized by cells expressing Fc receptors. In the case of placental transfer properties, only IgG can cross the placenta since the placental cells bind C_{H1} and C_{H3} domains. The Fc region is not only essential for the antibody effector functions, but also for retaining a long serum half-life\textsuperscript{56,57,59–62}. The structure of antibodies relates to its binding versatility, specificity and biological activity.

Antibodies can be fragmented by protein digestion such as with papain and pepsin, to create Fab (fragment antigen binding), Fv (fragment variable) or scFv (single-chain) fragments. Full antibodies that have had their heavy and light chains isolated\textsuperscript{63,64} have been shown to retain their antigen-binding specificity, but have lower affinity and solubility\textsuperscript{65}. However, monovalent antibody fragments (Fab) or single Fv (scFc) consisting of heavy (VH) and light (VL) chains can efficiently bind its antigen\textsuperscript{66}. One reason to develop smaller antibody fragments is the advantage for greater tissue penetration and rapid clearance from kidneys or blood\textsuperscript{56}.

1.6 MONOCLONAL ANTIBODIES TARGETING 

The potential for anti-PcrV antibodies to mediate protection was demonstrated by Sawa et al.\textsuperscript{23} where mice immunized with recombinant PcrV (rPcrV) had complete survival against a fatal dose of \textit{P. aeruginosa} strain PA103. They also demonstrated the same protection through passive immunization of 100 ug per mouse of rabbit anti-PcrV IgG. Other immunogens were tested such as PopD and ExoU, but only PcrV was found to be the most protective against the highly cytotoxic strain, PA103\textsuperscript{23,44}.

Rabbit-derived polyclonal antibodies (antibodies made from different immune cells) were then shown to improve survival against PA103 infection in mouse pneumonia and rabbit septic shock models\textsuperscript{55}. Mice were treated intravenous (i.v) with 50 ug or more of anti-PcrV IgG 4 hours post infection had complete survival and mice treated 1 hour after infection with 10 ug had 80% survival. Rabbit septic models treated i.v or intra-tracheal 1 hour post infection proved to have stable hemodynamics and did not develop metabolic acidosis, markers indicative of septic shock. The treated rabbits also had lower lung and blood
bacteremia. It was also shown that anti-PcrV Fab’2 had comparable protective effects indicating protection is Fc independent.

The generation of a murine monoclonal antibody (Mab) against PcrV, Mab166, demonstrated to be protective against PA103 in co-instillation infection and passive protection models. Rabbit polyclonal anti-PcrV IgG which has multiple binding sites on PcrV was used as the standard when screening for protective monoclonal antibody and its superior protective effect indicates that a combination of monoclonal may have a synergistic protective effect. The Fab fragments were further characterized in rats by Faure et al. and showed their potential for adjuvant therapies in P. aeruginosa acute lung injury.

The humanized version of Mab166 known as KB001 has undergone phase II clinical trial aimed to reduce pneumonia lung infections from pre-colonized cystic fibrosis patients by administering one single antibody dose of either 3 or 10 mg/kg. Results showed that the untreated group experienced 60% of ventilated associated pneumonia (VAP) compared to only 33% and 31% of the 3 or 10 mg/kg respectively. The clinical trial was shown to be safe and tolerable, but no significant effect on pulmonary function or exacerbation has been concluded. Recently, Warrener et al. developed a murine-derived monoclonal anti-PcrV antibody (V2L2MD or V2L2) that demonstrated strong prophylactic protection of P. aeruginosa infected mice compared to MAb166. Since V2L2MD has shown to be most promising anti-PcrV antibody, it is the comparator to future engineered antibodies.

### 1.7 Camelid Antibodies

Camelid antibodies, discovered in 1993 by Hamers-Casterman et al., are single-domain antibodies which are capable of antigen binding by the heavy-chain variable domains (VHVs). These antibodies have a molecular weight of approximately 95 kDa and are found in camels, dromedaries and llamas. The small size of the VHVs (15 kDa) allow for fast tissue penetration, and are able to be rapidly cleared by the renal system which have a minimum cut off of around 60 kDa. VHVs have also been shown to bind and migrate across the blood brain barrier (BBB) in vitro. Similar to full IgG VH domains, VHVs have four framework regions (FRs) and three CDRs, but the FRs have high sequence homology (> 80%). The FR2 area of VHVs have four amino acid substitutions in place where
conventional antibodies remain conserved as it is where the VH domains bind with the VL domains\textsuperscript{73}.

