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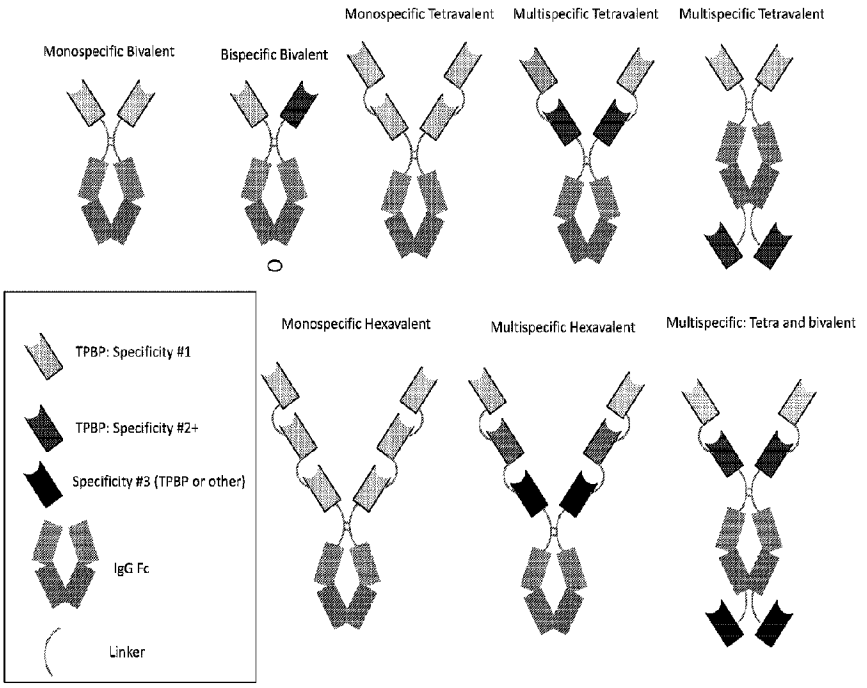

(57) Abrégé/Abstract:
This invention relates generally to molecules that specifically bind bacterial V-tip proteins of the type III secretion system of Gram negative bacteria such as PcrV from Pseudomonas aeruginosa. More specifically, this invention relates to molecules that block the injection of effector molecules into target cells. This invention also relates to molecules that specifically bind to bacterial lipoproteins, such as Oprl. The molecules of the present invention are monospecific or multispecific and can bind their target antigen in a monovalent or multivalent manner. The invention also relates generally to molecules that specifically bind bacterial cell surface proteins such as Oprl, and to methods of use these molecules in a variety of therapeutic, diagnostic, and/or prophylactic indications.

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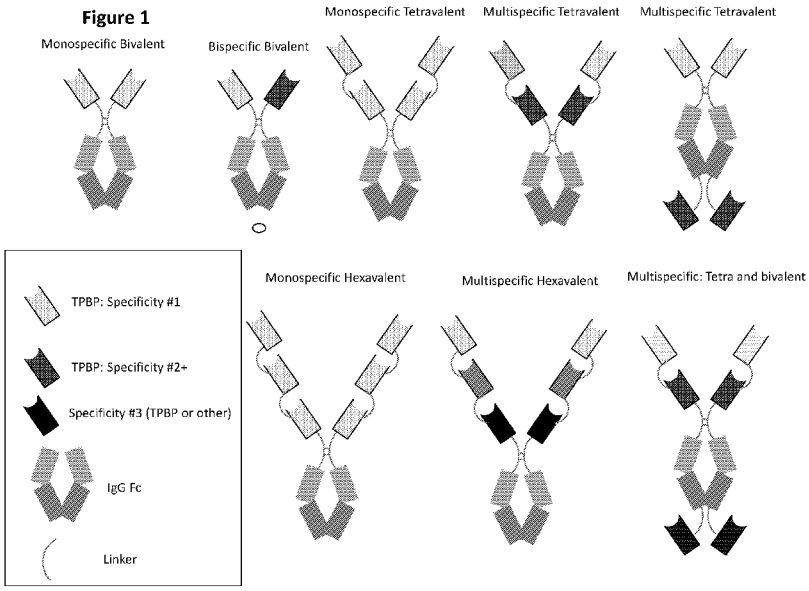
[Continued on next page]

(54) Title: TYPE III SECRETION SYSTEM TARGETING MOLECULES

Figure 1



(57) Abstract: This invention relates generally to molecules that specifically bind bacterial V-tip proteins of the type III secretion system of Gram negative bacteria such as PcrV from Pseudomonas aeruginosa. More specifically, this invention relates to molecules that block the injection of effector molecules into target cells. This invention also relates to molecules that specifically bind to bacterial lipoproteins, such as Oprl. The molecules of the present invention are monospecific or multispecific and can bind their target antigen in a monovalent or multivalent manner. The invention also relates generally to molecules that specifically bind bacterial cell surface proteins such as Oprl, and to methods of use these molecules in a variety of therapeutic, diagnostic, and/or prophylactic indications.

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TYPE III SECRETION SYSTEM TARGETING MOLECULES

RELATED APPLICATIONS

[0001] This application claims priority from U.S. Provisional Application No. 62/155,967, filed May 1, 2015 and U.S. Provisional Application No. 62/254,992, filed November 13, 2015.

FIELD OF THE INVENTION

[0002] This invention relates generally to molecules that specifically bind bacterial V-tip proteins of the type III secretion system of Gram negative bacteria such as PcrV from *Pseudomonas aeruginosa*. More specifically, this invention relates to molecules that block or otherwise inhibit the injection of effector molecules into target cells. The molecules of the present invention are monospecific or multispecific and can bind their target antigen(s) in a monovalent or multivalent manner. In some embodiments, these antibodies may be combined with a bacterial surface targeting component, binding to the *Pseudomonas* protein, OprI. The invention also relates generally to molecules that specifically bind bacterial cell surface proteins such as OprI, and to methods of use these molecules in a variety of therapeutic, diagnostic, and/or prophylactic indications.

BACKGROUND OF THE INVENTION

[0003] V-tip proteins of *Pseudomonas aeruginosa* (PcrV) are essential components of the bacterial type III secretion system (T3SS) that is capable of injecting toxic effector molecules into eukaryotic cells. The V-tip proteins are localized at the extreme end of the T3SS apparatus and oligomerization is thought to be necessary for the functional translocalization of the effector molecules across target cell membranes.

[0004] OprI is an outer membrane lipoprotein in *Pseudomonas aeruginosa* and other *Pseudomonas* species. OprI is highly conserved in *P. aeruginosa* strains and therefore represents an excellent candidate for cell-surface targeting of *Pseudomonas* bacteria.

[0005] Accordingly, there exists a need for compositions and therapies that target V-

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tip proteins of Gram negative bacteria and cell-surface components such as OprI.

SUMMARY OF THE INVENTION

[0006] The disclosure provides molecules, e.g., polypeptides including antibodies, antigen-binding antibody fragments, antibody-like polypeptides, and/or fusion polypeptides, and compositions that bind bacterial V-tip proteins of the type III secretion system of Gram negative bacteria, such as PcrV from *Pseudomonas aeruginosa*, and/or cell-surface proteins such as the OprI protein of *Pseudomonas aeruginosa*, and methods of making and using these compositions in a variety of therapeutic, diagnostic, and/or prophylactic indications. The V-tip protein targeting molecules of the present invention are monospecific or multispecific and can bind their target antigen(s) in a monovalent or multivalent manner.

[0007] These molecules are useful in binding and neutralizing or otherwise inhibiting at least one biological activity of one or more bacterial V-tip proteins of the type III secretion system of Gram negative bacteria.

[0008] *Pseudomonas aeruginosa* and other drug resistant Gram negative bacteria are a major health concern, causing community acquired and nosocomial infections. Infections with such bacteria can be serious and life-threatening. An important virulence factor is the type 3 secretion system (T3SS). The T3SS of Gram negative bacteria is responsible for translocation of toxins into eukaryotic cells, causing cell death and lysis, thereby allowing the bacterium to establish infection.

[0009] PcrV of *Pseudomonas aeruginosa*, is an example of a V-tip protein common to many Gram negative bacterial T3SSs. The V-tip protein is located at the extreme end of the T3SS apparatus and oligomerization is thought to be necessary for the functional translocalization of the effector molecules across target cell membranes. PcrV of *P. aeruginosa* is required for injection of effector molecules (ExoS, ExoT, ExoU, and ExoY) into the eukaryotic cell cytosol, resulting in cell death and lysis. PcrV of *P. aeruginosa* has been shown to be a protective antigen, suggesting that targeting the V-tip proteins of numerous Gram negative bacteria will provide an effective therapeutic option.

[0010] In some embodiments, the V-tip protein targeting molecules are antibodies and antibody-like molecules that specifically bind Gram negative bacterial V-tip proteins of the type 3 secretion system (T3SS) apparatus and block the cytotoxicity toward eukaryotic cells. In some embodiments, the V-tip protein targeting antibody, referred to as the V-tip protein binding proteins (VPBP) are derived from antibodies or antigen-binding antibody fragments including, for example, single-chain variable fragments (scFv), Fab fragments,

single domain antibodies (sdAb), V_{NAR}, or VHHs. In preferred embodiments, the VPBPs are human or humanized sdAb. The sdAb fragments can be derived from VHH, V_{NAR}, engineered VH or VK domains. VHHs can be generated from camelid heavy chain only antibodies. V_{NAR}s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric VH and VK domains, including interface engineering and selection of specific germline families.

[0011] In other embodiments, the VPBPs are derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimer, anticalin/lipocalins, centyrins and fynomers.

[0012] In preferred embodiments, the V-tip protein is or is derived from the *Pseudomonas aeruginosa* PcrV, and the VPBP specifically binds PcrV. In some embodiments, the VPBP is able to bind 2 or more VPBPs of various Gram negative bacteria, including at least PcrV from *Pseudomonas aeruginosa*,. In some embodiments, the VPBP binds to a V-tip protein from a single Gram negative bacterial species such as PcrV from *Pseudomonas aeruginosa*,. In some embodiments, the VPBP binds to a V-tip protein from more than one Gram negative bacterial species, including at least PcrV from *Pseudomonas aeruginosa*, and is thereby considered species cross-reactive.

[0013] In some embodiments, the V-tip protein targeting molecule is a fusion protein. Unexpectedly, it was discovered that enhancing the valency of the VPBP greatly enhanced the efficacy of cyto-protection from *Pseudomonas aeruginosa* both *in vitro* and *in vivo*. More surprisingly, it was found that targeting two distinct epitopes on PcrV with the distinct VPBPs in a single multispecific fusion protein resulted in an even greater protection from cytotoxicity caused by *Pseudomonas aeruginosa*. The later finding held true, even when the individual monospecific VPBP was only weakly protective. In fact, it was found that when VPBP recognizing distinct epitopes on PCRV were incorporated into a single fusion protein, they were more potent at cyto-protection compared to VPBP-containing fusion proteins that were multivalent to the same epitope on PCRV or to the combination to two separate monospecific VPBP-containing fusion proteins each recognizing a distinct epitope on PCRV.