Another characteristic of VHHs is found in the CDRs where the CDR1 is found to be more variable\textsuperscript{78–80} and there are many dromedary VHHs with a longer CDR3 with added disulfide bonds for stabilization\textsuperscript{81}. Llamas, also have VHHs with the extended CDR3, but are more uncommon as most VHHs are similar in length to human VHs\textsuperscript{73}. The longer CDR3, greater variability and small fragment size of VHHs benefit the diverse antigenic recognition sites that conventional antibodies don’t posses such as better access to cryptic epitopes\textsuperscript{82} and recognition of enzyme active sites\textsuperscript{83,84}.

In terms of production levels, VHHs are produced in higher levels than conventional IgG even if engineered to have multiple VHHs for the recognition of different antigens or improved affinity\textsuperscript{73}. Unlike conventional antibodies, VHHs have been shown to have greater overall stability and still retain function at high temperatures\textsuperscript{85} and at extreme pH\textsuperscript{86}. Due to their unique characteristics and stability, VHHs have been used in therapeutic applications ranging from bacteria diarrhea, cancer, brain disorders and continued to be further explored and engineered for the benefit treatments world-wide.

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Camelid antibodies also known as heavy chain IgG consists of a variable heavy-chain fragment (VHH) and constant domains (CH2 and CH3). The full antibody weighs approximately 95 kDa while the VHH is around 15 kDa. Source:\textsuperscript{56}}
\end{figure}

1.8 \textbf{Experimental Strategy}

Identification and production of candidate antibodies was performed by Inhibrx. Briefly, llamas were immunized with a recombinant PcrV and serum was screen monthly for antibody titers against PcrV by Enzyme Linked Immunosorbent Assay (ELSA). When llamas
displayed increased titers against PcrV, peripheral blood mononuclear cells (PBMCs) were isolated, RNA extracted, and converted to cDNA. The cDNA was then used to amplify the VHH domains by PCR amplification which were enzyme digested and ligated into a yeast plasmid. The plasmid was then transformed into yeast which were screened and sorted for binding to labeled PcrV using fluorescence-activated cell sorting (FACS). Positive yeast (those that bind rPcrV antigen) had the VHH domains subcloned into a human Fc expression vector and expressed in eukaryotic cells where they were mass produced and purified. Twenty-four candidate antibodies with unique VHH sequences were provided for screening.
CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

All chemical reagents were obtained from Sigma Alrich®, (St. Louis, MO) unless otherwise noted. Sterile cell culture supplies including plastic ware, TMB, FBS, 1X DPBS, 0.05 % Trypsin-EDTA, DMEM and RPMI, HBSS media were from Thermo Fisher Scientific (Waltham, MA). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

2.2 BACTERIA STRAINS AND GROWTH CONDITIONS

*P. aeruginosa* strain PA103 (ATCC29260), which produces ExoU and ExoT effector proteins, was used for all in *vitro* and in *vivo* experiments. The bacteria were grown in Lysogeny broth (LB), also known as Luria broth. Bacteria was stored in 20% glycerol-LB and frozen at -80°C. Every two weeks, bacteria were streaked out from frozen stocks onto a Lysogeny agar (LA) plate, and grown overnight (≤ 16 hours) at 37°C. Colonies were picked into 10 mL LB for overnight cultures (ONC) and grown overnight at 37°C shaking (200 RPM). On the day of the assay, ONC were washed in 1X PBS by centrifugation at 3220 x g for 10 minutes at room temperature and diluted in equal volume of 1 X PBS. Day cultures consisted of 1/50-1/25 of washed ONC in T3SS inducing media (unless otherwise noted) and grown at 37°C and shaking to an OD$_{600nm}$ 0.3-0.5. Bacteria were then washed and diluted in assay media to an OD$_{600nm}$ 0.4 corresponding to approximately 2.5 X 10$^8$ bacteria / mL. The T3SS inducing media consist of, 5 mM EGTA and 20 mM MgCl$_2$ as previously described$^{26,38,41,87}$. For counting the colony-forming units (CFU), bacteria were diluted 100-fold for 8 serial dilutions in 1X PBS and plated in 25 uL volume in triplicate on LA plates.
2.3 Cell Lines

To model bacterial lung damage in vitro, lung epithelial cells were used. Adenocarcinomic human alveolar basal epithelial cells also known as A549 were cultured in DMEM with 10% FBS. For passaging A549 cells, 0.05% Trypsin-EDTA was used. Non-adherent human monocyte cell lines include THP-1 (Leukemic monocyte) and U937 (Lymphoblast lung) cultured in RPMI with 10% FBS. All cells were incubated at 37°C and 5% CO₂ and passaged every 2-3 days depending on confluence. For THP-1 differentiation into adherent macrophages, a final concentration of 5 ng/mL of phorphol 12-myristate 13-acetate (PMA) was used as previously described. Human derived macrophages were previously isolated at InhibRx and frozen until use. Cell stocks were made in 10% DMSO and 90% FBS and stored at -80°C.

2.4 Enzyme-Linked Immunosorbent Assay

To determine which antibodies bind PcrV, 96-well high binding plates (Corning® 3361) were coated overnight at 4°C with 2 ug/mL of recombinant PcrV diluted in coating buffer as described in abcam® Direct ELISA Protocol. On the day of the assay, the ELISA plate was washed three times with 0.05% PBS-Tween (PBS-T). The plate was then blocked in a 2X solution of Diluent Reagent (841390, R&D) in PBS-T for one hour at 37°C. A round bottom 96-well plate (3788, Corning®) was used to serially dilute the antibodies in duplicate, 5-fold starting at 10 ug/mL in 0.2X Diluent Reagent PBS-T. Diluted antibody samples were transferred to the pre-coated ELISA plate and incubated at 37°C for 1 hour. The ELISA plate was washed as previously described and a secondary antibody (109036097, Jackson ImmunoResearch) anti-human heavy and light chain HRP was diluted 1/10000 in the 0.2X Diluent Reagent PBS-T buffer and added to the plate for 45 minutes at 37°C. The ELISA plate was washed as previously described and TMB (555214, BD) substrate was prepared and added to the plate per manufacturer’s instructions. If necessary, the reaction was stopped using an acid (stop solution) and read at absorbance 450 nm (yellow pigment) or if no stop solution was used, read at 605 nm (blue pigment).
2.5 EPITOPE BINNING

The Forté BIO Octet RED96 System (Pall Corporation ©) was used to determine the preference of antibody for the same or different epitope. In a 96-well plate, the sequence of sample was added per column: 0.1% PBS-T for wash (step 1), biotinylated PcrV (7 ug/mL) as the antigen (step 2), biotin (10 mg/mL) for quenching (step 3), 0.1% PBS-T for wash (step 4), 100 nM of first antibody (step 5), 100 nM each of first and second antibody (step 6) and final wash in 0.1% PBS-T (step 7). The steps coordinate with epitope binning figures in the results page. Streptavidin (SA) sensors were used and the following experimental set up: the first and second washes with 0.1% PBS-T, loading of the antigen and quenching assay time was 60 seconds followed by the association of the first antibody and first and second antibody for 120 seconds and a final dissociation in 0.1% PBS-T of 240 seconds.