[0014] In some embodiments, the present invention includes fusion proteins incorporating more than one VPBP and are referred to herein as multivalent. In some embodiments, the VPBPs of the fusion protein recognize the same epitope on the target V-

protein and are referred to herein as monospecific-multivalent. In other embodiments, the VPBPs of the fusion protein recognize distinct epitopes on the target V-protein and are referred to herein as multispecific-multivalent. In some embodiments, the VPBP-containing fusion protein includes two VPBPs and has a bivalent binding capacity toward the target V-tip protein. In some embodiments, the VPBP-containing fusion protein includes three VPBPs and has a trivalent binding capacity toward the target V-tip protein. In some embodiments, the VPBP-containing fusion protein includes four VPBPs and has a tetravalent binding capacity toward the target V-tip protein. In some embodiments, the VPBP-containing fusion protein includes six VPBPs and has a hexavalent binding capacity toward the target V-tip protein. In some embodiments, the VPBP-containing fusion protein includes eight VPBPs and has an octavalent binding capacity toward the target V-tip protein. In these embodiments, the VPBPs incorporated into the fusion protein of the present invention can be monospecific or multispecific.

[0015] Generally the fusion proteins of the present invention consist of at least two or more VPBPs operably linked via a linker polypeptide. The utilization of sdAb fragments as the specific VPBP within the fusion the present invention has the benefit of avoiding the heavy chain : light chain mis-pairing problem common to many bi/multispecific antibody approaches. In addition, the fusion proteins of the present invention avoid the use of long linkers necessitated by many bispecific antibodies. Furthermore, the fusion proteins of the present invention are generally smaller in size (ranging approximately from 75 to 125kDa) than a conventional antibody. This reduced molecular weight maybe enable better penetration into site of infection compared to conventional antibodies.

[0016] In some embodiments, the fusion protein of the present invention is composed of a single polypeptide. In other embodiments, the fusion protein of the present invention is composed of more than one polypeptide. For example, wherein a heterodimerization domain is incorporated into the fusion protein so as the construct an asymmetric fusion protein. For example if an immunoglobulin Fc region is incorporated into the fusion protein the CH3 domain can be used as homodimerization domain, or the CH3 dimer interface region can be mutated so as to enable heterodimerization.

[0017] In some embodiments, the fusion protein contains the VPBPs on opposite ends. For example the VPBPs are located on both the amino-terminal (N-terminal) portion of the fusion protein and the carboxy-terminal (C-terminal) portion of the fusion protein. In other embodiments, all the VPBPs reside on the same end of the fusion protein. For

example, VPBPs reside on either the amino or carboxyl terminal portions of the fusion protein.

[0018] In some embodiments, the fusion protein lacks an Fc region.

[0019] In some embodiments, the fusion protein contains an immunoglobulin Fc region. In some embodiments, the immunoglobulin Fc region is an IgG isotype selected from the group consisting of IgG1 subclass, IgG2 subclass, IgG3 subclass, and IgG4 subclass.

[0020] In some embodiments, the immunoglobulin Fc region or immunologically active fragment thereof is an IgG isotype. For example, the immunoglobulin Fc region of the fusion protein is of human IgG1 subclass, having an amino acid sequence:

PAP**E**LLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV
DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP
APIEKTISKA KGQPREPQVY TLPPSRDELT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH
EALHNHYTQK SLSLSPGK (SEQ ID NO: 1)

[0021] In some embodiments, the immunoglobulin Fc region or immunologically active fragment thereof comprises a human IgG1 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 1.

[0022] In some embodiments, the human IgG1 Fc region is modified at amino acid Asn297 (Boxed, Kabat Numbering) to prevent glycosylation of the fusion protein, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the Fc region of the fusion protein is modified at amino acid Leu235 (Boxed, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E) or Leu235Ala (L235A). In some embodiments, the Fc region of the fusion protein is modified at amino acid Leu234 (Boxed, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu234Ala (L234A). In some embodiments, the Fc region of the fusion protein is altered at both amino acid 234 and 235, *e.g.*, Leu234Ala and Leu235Ala (L234A/L235A) or Leu234Val and Leu235Ala (L234V/L235A). In some embodiments, the Fc region of the fusion protein is lacking an amino acid at one or more of the following positions to reduce Fc receptor binding: Glu233 (E233, Bold in SEQ ID NO: 1), Leu234 (L234), or Leu235 (L235). In some embodiments, the Fc region of the fusion protein is altered at Gly235 to reduce Fc receptor binding. For

example, wherein Gly235 is deleted from the fusion protein. In some embodiments, the human IgG1 Fc region is modified at amino acid Gly236 to enhance the interaction with CD32A, e.g., Gly236Ala (G236A, Boxed in SEQ ID NO: 1). In some embodiments, the human IgG1 Fc region is lacks Lys447, which corresponds to residue 218 of SEQ ID NO: 1 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0023] In some embodiments, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG2 subclass, having an amino acid sequence:

PAPPVAGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVQFNWYVD
GVEVHNAKTK PREEQFNSTF RVVSVLTVVH QDWLNGKEYK CKVSNKGLPA
PIEKTISKTK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDISVE
WESNGQPENN YKTTPPMLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE
ALHNHYTQKS LSLSPGK (SEQ ID NO: 2)

[0024] In some embodiments, the fusion or immunologically active fragment thereof comprises a human IgG2 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 2.

[0025] In some embodiments, the human IgG2 Fc region is modified at amino acid Asn297 (Boxed, to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG2 Fc region lacks Lys447, which corresponds to residue 217 of SEQ ID NO: 2 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0026] In some embodiments, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG3 subclass, having an amino acid sequence:

PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVQFKWYV
DGVEVHNAKT KPREEQYNST FRVVSVLTVL HQDWLNGKEY KCKVSNKALP
APIEKTISKT KGQPREPQVY TLPPSREEMT KNQVSLTCLV KGFYPSDIAV
EWESSGQPEN NYNTTPPMLD SDGSFFLYSK LTVDKSRWQQ GNIFSCSVMH
EALHNRFTQK SLSLSPGK (SEQ ID NO: 3)

[0027] In some embodiments, the antibody or immunologically active fragment thereof comprises a human IgG3 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 3.

[0028] In some embodiments, the human IgG3 Fc region is modified at amino acid Asn297 (Boxed, Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG3 Fc region is modified at amino acid 435 to extend the half-life, *e.g.*, Arg435His (R435H, boxed in SEQ ID NO: 3). In some embodiments, the human IgG3 Fc region is lacks Lys447, which corresponds to residue 218 of SEQ ID NO: 3 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0029] In some embodiments, the immunoglobulin Fc region or immunologically active fragment of the fusion protein is of human IgG4 subclass, having an amino acid sequence:

PAPEFLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV
DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP
SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV
EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH
EALHNHYTQK SLSLSLGK (SEQ ID NO: 4)

[0030] In some embodiments, the antibody or immunologically active fragment thereof comprises a human IgG4 polypeptide sequence that is at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 4.

[0031] In other embodiments, the human IgG4 Fc region is modified at amino acid 235 to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E). In some embodiments, the human IgG4 Fc region is modified at amino acid Asn297 (Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG4 Fc region is lacks Lys447, which corresponds to residue 218 of SEQ ID NO: 4 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0032] In some embodiments, the human IgG Fc region is modified to enhance FcRn binding. Examples of Fc mutations that enhance binding to FcRn are Met252Tyr,

Ser254Thr, Thr256Glu (M252Y, S254T, T256E, respectively) (Kabat numbering, Dall'Acqua *et al* 2006, *J. Biol Chem* Vol 281(33) 23514–23524), Met428Leu and Asn434Ser (M428L, N434S) (Zalevsky *et al* 2010 *Nature Biotech*, Vol 28(2) 157-159), Met252Ile, Thr256Asp, Met428Leu (M252I, T256D, M428L, respectively) or Met252Tyr, Met428Leu/Val (M252Y, M428L/V, respectively), (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*). Met252 corresponds to residue 23 in SEQ ID NOs: 1, 3, and 4 and residue 22 in SEQ ID NO: 2. Ser254 corresponds to corresponds to residue 25 in SEQ ID NOs: 1, 3, and 4 and residue 24 in SEQ ID NO: 2. Thr256 corresponds to residue 27 in SEQ ID NOs: 1, 3, and 4 and residue 26 in SEQ ID NO: 2. Met428 corresponds to residue 199 in SEQ ID NOs: 1, 3, and 4 and residue 198 in SEQ ID NO: 2. Asn434 corresponds to residue 205 in SEQ ID NOs: 1, 3, and 4 and residue 204 in SEQ ID NO: 2.

[0033] In some embodiments, where the fusion protein of the invention includes an Fc polypeptide, the Fc polypeptide is mutated or modified. In these embodiments, the mutated or modified Fc polypeptide includes the following mutations: Met252Tyr and Met428Leu (M252Y, M428L) using the Kabat numbering system.

[0034] In some embodiments, the human IgG Fc region is modified to alter antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), *e.g.*, the amino acid modifications described in Natsume et al., 2008 Cancer Res, 68(10): 3863-72; Idusogie et al., 2001 J Immunol, 166(4): 2571-5; Moore et al., 2010 mAbs, 2(2): 181-189; Lazar et al., 2006 PNAS, 103(11): 4005–4010, Shields et al., 2001 JBC, 276(9): 6591–6604; Stavenhagen et al., 2007 Cancer Res, 67(18): 8882-8890; Stavenhagen et al., 2008 Advan. Enzyme Regul., 48: 152-164; Alegre et al, 1992 J Immunol, 148: 3461-3468; Reviewed in Kaneko and Niwa, 2011 Biodrugs, 25(1):1-11. Examples of mutations that enhance ADCC include modification at Ser239 and Ile332, for example Ser239Asp and Ile332Glu (S239D, I332E). Examples of mutations that enhance CDC include modifications at Lys326 which corresponds to residue 97 of SEQ ID NOs: 1, 3, and 4 and residue 96 of SEQ ID NO: 2, and Glu333, which corresponds to residue 104 of SEQ ID NOs: 1, 3, and 4 and residue 103 of SEQ ID NO: 2. In some embodiments, the Fc region is modified at one or both of these positions, for example Lys326Ala and/or Glu333Ala (K326A and E333A) using the Kabat numbering system.

[0035] In some embodiments, the human IgG Fc region is modified to induce heterodimerization. For example, having an amino acid modification within the CH3

domain at Thr366, which when replaced with a more bulky amino acid, *e.g.*, Try (T366W), is able to preferentially pair with a second CH3 domain having amino acid modifications to less bulky amino acids at positions Thr366, which corresponds to residue 137 of SEQ ID NOs: 1, 3, and 4 and residue 136 of SEQ ID NO: 2, Leu368, which corresponds to residue 139 of SEQ ID NOs: 1, 3, and 4 and residue 138 of SEQ ID NO: 2, and Tyr407, which corresponds to residue 178 of SEQ ID NOs: 1, 3, and 4 and residue 177 of SEQ ID NO: 2, *e.g.*, Ser, Ala and Val, respectively (T366S/L368A/Y407V). Heterodimerization via CH3 modifications can be further stabilized by the introduction of a disulfide bond, for example by changing Ser354, which corresponds to residue 125 of SEQ ID NOs: 1, 3, and 4 and residue 124 of SEQ ID NO: 2, to Cys (S354C) and Y349, which corresponds to residue 120 of SEQ ID NOs: 1, 3, and 4 and residue 119 of SEQ ID NO: 2, to Cys (Y349C) on opposite CH3 domains (Reviewed in Carter, 2001 Journal of Immunological Methods, 248: 7–15). In some of these embodiments, the Fc region may be modified at the protein-A binding site on one member of the heterodimer so as to prevent protein-A binding and thereby enable more efficient purification of the heterodimeric fusion protein. An exemplary modification within this binding site is Ile253, which corresponds to residue 24 of SEQ ID NOs: 1, 3, and 4 and residue 23 of SEQ ID NO: 2, for example Ile253Arg (I253R). For example the I253R modification maybe combined with either the T366S/L368A/Y407V modifications or with the T366W modifications. The T366S/L368A/Y407V modified Fc is capable of forming homodimers as there is no steric occlusion of the dimerization interface as there is in the case of the T336W modified Fc. Therefore, in preferred embodiments the I253R modification is combined with the T366S/L368A/Y407V modified Fc to disallow purification any homodimeric Fc that may have formed.