2.6 HEMOLYSIS ASSAY

Human heparinized blood was collected and stored at 4°C for up to one week. One milliliter of whole blood was washed in 1X PBS at 600 x g for 5 minutes at room temperature until supernatant was clear and resuspended in a final dilution of 1/50 in 1X PBS and stored at 4°C until use. In a round bottom 96-well plate (3788, Corning®), antibodies were 3-fold serially diluted in duplicate, at four times the final concentration in RPMI no phenol (11835-030, Life Technologies) for a final volume of 60 uL per well. An equal volume of OD_{600nm} 0.4 bacteria day culture in RPMI no phenol was added to the antibody dilution plate and incubated at 4°C for 20 minutes. In a 96-well V-bottom plate, 100 uL of washed red blood cells were combined with an equal volume of experimental solution (bacteria and antibody) for a final volume of 200 uL per well. The included experimental controls consisted of bacteria only, buffer only and 0.1% Triton-X. The plate was then incubated at 37°C for 2 hours, followed by a 30-minute incubation at 4°C and a final spin of 3200 x g for 5 minutes to complete red blood cell lysis. The supernatant (100 uL) was then transferred to a flat-bottom 96 well plate (3370, Corning®) and the released hemoglobin, indicative of red blood cell lysis, was read in a plate reader at an absorbance of 405 nm. The percent hemolysis inhibition was normalized to the bacteria and cell only controls.
2.7 Cytotoxicity Assay

On the day prior to the assay, eukaryotic cells were washed in 1X DPBS at 300 x g, trypsinized if cells are adherent and resuspended in proper media for counting. They were then counted using a hemocytometer or Scepter™ Cell Counter (EMD Millipore, Darmstadt, Germany) and seeded onto 96-well tissue culture treated plates (353072, BD Falcon). On the day of the assay, cells were stained with 5-10 uM of calcein AM (65-0853-39, eBioscience) in RPMI no phenol (11835-030, Life Technologies) for 30 minutes to 1 hour at 37°C and 5% CO₂. The experimental mixture was prepared as previously described in the hemolysis assay with the exception that the antibody solution was prepared at 2 X the final starting concentration and the bacteria was diluted for a final MOI (multiplicity of infection) of 10. Stained eukaryotic cells were washed with 1X DPBS and 100 uL of experimental solution was added. For adherent cells, the plate was spun at 300 x g for 5 minutes for bacteria-host cell contact, for non-adherent cells, plate was briefly shaken using a plate reader to ensure even distribution of bacteria and host cell suspension followed by a 1-hour incubation at 37°C and 5% CO₂. The plate was then shaken and centrifuged as previously described and 70 uL of supernatant were transferred to a flat-bottom 96-well plate. The assay plate was washed twice and resuspended in 100 uL 1X DPBS. Bacterial cytotoxicity was measured by reading the released calcein AM of the supernatant and the cell viability by the contained calcein AM using a plate reader at an excitation of 495 nm and emission 515 nm. Percent cytotoxicity inhibition was normalized to bacteria and host cell controls. The

2.8 Fluoresce-Activated Cell Sorting (FACS)

T3SS induced bacteria (12.5 X 10⁶) was incubated with primary antibody at a final concentration of 100 nM in HBSS +/- for 1 hour at 4°C. Samples were washed twice with HBSS +/- and incubated with secondary anti-human Alexa 647 (209-605-098, JacksonImmunoResearch) at 1/200 for 1 hour at 4°C. Samples were washed and ran using the BD FACSCalibur™ flow cytometer platform. For opzonization control, hIVIG (13533-800-71, Grifols) was used as the primary antibody. A secondary only control was used for both groups to confirm false/unspecific signal.
2.9 Mouse Strains and Infection Models

All animal studies were performed under an approved Animal Care and Use protocol by the University of California San Diego Institutional Animal Care and Use Committee (IACUC). CD-1® IGS mouse model from Charles River were used at 7-8 weeks of age and equally and randomly assigned an experimental group. Antibodies were diluted in proper buffer to equimolar concentrations of 1 mg/kg (unless otherwise noted) and sterile filtered using a sterile syringe and 0.22 um syringe filter unit into a 50 mL tube and kept on ice. Mice were weighed and appropriate treatment volume administered. For prophylaxis experiments, antibodies were administered intraperitoneal injection (IP) using 1 mL insulin syringes, 24 hours before infection. For treatment models, antibody was administered intravenous (i.v.) using a 30-gauge needle, by lateral tail vein 1-hour post-infection in ≤ 100 uL volume.

Murine pneumonia infection was performed by intra-tracheal inoculation (IT) as previously described with slight modifications. Day culture bacteria was diluted to an OD 0.4 in 1X DPBS and further diluted for an infection dose of 1 million CFU per mouse administered in 40 uL volume. Bacteria was kept on ice and plated out for CFU counts. Prior to infection, mice were anesthetized with 100 mg/kg ketamine and tails were labeled with lab markers according to experimental group and mouse number. Mice were monitored at least twice a day for a period of four days and fatality recorded. Mice were then sacrificed with CO₂. Minimal potential pain and distress was ensured throughout experiments.

2.10 Data Analysis

Graphpad Prism ® version 5.0 will be used for all data analysis. FACS analysis was performed using FlowJo Single Cell Analysis Software.
CHAPTER 3

RESULTS

3.1 ANTI-PCrV ANTIBODIES BINDING CHARACTERIZATION

After potential antibodies were identified and engineered (InhibRx), an ELISA coated with purified recombinant PcrV (rPcrV) antigen portrayed 18 out of 24 antibodies with superior binding (Figure 3.1). The antibodies respective $K_D$ values, which is the equilibrium dissociation constant between antibody and antigen relating to the amount of antibody needed to bind rPcrV (2 ug/mL) was calculated in GraphadPrism and shown in Table 3.1.

![Graphs showing binding of PcrV antibodies by ELISA to rPcrV coated plates](image)

Figure 3.1. Binding of PcrV antibodies by ELISA to rPcrV coated plates (2 ug/mL). This is preliminary data of n=1 in duplicate.
Table 3.1. Anti-PcrV antibody equilibrium dissociation constants (K_D)

<table>
<thead>
<tr>
<th>Anti-PcrV</th>
<th>K_D</th>
<th>Anti-PcrV</th>
<th>K_D</th>
<th>Anti-PcrV</th>
<th>K_D</th>
<th>Anti-PcrV</th>
<th>K_D</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcrV 1</td>
<td>0.0151</td>
<td>PcrV 7</td>
<td>0.0159</td>
<td>PcrV 13</td>
<td>0.7478</td>
<td>PcrV 19</td>
<td>0.0170</td>
</tr>
<tr>
<td>PcrV 2</td>
<td>0.0107</td>
<td>PcrV 8</td>
<td>0.0141</td>
<td>PcrV 14</td>
<td>0.0159</td>
<td>PcrV 20</td>
<td>0.0119</td>
</tr>
<tr>
<td>PcrV 3</td>
<td>~180.8</td>
<td>PcrV 9</td>
<td>0.0062</td>
<td>PcrV 15</td>
<td>0.0090</td>
<td>PcrV 21</td>
<td>0.0343</td>
</tr>
<tr>
<td>PcrV 4</td>
<td>0.3819</td>
<td>PcrV 10</td>
<td>0.0126</td>
<td>PcrV 16</td>
<td>0.0140</td>
<td>PcrV 22</td>
<td>0.0136</td>
</tr>
<tr>
<td>PcrV 5</td>
<td>1.1560</td>
<td>PcrV 11</td>
<td>-0.0015</td>
<td>PcrV 17</td>
<td>0.0152</td>
<td>PcrV 23</td>
<td>0.0135</td>
</tr>
<tr>
<td>PcrV 6</td>
<td>0.0137</td>
<td>PcrV 12</td>
<td>NA</td>
<td>PcrV 18</td>
<td>0.0113</td>
<td>PcrV 24</td>
<td>0.0129</td>
</tr>
</tbody>
</table>

Note: Kd is in ug/mL. The lower the Kd value, the higher the affinity of the antibody.

The antibodies were then tested for their ability to block hemolysis (Figure 3.2) and protect epithelium cell damage (Figure 3.3) and therefore confirm binding specificity to rPcrV.

![Figure 3.2](image_url)

Figure 3.2. PcrV antibodies ability to block hemolysis. The figure is representative of n=3 experiments.
Figure 3.3. PcrV antibodies ability to block lung epithelium cell (A549) damage. This is preliminary data (n=1) that supports hemolysis experiment.

Two antibodies, PcrV15 and PcrV20 demonstrated superior blocking ability (Figure 3.4). Even though PcrV21 and PcrV24 showed signs of medium hemolysis blocking (Figure 3.2), they are subfamilies of the more superior PcrV20, meaning they share CDR sequence similarities, but were not as effective blockers.