[0036] In some embodiments, the human IgG Fc region is modified to prevent dimerization. In these embodiments, the fusion proteins of the present invention are monomeric. For example modification at residue Thr366 to a charged residue, e.g. Thr366Lys, Thr366Arg, Thr366Asp, or Thr366Glu (T366K, T366R, T366D, or T366E, respectively), prevents CH3-CH3 dimerization.

[0037] In some embodiments, the fusion protein contains a polypeptide derived from an immunoglobulin hinge region. The hinge region can be selected from any of the human IgG subclasses. For example the fusion protein may contain a modified IgG1 hinge having the sequence of EPKSSDKTHTCPPC (SEQ ID NO: 5), where in the Cys220 that forms a disulfide with the C-terminal cysteine of the light chain is mutated to serine, e.g.,

Cys220Ser (C220S). In other embodiments, the fusion protein contains a truncated hinge having a sequence DKTHTCPPC (SEQ ID NO: 6). In some embodiments, the fusion protein has a modified hinge from IgG4, which is modified to prevent or reduce strand exchange, *e.g.*, Ser228Pro (S228P), having the sequence ESKYGPPCPPC (SEQ ID NO: 7). In some embodiments, the fusion protein contains one or more linker polypeptides. In other embodiments, the fusion protein contains one or more linker and one or more hinge polypeptides.

[0038] In some embodiments, the fusion proteins of the present invention lack or have reduced Fucose attached to the N-linked glycan-chain at N297. There are numerous ways to prevent fucosylation, including but not limited to production in a FUT8 deficient cell line; addition inhibitors to the mammalian cell culture media, for example Castanospermine, 2-deoxy-fucose, 2-flurofucose; the use of production cell lines with naturally reduced fucosylation pathways, and metabolic engineering of the production cell line.

[0039] In some embodiments, the VPBP is engineered to eliminate recognition by pre-existing antibodies found in humans. In some embodiments, single domain antibodies of the present invention are modified by mutation of position Leu11, for example Leu11Glu (L11E) or Leu11Lys (L11K). In other embodiments, single domain antibodies of the present invention are modified by changes in carboxy-terminal region, for example the terminal sequence consists of GQGTLVTVKPGG (SEQ ID NO: 8) or GQGTLVTVEPGG (SEQ ID NO: 9) or modification thereof. In some embodiments, the single domain antibodies of the present invention are modified by mutation of position 11 and by changes in carboxy-terminal region.

[0040] In some embodiments, the VPBPs of the fusion proteins of the present invention are operably linked via amino acid linkers. In some embodiments, these linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present invention can be of various lengths, for example 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids in length.

[0041] In some embodiments, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, i.e., $(GGS)_2$ (SEQ ID NO: 75); GGSGGSGGS, i.e., $(GGS)_3$ (SEQ ID NO: 76); GGSGGSGGSGGS, i.e., $(GGS)_4$ (SEQ ID NO: 77); GGSGGSGGSGGSGGS, i.e., $(GGS)_5$ (SEQ ID NO: 45), GGGGS (SEQ ID NO:

78); GGGGSGGGGS, i.e., ($GGGGS_2$) (SEQ ID NO: 79), and GGGGSGGGGSGGGGS, i.e., ($GGGGS_3$) (SEQ ID NO: 80).

[0042] In some embodiments, the fusion protein is tetravalent. In some embodiments, the tetravalent fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some embodiments, the tetravalent fusion protein has the following structure: VHH-Linker-Hinge-Fc-Linker-VHH, where the VHH is a humanized or fully human VHH sequence.

[0043] In some embodiments, the fusion protein is hexavalent. In some embodiments, the hexavalent fusion protein has the following structure: VHH-Linker-VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some embodiments, the hexavalent fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc-Linker-VHH, or VHH-Linker-Hinge-Fc-Linker-VHH-Linker-VHH where the VHH is a humanized or fully human VHH sequence.

[0044] In some embodiments, the fusion protein lacks an Fc region. In these embodiments, wherein the fusion protein is tetravalent, the protein has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker. In these embodiments, wherein the fusion protein is pentavalent, the protein has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH. In these embodiments, wherein the fusion protein is hexavalent, the protein has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH. In these embodiments, the VHH is a humanized or fully human VHH sequence.

[0045] In some embodiments, the VPBP-containing fusion protein may also contain additional binding domains that recognize non-V-tip proteins of gram negative bacteria such as PcrV from *Pseudomonas aeruginosa*,. These additional bacterial binding domains may confer additional functionality to the fusion protein of the present invention. These additional functionalities may include neutralization of additional bacterial virulence or growth factors or enable opsono-phagocytosis of the bacteria by host phagocytic cells. In some embodiments, the VPBP-containing fusion protein may also contain additional binding domains that recognize non-bacterial proteins. These additional non-bacterial binding domains, may confer additional functionality to the fusion protein of the present invention. These additional functionalities may enhance immune cell recruitment or activation, including neutrophils, natural killer cells, macrophages, monocytes, dendritic cells and T-cells.

[0046] In some embodiments, the VPBP-containing fusion protein may also contain additional binding domains that recognize the outer membrane protein I (OprI) protein or a fragment thereof. In a preferred embodiment, the VPBP-containing fusion protein includes at least a first domain that binds PcrV or a fragment thereof and a second domain that binds OprI or a fragment thereof. These bispecific fusion proteins are referred to herein as "PcrV x OprI bispecific fusion proteins," "PcrV x OprI fusion proteins" and/or "PcrV x OprI fusions." OprI is a cell surface protein that is highly conserved amongst *P. aeruginosa* strains. OprI is anchored to outer membrane via N-term Cys-lipidation, is present in 100% of *P. aeruginosa* strains tested, and is 100% conserved in genome sequenced *P. aeruginosa* strains.

[0047] In some embodiments, the first domain comprises one or more sequences from the PcrV sequences shown in Table 1. In some embodiments, the second domain comprises one or more sequences that bind OprI. In some embodiments, the second domain comprises one or more sequences from the OprI sequences shown in Table 2.

[0048] Dual targeting of PcrV and OprI allows the fusion polypeptides to tether or otherwise attach and/or bind to the bacteria cell surface, and it provides enhanced protection *in vivo*.

[0049] In some embodiments, the V-tip protein targeting molecule comprises one or more sequences from the PcrV sequences shown in Table 1.

[0050] The molecules provided herein exhibit inhibitory activity, for example by inhibiting at least one biological activity of one or more V-tip proteins of Gram negative bacteria, such as for example, functional translocalization of the effector molecules across target cell membranes. The molecules provided herein completely or partially reduce or otherwise modulate expression or activity of one or more V-tip proteins of Gram negative bacteria upon binding to, or otherwise interacting with, the V-tip protein(s) such as PcrV from *Pseudomonas aeruginosa*,. The reduction or modulation of a biological function of one or more V-tip proteins of Gram negative bacteria is complete or partial upon interaction between the molecules and the V-tip protein(s). The molecules are considered to completely inhibit expression or activity of one or more V-tip proteins of Gram negative bacteria when the level of expression or activity of the V-tip protein(s) in the presence of the molecule is decreased by at least 95%, *e.g.*, by 96%, 97%, 98%, 99% or 100% as compared to the level of expression or activity of the V-tip protein(s) in the absence of interaction, e.g., binding, with a molecule described herein. The molecules are considered to partially inhibit

expression or activity one or more V-tip proteins of Gram negative bacteria when the level of expression or activity of the V-tip protein(s) in the presence of the molecule is decreased by less than 95%, *e.g.*, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 75%, 80%, 85% or 90% as compared to the level of expression or activity of the V-tip protein(s) in the absence of interaction, e.g., binding, with a molecule described herein.

[0051] The V-tip protein targeting molecules provided herein are useful in treating, alleviating a symptom of, ameliorating and/or delaying the progression of a disease or disorder in a subject suffering from or identified as being at risk for a disease or disorder associated with at least one biological activity of one or more V-tip proteins of Gram negative bacteria such as PcrV from *Pseudomonas aeruginosa*, such as, for example, functional translocalization of the effector molecules across target cell membranes.

[0052] The disclosure provides molecules, e.g., polypeptides including antibodies, antigen-binding antibody fragments, antibody-like polypeptides, and/or fusion polypeptides, and compositions that bind bacterial non-V-tip proteins of the type III secretion system of Gram negative bacteria, such as OprI from *Pseudomonas aeruginosa*, and methods of making and using these compositions in a variety of therapeutic, diagnostic, and/or prophylactic indications. The OprI-targeting molecules of the present invention are monospecific or multispecific and can bind their target antigen(s) in a monovalent or multivalent manner.

[0053] In some embodiments, the OprI-protein targeting molecules are antibodies and antibody-like molecules that specifically bind OprI. In some embodiments, the antibody or antigen-binding fragment thereof are derived from antibodies or antigen-binding antibody fragments including, for example, single-chain variable fragments (scFv), Fab fragments, single domain antibodies (sdAb), V_{NAR}, or VHHs. In some embodiments, the anti-OprI antibodies are human or humanized sdAb. The sdAb fragments can be derived from VHH, V_{NAR}, engineered VH or VK domains. VHHs can be generated from camelid heavy chain only antibodies. V_{NAR}s can be generated from cartilaginous fish heavy chain only antibodies. Various methods have been implemented to generate monomeric sdAbs from conventionally heterodimeric VH and VK domains, including interface engineering and selection of specific germline families.

[0054] In other embodiments, the anti-OprI targeting molecules are derived from non-antibody scaffold proteins for example but not limited to designed ankyrin repeat proteins (darpins), avimer, anticalin/lipocalins, centyrins and fynomers.

[0055] In some embodiments, the anti-OprI targeting molecule is an antibody or antigen-binding fragment thereof comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 46-70 and 88. In some embodiments, the anti-OprI targeting antibody or antigen-binding fragment thereof also comprises an immunoglobulin Fc region or immunologically active fragment thereof. In some embodiments, the anti-OprI targeting antibody or antigen-binding fragment thereof also comprises an immunoglobulin Fc region or immunologically active fragment thereof comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-4.

[0056] In some embodiments, the fusion protein of the present invention is composed of a single polypeptide. In other embodiments, the fusion protein of the present invention is composed of more than one polypeptide. For example, wherein a heterodimerization domain is incorporated into the fusion protein so as the construct an asymmetric fusion protein. For example if an immunoglobulin Fc region is incorporated into the fusion protein the CH3 domain can be used as homodimerization domain, or the CH3 dimer interface region can be mutated so as to enable heterodimerization.

[0057] In some embodiments, the fusion protein contains the VPBPs on opposite ends. For example the VPBPs are located on both the amino-terminal (N-terminal) portion of the fusion protein and the carboxy-terminal (C-terminal) portion of the fusion protein. In other embodiments, all the VPBPs reside on the same end of the fusion protein. For example, VPBPs reside on either the amino or carboxyl terminal portions of the fusion protein.