Figure 3.4. Leading antibodies PcrV15 and PcrV20 demonstrated superior blocking ability as shown in their ability to block cytotoxicity in A549 cells.
In general, there were high blocking antibodies, PcrV15 and PcrV20, medium blocking, PcrV18 and low blockers such as PcrV17 with unique amino acid sequences (Figure 3.5). PcrV18 and PcrV17 were further investigated for their blocking ability.

![Medium Hemolysis Blocking](image)

**Figure 3.5. General antibody hemolysis blocking levels.** PcrV15 and PcrV20 are high binding, PcrV18 and PcrV17 demonstrated to be medium and low hemolysis blockers respectively.

### 3.2 Epitope Binning

Using the epitope binning assay, PcrV15, PcrV17, PcrV18 and PcrV20 were tested to see if they compete for the same antigenic determinant (have similar target). The binning assay showed that both PcrV15 and PcrV20 bind to the same epitope as indicative of Figure 3.6 (violet line). Since there was no binding of PcrV15 onto the streptavidin sensor in step 6 (no change in slope), it means that the sensor was fully quenched by the previous antibody in step 5 (PcrV20). PcrV15 challenged against PcrV15 (red line) serves as a control since they bind the same epitope and should not have a change in slope in step 6. The same experiment was repeated with the addition of PcrV15 first and PcrV20 as the challenger which had similar result (not shown). See Figure 3.6 for assay details.
Figure 3.6. Epitope binning of high binding PcrV15 and PcrV20. PcrV15 was challenged against itself (red line) and PcrV20 (violet line) which proved to bind the same epitope. Step 1 represents washing of the sensor. Step 2 is the coating of the sensor with biotinylated rPcrV. Step 3 is the quenching of empty space on the sensor with biotin. Step 4 is another washing and removing of unbound protein. Step 5 is the addition of the first antibody: red line is of PcrV15 and violet line is of PcrV20. Step 6 is the addition of PcrV15 and step 5 antibody. Step 7 is the last wash to determine dissociation rate.

On the contrary to PcrV15 and PcrV20 epitope binning, there is a clear difference in epitope binding between PcrV18 and PcrV15 as shown in Figure 3.7 where the green line in step 5 represents the addition and binding of PcrV18 to the sensor coated with biotinylated rPcrV antigen. However, in step 6 we see binding of PcrV15 (increase in slope) meaning that both proteins do not compete for the same epitope. PcrV17 behaved similarly to PcrV18 (data not shown), except the binding of PcrV17 to the antigen as shown in step 5, was not as robust (low slope).

Figure 3.7. PcrV15 and PcrV18 epitope binning challenge. Red line indicates PcrV15 against PcrV15 and green line represents PcrV18 against PcrV15. The respective steps are the same as previously described in Figure 3.6.
3.3 New Format Antibody Characterization

We then investigated the combination effects of PcrV18 with our leading antibodies and found a synergy, meaning that a combination of these two VHHs was able to more effectively block bacteria from lysing eukaryotic cells as shown in Figure 3.8.

![Figure 3.8. Antibody combination of leading antibodies, PcrV15 and PcrV20 with PcrV18 showed synergistic effect for improved cytotoxicity inhibition.](image)

This led to the engineering of new antibody formats as shown in Table 3.2, with single and combinational epitopes which were tested for their ability to block cell lysis.

Table 3.2. : New format antibody structures.

<table>
<thead>
<tr>
<th>VHH</th>
<th>Tandem</th>
<th>Top-and-Tail</th>
<th>Tandem Biepitopic</th>
<th>Tandem-Top-and-Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monovalent-monospecific</td>
<td>Tetravalent-monospecific</td>
<td>Tetravalent-monospecific</td>
<td>Bivalent-Biepitopic</td>
<td>Tetravalent-biepitopic</td>
</tr>
</tbody>
</table>

Note: The different color VHHs denotes a different binding epitope. The monospecific formats can also exist in bispecific form.