[0058] In some embodiments, the present invention includes fusion proteins incorporating more than one OprI targeting sequence and are referred to herein as multivalent. In some embodiments, the OprI targeting sequences of the fusion protein recognize the same epitope on OprI and are referred to herein as monospecific-multivalent. In other embodiments, the OprI targeting sequences of the fusion protein recognize distinct epitopes on OprI and are referred to herein as multispecific-multivalent. In some embodiments, the OprI targeting sequence-containing fusion protein includes two OprI targeting sequences and has a bivalent binding capacity toward the OprI. In some embodiments, the OprI targeting sequence-containing fusion protein includes three OprI targeting sequences and has a trivalent binding capacity toward the OprI. In some embodiments, the OprI targeting sequence-containing fusion protein includes four OprI targeting sequences and has a tetravalent binding capacity toward the OprI. In some

embodiments, the OprI targeting sequence-containing fusion protein includes six OprI targeting sequences and has a hexavalent binding capacity toward the OprI. In some embodiments, the OprI targeting sequence-containing fusion protein includes eight OprI targeting sequences and has an octavalent binding capacity toward the OprI. In these embodiments, the OprI targeting sequences incorporated into the fusion protein of the present invention can be monospecific or multispecific.

[0059] In some embodiments, the fusion protein lacks an Fc region.

[0060] In some embodiments, the fusion protein comprises an immunoglobulin Fc region or immunologically active fragment thereof comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-4.

[0061] In some embodiments, the Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide includes a human IgG1 region. In some embodiments, human IgG1 Fc region is modified at amino acid Asn297 (Boxed, Kabat Numbering) to prevent glycosylation of the antibody and/or fusion protein, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the Fc region of the antibody and/or fusion protein is modified at amino acid Leu235 (Boxed, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E) or Leu235Ala (L235A). In some embodiments, the Fc region of the antibody and/or fusion protein is modified at amino acid Leu234 (Boxed, Kabat Numbering) to alter Fc receptor interactions, *e.g.*, Leu234Ala (L234A). In some embodiments, the Fc region of the antibody and/or fusion protein is altered at both amino acid 234 and 235, *e.g.*, Leu234Ala and Leu235Ala (L234A/L235A) or Leu234Val and Leu235Ala (L234V/L235A). In some embodiments, the Fc region of the antibody and/or fusion protein is lacking an amino acid at one or more of the following positions to reduce Fc receptor binding: Glu233 (E233, Bold in SEQ ID NO: 1), Leu234 (L234), or Leu235 (L235). In some embodiments, the Fc region of the antibody and/or fusion protein is altered at Gly235 to reduce Fc receptor binding. For example, wherein Gly235 is deleted from the antibody and/or fusion protein. In some embodiments, the human IgG1 Fc region is modified at amino acid Gly236 to enhance the interaction with CD32A, e.g., Gly236Ala (G236A, Boxed in SEQ ID NO: 1). In some embodiments, the human IgG1 Fc region is lacks Lys447, which corresponds to residue 218 of SEQ ID NO: 1 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0062] In some embodiments, the Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide includes a human

IgG2 region. In some embodiments, the human IgG2 Fc region is modified at amino acid Asn297 (Boxed, to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG2 Fc region lacks Lys447, which corresponds to residue 217 of SEQ ID NO: 2 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0063] In some embodiments, the Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide includes a human IgG3 region. In some embodiments, the human IgG3 Fc region is modified at amino acid Asn297 (Boxed, Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG3 Fc region is modified at amino acid 435 to extend the half-life, *e.g.*, Arg435His (R435H, boxed in SEQ ID NO: 3). In some embodiments, the human IgG3 Fc region is lacks Lys447, which corresponds to residue 218 of SEQ ID NO: 3 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0064] In some embodiments, the Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide includes a human IgG4 region. In other embodiments, the human IgG4 Fc region is modified at amino acid 235 to alter Fc receptor interactions, *e.g.*, Leu235Glu (L235E). In some embodiments, the human IgG4 Fc region is modified at amino acid Asn297 (Kabat Numbering) to prevent to glycosylation of the antibody, *e.g.*, Asn297Ala (N297A) or Asn297Asp (N297D). In some embodiments, the human IgG4 Fc region is lacks Lys447, which corresponds to residue 218 of SEQ ID NO: 4 (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*).

[0065] In some embodiments, the human IgG Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide is modified to enhance FcRn binding. Examples of Fc mutations that enhance binding to FcRn are Met252Tyr, Ser254Thr, Thr256Glu (M252Y, S254T, T256E, respectively) (Kabat numbering, Dall'Acqua *et al* 2006, *J. Biol Chem* Vol 281(33) 23514–23524), Met428Leu and Asn434Ser (M428L, N434S) (Zalevsky *et al* 2010 *Nature Biotech*, Vol 28(2) 157-159), Met252Ile, Thr256Asp, Met428Leu (M252I, T256D, M428L, respectively) or Met252Tyr, Met428Leu/Val (M252Y, M428L/V, respectively), (EU index of *Kabat et al* 1991 *Sequences of Proteins of Immunological Interest*). Met252 corresponds to residue 23 in SEQ ID NOs: 1, 3, and 4 and residue 22 in SEQ ID NO: 2. Ser254 corresponds to

corresponds to residue 25 in SEQ ID NOs: 1, 3, and 4 and residue 24 in SEQ ID NO: 2. Thr256 corresponds to residue 27 in SEQ ID NOs: 1, 3, and 4 and residue 26 in SEQ ID NO: 2. Met428 corresponds to residue 199 in SEQ ID NOs: 1, 3, and 4 and residue 198 in SEQ ID NO: 2. Asn434 corresponds to residue 205 in SEQ ID NOs: 1, 3, and 4 and residue 204 in SEQ ID NO: 2.

[0066] In some embodiments, the Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide is mutated or modified. In these embodiments, the mutated or modified Fc polypeptide includes the following mutations: Met252Tyr and Met428Leu (M252Y, M428L) using the Kabat numbering system.

[0067] In some embodiments, the human IgG Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide is modified to alter antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), *e.g.*, the amino acid modifications described in Natsume et al., 2008 Cancer Res, 68(10): 3863-72; Idusogie et al., 2001 J Immunol, 166(4): 2571-5; Moore et al., 2010 mAbs, 2(2): 181-189; Lazar et al., 2006 PNAS, 103(11): 4005–4010, Shields et al., 2001 JBC, 276(9): 6591–6604; Stavenhagen et al., 2007 Cancer Res, 67(18): 8882-8890; Stavenhagen et al., 2008 Advan. Enzyme Regul., 48: 152-164; Alegre et al, 1992 J Immunol, 148: 3461-3468; Reviewed in Kaneko and Niwa, 2011 Biodrugs, 25(1):1-11. Examples of mutations that enhance ADCC include modification at Ser239 and Ile332, for example Ser239Asp and Ile332Glu (S239D, I332E). Examples of mutations that enhance CDC include modifications at Lys326 which corresponds to residue 97 of SEQ ID NOs: 1, 3, and 4 and residue 96 of SEQ ID NO: 2, and Glu333, which corresponds to residue 104 of SEQ ID NOs: 1, 3, and 4 and residue 103 of SEQ ID NO: 2. In some embodiments, the Fc region is modified at one or both of these positions, for example Lys326Ala and/or Glu333Ala (K326A and E333A) using the Kabat numbering system.

[0068] In some embodiments, the human IgG Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide is modified to induce heterodimerization. For example, having an amino acid modification within the CH3 domain at Thr366, which when replaced with a more bulky amino acid, *e.g.*, Try (T366W), is able to preferentially pair with a second CH3 domain having amino acid modifications to less bulky amino acids at positions Thr366, which corresponds to residue 137 of SEQ ID NOs: 1, 3, and 4 and residue 136 of SEQ ID NO: 2, Leu368, which

corresponds to residue 139 of SEQ ID NOs: 1, 3, and 4 and residue 138 of SEQ ID NO: 2, and Tyr407, which corresponds to residue 178 of SEQ ID NOs: 1, 3, and 4 and residue 177 of SEQ ID NO: 2, *e.g.*, Ser, Ala and Val, respectively (T366S/L368A/Y407V). Heterodimerization via CH3 modifications can be further stabilized by the introduction of a disulfide bond, for example by changing Ser354, which corresponds to residue 125 of SEQ ID NOs: 1, 3, and 4 and residue 124 of SEQ ID NO: 2, to Cys (S354C) and Y349, which corresponds to residue 120 of SEQ ID NOs: 1, 3, and 4 and residue 119 of SEQ ID NO: 2, to Cys (Y349C) on opposite CH3 domains (Reviewed in Carter, 2001 Journal of Immunological Methods, 248: 7–15). In some of these embodiments, the Fc region may be modified at the protein-A binding site on one member of the heterodimer so as to prevent protein-A binding and thereby enable more efficient purification of the heterodimeric fusion protein. An exemplary modification within this binding site is Ile253, which corresponds to residue 24 of SEQ ID NOs: 1, 3, and 4 and residue 23 of SEQ ID NO: 2, for example Ile253Arg (I253R). For example the I253R modification maybe combined with either the T366S/L368A/Y407V modifications or with the T366W modifications. The T366S/L368A/Y407V modified Fc is capable of forming homodimers as there is no steric occlusion of the dimerization interface as there is in the case of the T336W modified Fc. Therefore, in preferred embodiments the I253R modification is combined with the T366S/L368A/Y407V modified Fc to disallow purification any homodimeric Fc that may have formed.

[0069] In some embodiments, the human IgG Fc region of the OprI targeting antibody or antigen-binding fragment thereof or the OprI-targeting fusion polypeptide is modified to prevent dimerization. In these embodiments, the antibodies and/or fusion proteins of the present invention are monomeric. For example modification at residue Thr366 to a charged residue, e.g. Thr366Lys, Thr366Arg, Thr366Asp, or Thr366Glu (T366K, T366R, T366D, or T366E, respectively), prevents CH3-CH3 dimerization.

[0070] In some embodiments, the fusion proteins of the present invention are operably linked via amino acid linkers. In some embodiments, these linkers are composed predominately of the amino acids Glycine and Serine, denoted as GS-linkers herein. The GS-linkers of the fusion proteins of the present invention can be of various lengths, for example 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acids in length.

[0071] In some embodiments, the GS-linker comprises an amino acid sequence selected from the group consisting of GGSGGS, i.e., $(GGS)_2$ (SEQ ID NO: 75);

GGSGGSGGS, i.e., $(GGS)_3$ (SEQ ID NO: 76); GGSGGSGGSGGS, i.e., $(GGS)_4$ (SEQ ID NO: 77); GGSGGSGGSGGSGGS, i.e., $(GGS)_5$ (SEQ ID NO: 45), GGGGS (SEQ ID NO: 78); GGGGSGGGGS, i.e., $(GGGGS_2)$ (SEQ ID NO: 79), and GGGGSGGGGSGGGGS, i.e., $(GGGGS_3)$ (SEQ ID NO: 80).

[0072] In some embodiments, the fusion protein is tetravalent. In some embodiments, the tetravalent fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some embodiments, the tetravalent fusion protein has the following structure: VHH-Linker-Hinge-Fc-Linker-VHH, where the VHH is a humanized or fully human VHH sequence.

[0073] In some embodiments, the fusion protein is hexavalent. In some embodiments, the hexavalent fusion protein has the following structure: VHH-Linker-VHH-Linker-VHH-Linker-Hinge-Fc, where the VHH is a humanized or fully human VHH sequence. In some embodiments, the hexavalent fusion protein has the following structure: VHH-Linker-VHH-Linker-Hinge-Fc-Linker-VHH, or VHH-Linker-Hinge-Fc-Linker-VHH-Linker-VHH where the VHH is a humanized or fully human VHH sequence.

[0074] In some embodiments, the fusion protein lacks an Fc region. In these embodiments, wherein the fusion protein is tetravalent, the protein has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker. In these embodiments, wherein the fusion protein is pentavalent, the protein has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH. In these embodiments, wherein the fusion protein is hexavalent, the protein has the following structure VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH-Linker-VHH. In these embodiments, the VHH is a humanized or fully human VHH sequence.

[0075] It will be appreciated that administration of therapeutic entities in accordance with the invention will be administered with suitable carriers, buffers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed, Mack Publishing Company, Easton, PA (1975)), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as Lipofectin™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-

solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in treatments and therapies in accordance with the present invention, provided that the active ingredient in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. *See* also Baldrick P. "Pharmaceutical excipient development: the need for preclinical guidance." Regul. Toxicol Pharmacol. 32(2):210-8 (2000), Wang W. "Lyophilization and development of solid protein pharmaceuticals." Int. J. Pharm. 203(1-2):1-60 (2000), Charman WN "Lipids, lipophilic drugs, and oral drug delivery-some emerging concepts." J Pharm Sci. 89(8):967-78 (2000), Powell *et al.* "Compendium of excipients for parenteral formulations" PDA J Pharm Sci Technol. 52:238-311 (1998) and the citations therein for additional information related to formulations, excipients and carriers well known to pharmaceutical chemists.

BRIEF DESCRIPTION OF FIGURES

[0076] Figure 1 is a series of schematic representations of exemplary VPBP-containing fusion proteins of the present disclosure. VPBP recognizing distinct epitopes are differentially shaded in these schematic representations.

[0077] Figures 2A, 2B, and 2C are a series of graphs depicting hemolysis analysis using various VPBP-containing fusion proteins of the disclosure.

[0078] Figures 3A and 3B are a series of graphs depicting cytotoxicity analysis using various VPBP-containing fusion proteins of the disclosure. The A549 cell line was used as the target cell line.

[0079] Figures 4A, 4B, and 4C are a series of graphs depicting survival analysis in an infection model using various VPBP-containing fusion proteins of the disclosure. The V2L2 mAb was used a positive control as PCRV blocking antibody.

[0080] Figure 5 is a graph depicting binding of an example OprI antibody to bind to a variety of *Pseudomonas aeruginosa* strains and to *Pseudomonas putida.*

[0081] Figure 6 is a graph depicting the ability of various VPBP-containing fusion proteins of the disclosure to bind *P. aeruginosa* via OprI.

[0082] Figure 7 is a graph depicting the ability of various VPBP-containing fusion proteins of the disclosure to provide superior protection *in vivo* in *P. aeruginosa* prophylaxis-pneumonia model. Included herein is an exemplary multispecific, PCRV-OprI (PCRV-18-15-OprI-7), fusion protein of the disclosure demonstrating enhanced protective

capacity over a bispecific targeting PCRV and PSL (disclosed in US20150284450 and DiGiandomenico *et al.*, "*A multifunctional bispecific antibody protects against Pseudomonas aeruginosa,*" Sci Transl Med., vol. 6(262): 262ra155 (2014)) at equivalent molar dose.

DETAILED DESCRIPTION

[0083] Unless otherwise defined, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art. Standard techniques are used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (*e.g.,* electroporation, lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. *See e.g.,* Sambrook *et al.* Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients. The term patient includes human and veterinary subjects.

[0084] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0085] As used herein, the terms "targeting fusion protein" and "antibody" can be synonyms. As used herein, the term "antibody" refers to immunoglobulin molecules and immunologically active portions of immunoglobulin (Ig) molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. By

"specifically bind" or "immunoreacts with" "or directed against" is meant that the antibody reacts with one or more antigenic determinants of the desired antigen and does not react with other polypeptides or binds at much lower affinity ($K_d > 10^{-6}$). Antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, dAb (domain antibody), single chain, Fab, Fab' and $F(ab')_2$ fragments, F_v, scFvs, an Fab expression library, and single domain antibody (sdAb) fragments, for example V_HH, V_{NAR}, engineered V_H or V_K.

[0086] The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. In general, antibody molecules obtained from humans relate to any of the classes IgG, IgM, IgA, IgE and IgD, which differ from one another by the nature of the heavy chain present in the molecule. Certain classes have subclasses (also known as isotypes) as well, such as IgG_1, IgG_2, and others. Furthermore, in humans, the light chain may be a kappa chain or a lambda chain.

[0087] The term "monoclonal antibody" (mAb) or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one molecular species of antibody molecule consisting of a unique light chain gene product and a unique heavy chain gene product. In particular, the complementarity determining regions (CDRs) of the monoclonal antibody are identical in all the molecules of the population. MAbs contain an antigen binding site capable of immunoreacting with a particular epitope of the antigen characterized by a unique binding affinity for it.

[0088] The term "antigen-binding site" or "binding portion" refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains, referred to as "hypervariable regions," are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus, the term "FR" refers to amino acid sequences which are naturally found between, and adjacent to, hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three-dimensional space to form an antigen-binding surface. The

antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs." The assignment of amino acids to each domain is in accordance with the definitions of Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk J. Mol. Biol. 196:901-917 (1987), Chothia *et al.* Nature 342:878-883 (1989).

[0089] The single domain antibody (sdAb) fragments portions of the fusion proteins of the present invention are referred to interchangeably herein as targeting polypeptides herein.

[0090] As used herein, the term "epitope" includes any protein determinant capable of specific binding to/by an immunoglobulin or fragment thereof, or a T-cell receptor. The term "epitope" includes any protein determinant capable of specific binding to/by an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. An antibody is said to specifically bind an antigen when the dissociation constant is ≤ 1 μM; *e.g.*, ≤ 100 nM, preferably ≤ 10 nM and more preferably ≤ 1 nM.

[0091] As used herein, the terms "immunological binding" and "immunological binding properties" and "specific binding" refer to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (k_{on}) and the "off rate constant" (k_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. (*See* Nature 361:186-87 (1993)). The ratio of k_{off} /k_{on} enables the cancellation of all parameters not related to affinity, and is equal to the dissociation constant K_d. (*See, generally*, Davies et al. (1990) Annual Rev Biochem 59:439-473). An antibody of the

present invention is said to specifically bind to an antigen, when the equilibrium binding constant (K_d) is ≤1 μM, preferably ≤ 100 nM, more preferably ≤ 10 nM, and most preferably ≤ 100 pM to about 1 pM, as measured by assays such as radioligand binding assays, surface plasmon resonance (SPR), flow cytometry binding assay, or similar assays known to those skilled in the art.

[0092] Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

[0093] Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide- containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur- containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine valine, glutamic- aspartic, and asparagine-glutamine.

[0094] As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules are contemplated as being encompassed by the present invention, providing that the variations in the amino acid sequence maintain at least 75%, more preferably at least 80%, 90%, 95%, and most preferably 99%. In particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic amino acids are aspartate, glutamate; (2) basic amino acids are lysine, arginine, histidine; (3) non-polar amino acids are alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and (4) uncharged polar amino acids are glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. The hydrophilic amino acids include arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and threonine. The hydrophobic amino acids include alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine. Other families of amino acids include (i) serine and threonine, which are the aliphatic-hydroxy family; (ii) asparagine and glutamine, which are the amide containing family; (iii) alanine, valine, leucine and

isoleucine, which are the aliphatic family; and (iv) phenylalanine, tryptophan, and tyrosine, which are the aromatic family. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein. Fragments or analogs of antibodies or immunoglobulin molecules can be readily prepared by those of ordinary skill in the art. Preferred amino- and carboxy-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. Bowie *et al.* Science 253:164 (1991). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in accordance with the invention.

[0095] Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (4) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various muteins of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally- occurring sequence (preferably in the portion of the polypeptide outside the domain(s) forming intermolecular contacts. A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (*e.g.*, a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden

and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton et al. Nature 354:105 (1991).

[0096] The term "polypeptide fragment" as used herein refers to a polypeptide that has an amino terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the naturally-occurring sequence deduced, for example, from a full length cDNA sequence. Fragments typically are at least 5, 6, 8 or 10 amino acids long, preferably at least 14 amino acids long' more preferably at least 20 amino acids long, usually at least 50 amino acids long, and even more preferably at least 70 amino acids long. The term "analog" as used herein refers to polypeptides which are comprised of a segment of at least 25 amino acids that has substantial identity to a portion of a deduced amino acid sequence and which has specific binding to CD47, under suitable binding conditions. Typically, polypeptide analogs comprise a conservative amino acid substitution (or addition or deletion) with respect to the naturally- occurring sequence. Analogs typically are at least 20 amino acids long, preferably at least 50 amino acids long or longer, and can often be as long as a full-length naturally-occurring polypeptide.

[0097] Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics". Fauchere, J. Adv. Drug Res. 15:29 (1986), Veber and Freidinger TINS p.392 (1985); and Evans *et al.* J. Med. Chem. 30:1229 (1987). Such compounds are often developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (*i.e.,* a polypeptide that has a biochemical property or pharmacological activity), such as human antibody, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -- CH_2NH--, --CH_2S-, --CH_2-CH_2--, --CH=CH--(cis and trans), --$COCH_2$--, $CH(OH)CH_2$--, and -CH_2SO--, by methods well known in the art. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (*e.g.,* D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch Ann. Rev. Biochem. 61:387

(1992)); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

[0098] The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, and/or an extract made from biological materials.

[0099] As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker, *e.g.*, by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (*e.g.*, streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods). In certain situations, the label or marker can also be therapeutic. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (*e.g.*, ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I), fluorescent labels (*e.g.*, FITC, rhodamine, lanthanide phosphors), enzymatic labels (*e.g.*, horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (*e.g.*, leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance. The term "pharmaceutical agent or drug" as used herein refers to a chemical compound or composition capable of inducing a desired therapeutic effect when properly administered to a patient.

[00100] As used herein, the terms "treat," treating," "treatment," and the like refer to reducing and/or ameliorating a disorder and/or symptoms associated therewith. By "alleviate" and/or "alleviating" is meant decrease, suppress, attenuate, diminish, arrest, and/or stabilize the development or progression of a disease such as, for example, a cancer. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

[00101] In this disclosure, "comprises," "comprising," "containing," "having," and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; the terms "consisting essentially of" or "consists essentially" likewise have the meaning ascribed in U.S. Patent law and these terms are open-ended, allowing for the presence of more than that which is recited so long as basic or novel

characteristics of that which is recited are not changed by the presence of more than that which is recited, but excludes prior art embodiments.

[00102] By "effective amount" is meant the amount required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

[00103] By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, rodent, ovine, primate, camelid, or feline.

[00104] The term "administering," as used herein, refers to any mode of transferring, delivering, introducing, or transporting a therapeutic agent to a subject in need of treatment with such an agent. Such modes include, but are not limited to, oral, topical, intravenous, intraperitoneal, intramuscular, intradermal, intranasal, and subcutaneous administration.

[00105] By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

[00106] Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

[00107] Unless specifically stated or obvious from context, as used herein, the terms "a," "an," and "the" are understood to be singular or plural. Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive.

[00108] Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise

clear from the context, all numerical values provided herein are modified by the term "about."