A family of PcrV15 antibodies were first engineered and tested for their blocking ability. As shown in Figure 3.9, an increase in binding regions also improved the antibodies protective effect.
Figure 3.9. PcrV15 antibody format lysis inhibition comparison. PcrV15VHH are single VHHs, PcrV15 have the normal camelid structure shown in Figure 1.4, PcrV15-15 is the tandem tetravalent-monospecific. There is an increase in protection with increasing epitope binding as shown by the left curve shift.

The biepitopic tandem antibodies consisting of PcrV18 and PcrV15 VHHs were tested for their hemolysis blocking ability and results further confirmed that a combination of PcrV15 and PcrV18 VHH antibodies gave optimal protection (Figure 3.10). It also demonstrated that directionality of the VHH is important as PcrV18-15 protects better than PcrV15-18, meaning that PcrV18VHH must be at the ends of the antibody.

Figure 3.10. Hemolysis inhibition by PcrV15 and PcrV18 tandem biepitopic antibodies.

A hemolysis experiment comparing the top-and-tail formats revealed that the more binding sites per antibody did not significantly improve protection as shown in Figure 3.11. Hence, the tandem biepitopic antibody version of PcrV18-15 and PcrV18-20 proved to be the most effective and efficient blockers.

The lead antibodies, PcrV18-15 and Pcrv18-20 were further tested for their ability to protect immune cell lines including differentiated THP-1, U937 monocytes and human derived macrophages. Results in Figure 3.12 show the antibodies ability to protect the phagocytic cells from bacterial lysis, which can therefore be able to fight off bacterial infections.
Figure 3.12. Cytotoxicity tests of phagocytic cells. Anti-PcrV antibody are able to protect cells against Pa103 lysis (MOI 10). Antibodies tested reflect their availability at the time of the assay.

3.4 *in vivo* Prophylaxis and Treatment Effect of PcrV Antibodies

Due to the *in vitro* evidence of protection, initial antibodies PcrV15 and PcrV20 were tested *in vivo* mimicking a pneumonia model with prophylaxis treatment. The antibodies were compared with the current leading anti-PcrV antibody by MedImmune, V2L2. Results show all groups were statistically significant against the control antibody and can confer some level of protection (Figure 3.13). Comparing the experimental groups PcrV15, PcrV20 and V2L2 at their equimolar level (0.53 mg/kg to 1 mg/kg and 2.7 mg/kg to 5 mg/kg) excluding controls, there was no statistical significance in survival.
Figure 3.13. There was 100% survival in the PcrV15, and 87.5% survival in V2L2 and PcrV20 group at the high concentration of 5 mg/kg or equimolar amount of 2.7mg/kg. For the lower antibody concentration, there was 25%, 62.5%, 50% survival for the lower concentration PcrV15, PcrV20 and V2L2 groups respectively. There was a 25% for the control, most likely indicative not no infection. Groups are n=8 for except the controls which are n=4.

There was significant improvement in survival comparing the biepitopic PcrV18-15 and V2L2. Using the log-rank (Mantel-Coxantibody) test, PcrV18-15 was statistically different from V2L2 (p<0.0001) as shown in Figure 3.14.

It was wonderful to know that *pseudomonas aeruginosa* lung infections could be better controlled using the new format antibody, PcrV18-15. The PcrV18-20 antibody was tested in a treatment model (Figure 3.15) which only had a final 12.5% survival, but there was a significant extended survival time compared to control.
In vivo mouse pneumonia model experiment, prophylaxis treatment. There was a final 87.5% survival for PcrV18-15 at n=8 per group. Both groups were significantly different compared to control.

Figure 3.15. In vivo mouse pneumonia model experiment, prophylaxis treatment. There was a final 87.5% survival for PcrV18-15 at n=8 per group.

3.5 Investigating Anti-PcrV Antibodies Blocking Effect

As previous results and research indicate the ability of anti-PcrV antibodies to block cell lysis and improve survival, the antibodies were tested for their ability to opsonize/coat bacteria (Pa103) using FACS. Using a positive control (human IVIG antibody), it was shown that the antibodies protection is most likely due to the removal of PcrV protein from the T3SS (Figure 3.16) as there was no indication of the antibody being attached to the
bacteria. This confirms the previous findings of anti-PcrV antibodies Fc independent protection.