[00109] Therapeutic formulations of the invention, which include a V-tip protein targeting molecule of the invention, are used to treat or alleviate a symptom associated with a disease or disorder associated with aberrant activity and/or expression of one or more V-tip proteins of Gram negative bacteria, such as PcrV from *Pseudomonas aeruginosa*, in a subject. A therapeutic regimen is carried out by identifying a subject, *e.g.*, a human patient suffering from (or at risk of developing) a disease or disorder associated with aberrant activity and/or expression of one or more V-tip proteins of Gram negative bacteria, such as PcrV from *Pseudomonas aeruginosa*, using standard methods, including any of a variety of clinical and/or laboratory procedures. The term patient includes human and veterinary subjects. The term subject includes humans and other mammals.

[00110] Efficaciousness of treatment is determined in association with any known method for diagnosing or treating the particular disease or disorder associated with aberrant activity and/or expression of one or more V-tip proteins of Gram negative bacteria, such as PcrV from *Pseudomonas aeruginosa*. Alleviation of one or more symptoms of the disease or disorder associated with aberrant activity and/or expression of one or more V-tip proteins of Gram negative bacteria, such as PcrV from *Pseudomonas aeruginosa*, indicates that the V-tip protein targeting molecule confers a clinical benefit.

[00111] Methods for the screening of V-tip protein targeting molecules that possess the desired specificity include, but are not limited to, enzyme linked immunosorbent assay (ELISA), enzymatic assays, flow cytometry, and other immunologically mediated techniques known within the art.

[00112] The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: Hemolysis Blocking

[00113] The ability of the VPBPs of the present invention to block bacterial induced hemolysis of red blood cells (RBCs) can be assessed by numerous protocols known in the art. For example, human RBCs were washed in PBS and resuspended at 2% (v/v) in DMEM. *Pseudomonas aeruginosa* bacteria plus serially diluted antibodies were added to RBCs in 96 well round bottom plates. The plates were incubated for 2 h at 37°C and then 2 h at 4°C. The plates were then spun to pellet intact RBCs, after which the supernatant was

transferred to a flat bottom 96-well plate for spectrophotometric observation of released hemoglobin.

[00114] As shown in Figures 2A-2D, both monospecific and multispecific multivalent VPBPs of the present invention are able to block bacterial induced hemolysis of RBCs.

Example 2: Cytotoxicity Blocking

[00115] The ability of the VPBPs of the present invention to block bacterial induced cytotoxicity of mammalian cells can be assessed by numerous protocols known in the art. For example, A confluent monolayer of A549 (lung epithelial) cells were grown in 96-well plates. Cells were loaded with Calcein AM and then washed to remove excess Calcein. *P. aeruginosa* and antibodies at varying concentrations were added to the A549 cells and incubated for 2 h at 37°C. Monolayers were then washed, after which the remaining cells were quantified by fluorescence.

[00116] As shown in Figures 3A-3B, both monospecific and multispecific multivalent VPBPs of the present invention are able to block bacterial induced cytotoxicity of mammalian cells.

Example 3: *Pseudomonas aeruginosa* Infection Model

[00117] The ability of the VPBPs of the present invention to protect against a bacterial infection can be assessed using a mouse model of *P. aeruginosa* infection. Mice were pre-treated with PcrV antibodies 24 h prior to infection with *P. aeruginosa*. At t=0 mice were intra-tracheally infected with *P. aeruginosa* and survival was monitored for 4 days. Importantly, it was discovered that the multispecific multivalent VPBP-containing fusion proteins conferred substantially more protection compared to monospecific multivalent VPBP-containing fusion proteins (Figures 4A-4C). The multispecific multivalent VPBP-containing fusions of the present invention also are substantially more potent than the anti-PCRV antibody, V2L2, known in the art to be a potent blocker of *P. aeruginosa* induced hemolysis (*see e.g.*, PCT/US2012/063639, published as WO 2013/0170565).

Example 4: OprI antibodies bind to multiple strains

[00118] The ability of OprI targeting antibodies to bind to *Pseudomonas* strains can

be assessed by whole cell bacterial ELISA. Bacterial cultures were grown to mid-logarithmic phase in standard bacteriologic media, then washed and resuspended in PBS. Equal volumes of bacterial suspension were placed in 96 well plates and incubated at 37C for 24 h. Plates were blocked with BSA and then serial dilutions of antibodies were added. After subsequent washing, HRP-conjugated anti-human Fc specific secondary antibody was added. Following incubation and washing, TMB substrate was added and absorbance at 600 nm was measured to detect binding of antibodies to bacteria. As shown in Figure 5, OprI antibodies were found to bind to all strains of *Pseudomonas aeruginosa* tested, as well as *Pseudomonas putida*.

Example 5: Bispecific Molecules in *Pseudomonas aeruginosa* Infection Model

[00119] The studies presented herein demonstrate the ability of the VPBPs of the present invention to protect against a bacterial infection can be assessed using a mouse model of *P. aeruginosa* infection. In particular, these studies use a VPBP that binds both PcrV and outer membrane protein I ("OprI"), also referred to herein as "PcrV x OprI bispecific fusion proteins," "PcrV x OprI fusion proteins" and/or "PcrV x OprI fusions." Dual targeting of PcrV and OprI allows the fusion polypeptides to tether or otherwise attach and/or bind to the bacteria cell surface, and the studies provided herein demonstrate that this dual-targeting also produces enhanced protection *in vivo*.

[00120] The PcrV x OprI bispecific fusions target the bacterial cell surface by targeting OprI. Figure 6 demonstrates that the PcrV x OprI bispecific fusions of the disclosure bind to *P. aeruginosa* by flow cytometry.

[00121] The PcrV x OprI bispecific fusions also are more potent *in vivo* than the bispecific molecule Bis4, which binds PcrV and PSL and is known in the art to be a blocker *P. aeruginosa* induced hemolysis (*see e.g.*, DiGiandomenico et al., "*A multifunctional bispecific antibody protects against Pseudomonas aeruginosa,*" Sci Transl Med., vol. 6(262): 262ra155 (2014)). Figure 7 demonstrates that the PcrV x OprI bispecific fusion proteins provide superior protection *in vivo* in a *P. aeruginosa* pneumonia-prophylaxis animal model.

Table 1. PcrV-VPBP Sequences

PcrV1A (1A7)
EVQLVQSGGGLVQAGGSLRLSCAASGRIFGTYGMGWFRQAPGKERVFVAAISKSG
PTTYYADSVKGRFTISRDNAENTVYLQMNSLKPEDTAVYYCGASSHSMLVVTTSQ
VDYWGRGTQVTVSS (SEQ ID NO: 10)

PcrV2A (1B9)
EVQLVQSGGGLVQPGGSLRLSCAVSGLIFDNYGIGWFRQAPEKEREGVSCIHESDGS
TYYTDSVKGRFAISRDNAKNTGYLEMNNLKPEDTAVYYCVVLSYVSRCPEGSKYD
YWGQGTQVTVSS (SEQ ID NO: 11)

PcrV3A (1B12)
EVQLVQSGGGLVQPGGSLRLSCAASGFTLDYYPIGWFRQAPGKEREGVSCISSSEGS
TYYADSVKGRFTISRDNAKNTVYLQMNNMKPEDTAVYYCATDFFTTGCPSGGGK
YDYWGQGTQVTVSS (SEQ ID NO: 12)

PcrV4A (1G9)
EVQLVQSGGGLVRAGGSLRLSCAPSERTFGSFGMGWFRQAPGKEREFVAALMWG
TSYTSYADSVKGRFTVSKDNAKNTLYLQMNSLKPEDTAVYYCAAGAVGADPRRY
DYWGQGTQVTVSS (SEQ ID NO: 13)

PcrV5A (2A5)
EVQLVQSGGGLVQAGGSLRLSCAASGLAFRNYRMGWFRQAPGKEREFVAAISGNI
GGSGVGTDYADSVKGRFTISRDNDKDTAYLQMNSLKPEDTAVYYCAADHHLTML
PGEYDFWGEGTQVTVSS (SEQ ID NO: 14)

PcrV6A (2A11)
EVQLVQSGGGLVQPGGSLRLSCAASGSTLDYYAIGWFRQAPGKEREGVACISSSDG
STDYADSMKGRFTISRDNAQKTVYLQMNSLKPEDTAVYSCAAVAFFCGSSWYLSS
GMDYWGKGTQVTVSS (SEQ ID NO: 15)

PcrV7A (2A12)
EVQLVQSGGGLVQAGGSLRLSCAASGGTFSSNAMYWYRQAPGKQRELVASISGTS
NANYPDSVKGRFTISRDNAKNTVTLQMNSLKPEDTAVYYCRAAPVSGPLIGRIFWG
QGTQVTVSS (SEQ ID NO: 16)

PcrV8A (2B4)
EVQLVQSGGGLVQAGGSLRLSCATSGLTFSVYAMGWFRQAPGKQREFVARITAGG
SGTYYADSMEGRFTISRDNARNTVYLQMNSLKPEDTAVYYCAAARHWTRGTEHL
PTAYDYWGQGTQVTVSS (SEQ ID NO: 17)

PcrV9A (2B7)
EVQLVQSGGGLVQAGGSLRLSCASSGSTFRTYGMGWFRQPPGKQREWVAGMAID
GLTTYADSAKGRFTASRDNARNIVYLQMNELKPEDTAVYYCYAAGYWGQGTQVT
VSS (SEQ ID NO: 18)

PcrV10A (2G6)
EVQLVQSGGGLVQAGGSLRLSCTTSGITFSDNAMYWYRQAPGKQRELVASISSGG
WTNYADSVKGRFTISRDNVKNTVTLQMNSLEPEDTALYYCRAAPVRGNFIGRVFW
GQGTQVTVSS (SEQ ID NO: 19)

PcrV11A (4H7)
EVQLVQSGGGLVQPGGSLRLSCAAFGSIFTIGTMGWYRQAPGKQRELVATITRGSS
TNYADSVKGRFTISIDSAKNTVYLQMNSLKSEDTAVYYCAADRGAVGPAMRVVA
DYWGQGTQVTVSS (SEQ ID NO: 20)

PcrV12A (3B7)
EVQLVQSGGGLVQAGGSLRLSCAASGSTFSSNAMYWYRQAPGKQRELVASISDGG
FTTYYADSVKGRFTISKDNAENTVYLQMNIMKPEDTAVYYCAASISSRVVVHTAQ
ADYWGQGTQVTVSS (SEQ ID NO: 21)

PcrV13A (4G2)
EVQLVQSGGGLVQPGGSLRLPCAASGSIFTIGTMGWYRQAPGKQRELVATITRGSS
TNYADSVKDRFTISRDNAKRTLHLQMNGLKAEDTAVYYCATDLFENSCPLKHDFW
GQGTQVTVSS (SEQ ID NO: 22)

PcrV14A (4G10)
EVQLVQSGGGLVQAGGSLRLSCAASRITFALYVIDWYRQTPESQRELVARIRPEGL
AVYADSVKGRFTISRDNGRNTAYLQMNSLQEEDTAVYYCHADPVFTPGRNDYWG
QGTQVTVSS (SEQ ID NO: 23)

PcrV15A (4G9)
EVQLVQSGGGLVQPGESLRLSCAASGSIFSINTMVWYRQVPGKQRELVASITNQGIP
HYADSVKGRFTISRENAKNTVNLQMNSLKPEDTAVYVCNAWIRSDGVSPYLNYW
GQGTQVTVSS (SEQ ID NO: 24)

PcrV16A (3B10)
EVQLVQSGGGLVQPGGSLGLSCVGSGSISGIHTMGWYRRAPGNQRELIATATSAGI
TNYSESVKGRFTISRDNAKSTVYLQMSSLKPEDTGVYYCNDVFGRTSWGQGTQVT
VSS (SEQ ID NO: 25)

PcrV17A (3G1)
EVQLVQSGGGLVQPGGSLRLSCAASGNIFGGNVMGWYRQAPGKQRELVAGIGSLG
RTTYADSVKGRFSISRDNAKNTVYLQMDSLKPEDTAVYYCNVVRLGGPDYWGQG
TQVTVSS (SEQ ID NO: 26)

PcrV18A (3F2)
EVQLVQSGGGLVQAGGSLRLSCTTSGNTFSDNAMYWYRQAPGKQREQVASISSGG
WTNYADSVKGRFTISRDNVKNTVTLQMDRLEPEDTALYYCRAAPVRGYLIGRVFW
GQGTQVTVSS (SEQ ID NO: 27)

PcrV19A (4E12)
EVQLVQSGGGLVQAGGSLRLSCSASGSNSIFNMGWYRQRPGRQRELVALISSGTGS
TSYAGSVKGRFAISRDNAKATVYLQMNSLKLEDTAVYYCRITTDNARLVYWGQGT
QVTVSS (SEQ ID NO: 28)

PcrV20A (3C1)
EVQLVQSGGGLVQPGGSLRLSCAASGRIFSVNNMGWYRQTPGKQRELVAVITVNG
ITTYSDSVKGRFTLSRDNAKNTIYLQMNSLKPEDTAVYSCYGYIRLAATNPYVQYW
GQGTQVTVSS (SEQ ID NO: 29)

PcrV21A (3C7)
EVQLVQSGGGLVQPGGSLRLSCAASGSIFSINTMGWYRQAPGNQRDIVATITMNGV
PHYADAVKGRFTISRDNAKNTVYLQMNGLKPEDTAVYYCNAWINLYGSPPLQNY
WGQGTQVTVSS (SEQ ID NO: 30)

PcrV22A (4H8)
EVQLVQSGGGLVQAGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVTSITNQGI
PHYADSVKGRFTISRENAKNTVNLQMNSLKPEDTAVYVCNAWIRGDGGSPYLNY
WGQGTQVTVSS (SEQ ID NO: 31)

PcrV23A (1G6)
EVQLVQSGGGLVQPGESLRLSCAASGSIFSINTMVWYRQVPGKQRELVASITNQGIP
HYADSVKGRFTISRENAKNTVNLQMNSLKPEDTAVYVCNAWIRSDGVPPYLNYW
GQGTQVTVSS (SEQ ID NO: 32)

PcrV24A (2E1)
EVQLVQSGGGLVQPGGSLRLSCAASGSIFNINSMHWYRQAPGNQRELVASISKGGI
TNYADSVKGRFAISRDDAQNTLYLQMNSLKPEDTAVYYCNAWISEIATGPILYNY
WGQGTQVTVSS (SEQ ID NO: 33)

PcrV25A (1F9)
EVQLVQSGGGLVQPGGSLSLSCAASGSVFSINRMAWYRQAPGKQRELVADIGTMG
ASDYADSVKGRFTISRDNAKKTVDLQMNSLKPEDTAVYFCNAWMRGAPDVAYTN
YWGQGTQVTVSS (SEQ ID NO: 34)

PcrV26A (4H1)
EVQLVQSGGGLVQPGGSLRLSCAASGRVVSINNMGWYQQTPGNQRELVAIITLNG
VTTYADSVKGRFTISRDNAKNTVYLQMASLKPEDTAIYYCNAWVRTVPGSAYSNY
WGQGTQVTVSS (SEQ ID NO: 35)

PcrV27A (1B4)
EVQLVQSGGDLVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKQRELVAVITMNG
VTTYEDSVKGRFTLSRDNAKNTIYLQMNSLKPEDTAVYFCYGYIRLAATNPYVQY
WGQGTQVTVSS (SEQ ID NO: 36)

hzPcrV15v1
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKQRELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSDGVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 37)

hzPcrV15v2
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKQRELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSYGVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 38)

hzPcrV15v3
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKQRELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSEGVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 39)

hzPcrV15v4
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKQRELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSQGVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 40)

hzPcrV15DAv5
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKQRELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSDAVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 41)

hzPcrV15DTv6
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKQRELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSDTVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 42)

hzPcrV15v7
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKGRELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSQGVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 81)

hzPcrV15v8
EVQLLESGGGEVQPGGSLRLSCAASGSIFSINTMVWYRQAPGKGLELVSSITNQGIP
HYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNAWIRSQGVSPYLNYWG
QGTLVTVKP (SEQ ID NO: 82)

hzPcrV18
EVQLLESGGGEVQPGGSLRLSCAASGNTFSDNAMYWYRQAPGKQRELVSSISSGG
WTNYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCRAAPVRGYLIGRVFW
GQGTLVTVKP (SEQ ID NO: 43)

hzPcrV18v2
EVQLLESGGGEVQPGGSLRLSCAASGNTFSDNAMYWYRQAPGKGRELVSSISSGG
WTNYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCRAAPVRGYLIGRVFW
GQGTLVTVKP (SEQ ID NO: 83)

hzPcrV18v3
EVQLLESGGGEVQPGGSLRLSCAASGNTFSDNAMYWYRQAPGKGLELVSSISSGG
WTNYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCRAAPVRGYLIGRVFW
GQGTLVTVKP (SEQ ID NO: 84)

hzPcrV20v1
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKQRELVSVITVNGI
TTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQYW
GQGTLVTVKP (SEQ ID NO: 44)

hzPcrV20v2
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKQRELVSVITVGGI
TTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQYW
GQGTLVTVKP (SEQ ID NO: 71)

hzPcrV20v3
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKQRELVSVITVQGI
TTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQYW
GQGTLVTVKP (SEQ ID NO: 72)

hzPcrV20v4
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKQRELVSVITNQGI
TTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQYW
GQGTLVTVKP (SEQ ID NO: 73)

hzPcrV20v5
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKQRELVSVITVSGI
TTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQYW
GQGTLVTVKP (SEQ ID NO: 74)

hzPcrV20v6
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKGRELVSVITNQGI
TTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQYW
GQGTLVTVKP (SEQ ID NO: 85)

hzPcrV20v7
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKGLELVSVITNQGI
TTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQYW
GQGTLVTVKP (SEQ ID NO: 86)

hzPcrV20v8
EVQLLESGGGEVQPGGSLRLSCAASGRIFSVNNMGWYRQAPGKGLEWVSVITNQG
ITTYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCYGYIRLAATNPYVQY
WGQGTLVTVKP (SEQ ID NO: 87)

Table 2. OprI Binding Protein Sequences

OprI-VHH-1 (also referred to herein as "OprI-1")
QLQLQESGGGLVQSGRSLRLSCSASGSLFRFDTVWWYRQAPGKQREWVAYITAGG
MTNYADSVKGRFTISKDNAKNMVYLQMDSLLPEDTAVYYCNVGRNWGQGTQVT
VSS (SEQ ID NO: 46)

OprI-VHH-2 (also referred to herein as "OprI-2")
EVQLVQSGGGLVQPGESLRLSCAASGNIFRFDTVWWYRQPPGEQREWVSYITAGSI
TNYADSVKGRFTISRDNAKNMVYLQMDNLKPEDTAVYYCRVGGSSWGQGTQVT
VSS (SEQ ID NO: 47)

OprI-VHH-3 (also referred to herein as "OprI-3")
EVQLVQSGGGLVQAGDSLRLSCAASGGISSTYAMGWFRQAPGKEREFVASIRLGSE
ATYYADSVKGRFTISRDNALKTIYLQMNSLKPDDTAVYYCAVDASLFLVTVDYWG
RGTQVTVSS (SEQ ID NO: 48)

OprI-VHH-4 (also referred to herein as "OprI-4")
QVQLVQSGGGLVQAGGSLRLSCAASGRTFSRCVMGWFRQAPGKEREFVATISWSG
ASTVYADSVKGRFTISRENAKNTVYLQMNSLKPEDTAVYYCAAAESSWNGDIRLK
GYDYWGQGTQVTVSS (SEQ ID NO: 49)

OprI-VHH-5 (also referred to herein as "OprI-5")
QVTLKESGGGLVQAGGSLRLSCAASGRSFRTYTMAWFRQPPGKEREFVAAITWSG
GSTFYADPVKGRFTISRDNAKNTVYLQMNTLKPEDTAVYYCAVETSISGRYTVFQP
RFYDSWGQGTQVTVSS (SEQ ID NO: 50)

OprI-VHH-6 (also referred to herein as "OprI-6")
QVQLQESGGGLVQPGESLRLSCAASGNIFRFDTVWWYRQPPGEQREWVSYITAGSI
TNYADSVKGRFIISRDNAKNMVYLQMDNLKPEDTAVYYCRVGGGSWGQGTQVTV
SS (SEQ ID NO: 51)

OprI-VHH-7 (also referred to herein as "OprI-7")
EVQLVQSGGGLVQPGGSLRLSCIASGSIFSTKTMGWYRQAPGKQREWVALITTGLS
TQYLDSLEGRFTISRDNANNRVFLQMNNLKPEDTGVYYCNVVPGRGATYWGKGT
QVTVSS (SEQ ID NO: 52)

OprI-VHH-8 (also referred to herein as "OprI-8")
QLQLQESGGGLVQPGRSLRLSCAGSGSIFRYDTVWWYRQAPGKQREWVAYVTAG
GITNYADSVKGRFTISKDNAKNMVYLQMDSLLPEDTAVYYCHVGRNWGQGTQVT
VSS (SEQ ID NO: 53)

OprI-VHH-9 (also referred to herein as "OprI-9")
QLQLQESGGGLVQAGGSLRLSCAASGRTFSSNVYSMGWFRQAPGKEREFVSAITW
RGGTTYYADSVKDRFTISKDNAKNTVYLQMNSLKSEDTAVYYCACSRMDSTRYD
YWGQGTQVTVSS (SEQ ID NO: 54)

OprI-VHH-11 (also referred to herein as "OprI-11")
EVQLVQSGGGLVQSGRSLRLSCSASGSLFRFDTVWWYRQAPGKQREWVAYITAGG
ITNYADSVKGRFTISKDNAKNMVYLQMDSLLPEDTAVYYCSVGRNWGQGTQVTV
SS (SEQ ID NO: 55)

OprI-VHH-12 (also referred to herein as "OprI-12")
QVQLQESGGGLVQPGGSLRLSCAASGITVRINTMGWYRQAPGKQRELVAYITSGGI
TNYVDSVKGRFTIARDDAKNTVYLQMNSLKPEDTAVYYCNVHGWRDFWGQGTQ
VTVSS (SEQ ID NO: 56)

OprI-VHH-13 (also referred to herein as "OprI-13")
QVQLVQSGGGLVQPGGSLRLSCAASGTIFRFNTMAWYRQAPGKQREFVAYITWAG
MTGYQDSVQDRFTISRDNAKNTVSLQMNNLKPEDTAVYFCNKHGSSFVRDYWGQ
GTQVTVSS (SEQ ID NO: 57)

OprI-VHH-14 (also referred to herein as "OprI-14")
EVQLVQSGGGLVQPGGSLRLSCAAAGSDFAIGAMGWYRQAPGKQRDFVAHITSGG
IPSFADSVKGRFTLSRDNAKNTVYLQMDSLKPDDTAVYYCYLRKRGSGTTTWGQG
TQVTVSS (SEQ ID NO: 58)