Figure 3.16. FACS analysis for antibody binding indicative of opzonization. Overlapping antibodies include V2L2, Control IgG, PcrV18, PcrV15, PcrV18-15, PcrV15-15, and no antibody control.
CHAPTER 4

CONCLUSION

It is with great excitement to share results that demonstrate the potential for further antibody development against pseudomonas aeruginosa infection. As previously mentioned, *P. aeruginosa* is the second most common cause of nosocomial pneumonia and the most common multidrug-resistant Gram-negative bacteria clinically isolated. It is therefore important to investigate and develop new therapies.

Previous research has shown *in vivo* protection using antibodies targeting the type three secretion system (T3SS) of virulent strains, particularly the PcrV protein. The most current anti-PcrV antibody, V2L2MD (V2L2) has been shown to be most effective by Warren et al. By using llama-derived antibodies created by Inhibrx, two particular antibodies: PcrV15, and PcrV20 demonstrated to mediate protection.

The lead antibodies in combination with an intermediate protective antibody, PcrV18 showed synergistic protection *in vitro*. Using epitope binning, we found that PcrV15 and PcrV20 share the same antigenic determinant although they have unique CDRs while PcrV18 targeted different binding site. Having two different binding epitopes would benefit therapeutic uses in cases of bacteria antibody resistance. The new format antibodies, consisting of PcrV VHHs, PcrV18, PcrV15 and PcrV20 in tandem biepitopic bivalent-biepitopic form (PcrV18-15 and PcrV18-20) have shown to have superior protection *in vitro*. More importantly, the same protection is reflected in mouse *in vivo* experiments in prophylaxis and treatment pneumonia models and has superior protection compared to V2L2 in pre-treatment models.

It is interesting to reflect back on preliminary ELISA data, particularly Table 3.1, which indicates that PcrV15, PcrV18 and PcrV20 had the lowest K_D. Upon further investigation of all twenty-four antibodies by hemolysis and cytotoxicity assays, it indicates...
that a low $K_D$ (such as that of PcrV2) and hence a high affinity is not necessarily reflective of its target function.

The function of the antibodies was briefly explored for its neutralizing function, particularly for opsonization. Results show that anti-PcrV antibodies are not present in T3SS induced bacteria and possibly strip the PcrV off resulting in a blunted needle. This would allow the host immune system a chance to clear the bacteria infection. As previous research indicates, anti-PcrV antibodies are Fc independent and therefore neutralize the pathogen. Further exploration of the antibody neutralization method would help understand if the antibody is merely blocking or perhaps stripping the protein off. What we do know is that having these antibodies present, increases the chances of survival and are therefore in the process of being modified for greater human antibody compatibility.

As the data suggest, single PcrV VHHs do not mediate protection, but we have yet to develop and test a multivalent VHH which may help the structure and function. This format would help in UTI models where the small VHHs can be excreted through the urinary system and be a more effective therapy.

The interplay of non-traditional antibody formats in particularly the exploration and characterization of antibodies from various species and the continued growth in bioinformatics and cutting edge methodologies has widened the spectra of antibody therapies. We hope to continue to optimize our lead PcrV antibodies and possibly test them against pathogens that have structurally and functionally conserved PcrV-like proteins including those of *Yersinia, Salmonella and Shigella*\(^{91,92}\).
ACKNOWLEDGEMENTS

I would like to thank Dr. Kelly Doran for allowing me to pursue my interest in microbiology in Dr. Victor Nizet’s lab. It was not easy having a split between UCSD and SDSU, but it was well worth it as I met wonderful people from both places.

To Victor: Thank you very much for accepting me into your lab family. It was exactly what I was looking for and more. I couldn’t have asked for a better lab “Dad”. Let’s schedule a meeting soon 😊

To Ross Corriden: We will publish. Signed, Julie.
To Andrew Hollands: Thank you for the opportunity and your support.

To the entire Nizet lab: Our paths will cross again. Thank you all for your support as colleagues and friends. I’ve learned from each one of you and appreciate all that you do.

To my family: Thank you for all of your support. It was not easy being in lab or school, knowing there was a husband, baby and dog that needed me at home. Los amo.
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