OprI-VHH-15 (also referred to herein as "OprI-15")
QVQLQESGGGLVQAGGSLRLSCAASGRIFSNCVMGWFRQAPGKEREFVAAISWSG
DTTHYADSLKGRFAISRDNANNTVFLQKDSLTPSDTAVYYCAASSRITSCQAMGVV
PLLQPWYDYWGRGTQVTVSS (SEQ ID NO: 59)

OprI-VHH-16 (also referred to herein as "OprI-16")
QVQLQESGGGLVQPGRSLRLSCAASGNIFRFDTVWWYRQAPGKQREWVAYVTAG
GITNYADSVKGRFTISKDNAKNIVYLHTDNLAPEDTAVYYCRVGQNWGQGTQVTV
SS (SEQ ID NO: 60)

OprI-VHH-17 (also referred to herein as "OprI-17")
QLQLQESGGGLVQPGGSPRLSCAASESIFRFNTMAWYRQAPGKQRELVAYITWAG
RTDYGDFVKGRFTISRDNAKNTVSLQMNSLKPEDTAVYYCNKHGSRFERDYWGQ
GTQVTVSS (SEQ ID NO: 61)

OprI-VHH-18 (also referred to herein as "OprI-18")
QVQLQESGGDLVQPGGSLRLSCVASETIFRFNTMAWYRQAPGKRRELVGYITWAG
RTGYGDFVEGRFTISRDNSKNTVSLQMNSLKPEDTAVYYCNKHGSSFTQDYWGQG
TQVTVSS (SEQ ID NO: 62)

OprI-VHH-19 (also referred to herein as "OprI-19")
QLQLQESGGDLVQPGGSLRLSCVASETIFRFNTMAWYRQAPGKRRELVGYITWAG
RTGYGDFVEGRFTVSRDNSKNTVSLQMNSLKPEDTAVYYCNKHGASFTQDYWGQ
GTQVTVSS (SEQ ID NO: 63)

OprI-VHH-21 (also referred to herein as "OprI-21")
QLQLQESGGGLVRPGSSLTLSCVASETIFRFNTMAWYRQAPGKRRELVGYITWAGR
TGYGDFVEGRFTISRDNSKNTVSLQMNSLEPEDTADYYCNKHGSSFLRDYWGQGT
QVTVSS (SEQ ID NO: 64)

OprI-VHH-22 (also referred to herein as "OprI-22")
QVQLQESGGGLVQPGRSLRLSCAGSGSMFRFDTVWWYRQAPGKQRDWVSYITAG
SIANYADSVKGRFTISRDNTKNMVYLQMDSLKPEDTAVYYCRVGGNSWGQGTQV
TVSS (SEQ ID NO: 65)

OprI-VHH-23 (also referred to herein as "OprI-23")
QVQLQQSGGGLVQPGGSLRLSCEASSNIFRFNTMAWYRQAPGKQREFAAYITWAG
LTGYGDSLKGRFIISRDNAKNIVTLQMNSLKPEDTAVYYCNKHGSDFVRDYWGQG
TQVTVSS (SEQ ID NO: 66)

hzOprI-7v1 (also referred to herein as "OprI-7v1")
EVQLLESGGGEVQPGGSLRLSCAASGSIFSTKTMGWYRQAPGKQREWVSLITTGLS
TQYAESVKGRFTISRDNANNTVYLQMSSLRAEDTAVYYCNVVPGRGATYWGQGT
LVTVKP (SEQ ID NO: 67)

hzOprI-7v2 (also referred to herein as "OprI-7v2")
EVQLLESGGGEVQPGGSLRLSCAASGSIFSTKTMGWYRQAPGKQREWVSLITTGLS
TQYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNVVPGRGATYWGQGT
LVTVKP (SEQ ID NO: 68)

hzOprIv3 (also referred to herein as "OprI-7v3")
EVQLLESGGGEVQPGGSLRLSCAASGSIFSTKTMGWYRQAPGKGLEWVSLITTGLS
TQYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNVVPGRGATYWGQGT
LVTVKP (SEQ ID NO: 69)

hzOprI-7v4 (also referred to herein as "OprI-7v4")
EVQLLESGGGEVQPGGSLRLSCAASGSIFSTKTMGWYRQAPGKGLEWVSLITTGLS
TQYAESVKGRFTISRDNAKNTVYLQMSSLRAEDTAVYYCNVVPGRGATYWGQGT
LVTVKP (SEQ ID NO: 70)

hzOprI-7v5
EVQLLESGGGEVQPGGSLRLSCAASGSIFSTKTMGWYRQAPGKGREWVSLITTGLS
TQYAESVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCNVVPGRGATYWGQGT
LVTVKP (SEQ ID NO: 88)

What is claimed is:

1. An isolated polypeptide comprising a first binding domain that binds PcrV from *Pseudomonas aeruginosa* and a second binding domain that binds OprI from *Pseudomonas aeruginosa* wherein the first and second binding domains are antibodies or antigen-binding fragments thereof, and wherein the polypeptide inhibits the functional translocalization of effector molecules across target cell membranes.

2. The isolated polypeptide of claim 1, wherein the first binding domain is a first VHH domain and the second binding domain is a second VHH domain.

3. The isolated polypeptide of claim 2, wherein the first VHH domain comprises the CDRs of SEQ ID NO: 41 as determined by Chothia numbering.

4. The isolated polypeptide of claim 2 or claim 3, wherein the second VHH domain comprises the CDRs of SEQ ID NO: 62 as determined by Chothia numbering

5. The isolated polypeptide of claim 3 or claim 4, wherein the first VHH domain is humanized.

6. The isolated polypeptide of any one of claims 3-5, wherein the second VHH domain is humanized.

7. The isolated polypeptide of any one of claims 1-6, wherein the polypeptide comprises a third binding domain that binds PcrV from *Pseudomonas aeruginosa*, wherein the first binding domain and the third binding domain bind different epitopes of PcrV.

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8. The isolated polypeptide of claim 7, wherein the third binding domain is a VHH domain.

9. The isolated polypeptide of any one of claims 1-8, wherein the polypeptide comprises an immunoglobulin Fc region polypeptide.

10. The isolated polypeptide of claim 9, wherein the immunoglobulin Fc region polypeptide comprises the amino acid sequence of SEQ ID NOs: 1, 2, 3, or 4.

11. The isolated polypeptide of any one of claims 1-10, wherein the polypeptide is able to block *Pseudomonas aeruginosa* induced hemolysis of red blood cells in an *in vitro* assay.

12. The isolated polypeptide of any one of claims 1-11, wherein the polypeptide is able to block *Pseudomonas aeruginosa* induced cytotoxicity of mammalian cells in an *in vitro* assay.

13. The isolated polypeptide of any one of claims 1-12 for use in the treatment of *Pseudomonas aeruginosa* infection in a subject in need thereof.

14. The isolated polypeptide for use of claim 13, wherein the *Pseudomonas aeruginosa* infection is a community acquired infection.

15. The isolated polypeptide for use of claim 13, wherein the *Pseudomonas aeruginosa* infection is a nosocomial infection.

16. The isolated polypeptide of any one of claims 1-12 for use in the prevention of *Pseudomonas aeruginosa* infection in a subject at risk of developing *Pseudomonas aeruginosa* infection.

17. The isolated polypeptide for use of claim 16, wherein the *Pseudomonas aeruginosa* infection is a community acquired infection.

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18. The isolated polypeptide for use of claim 16, wherein the *Pseudomonas aeruginosa* infection is a nosocomial infection.

Date Reçue/Date Received 2022-08-30

Figure 1

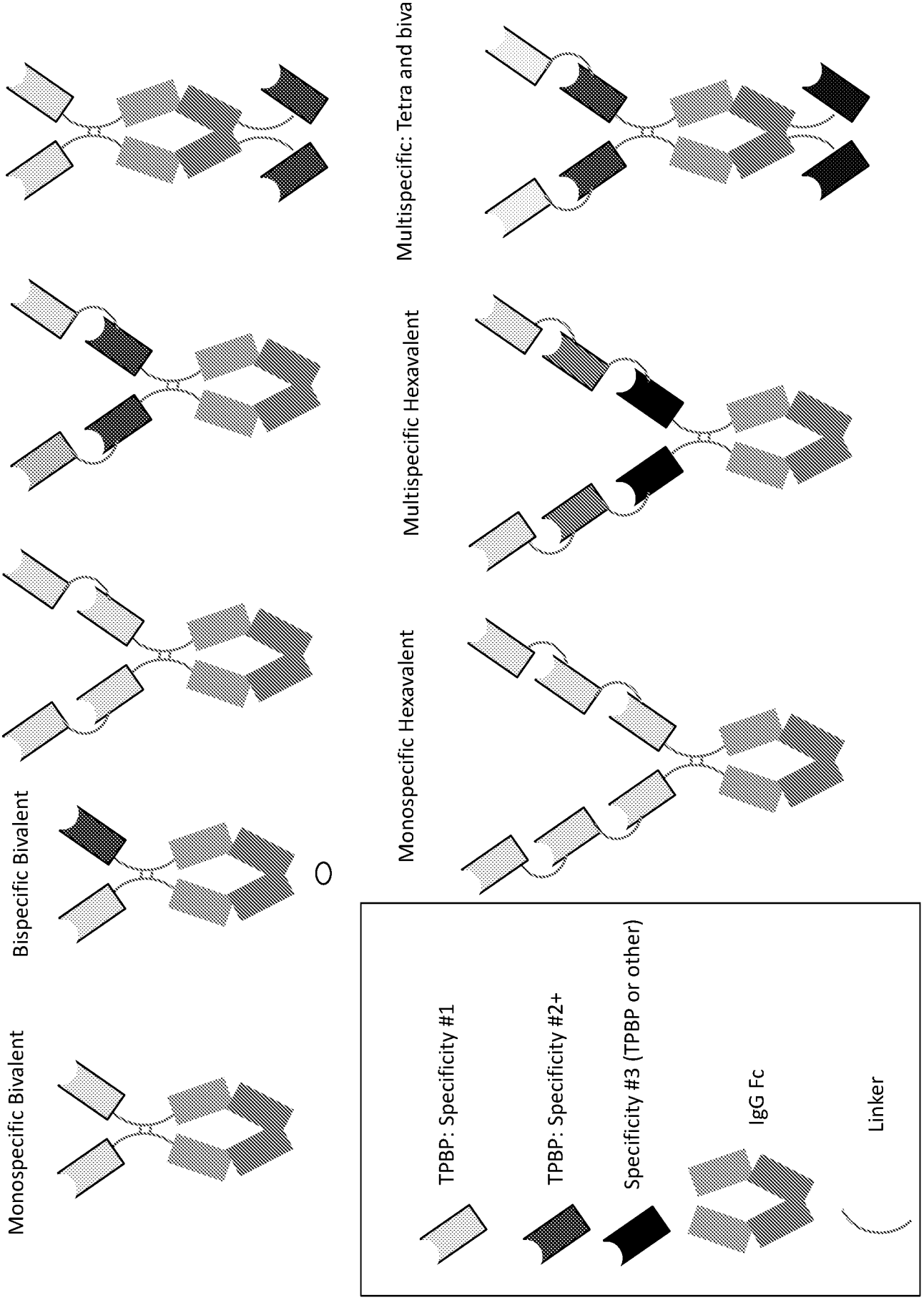

Monospecific Bivalent
Bispecific Bivalent
Monospecific Tetravalent
Multispecific Tetravalent
Multispecific Tetravalent
Monospecific Hexavalent
Multispecific Hexavalent
Multispecific: Tetra and bivalent
TPBP: Specificity #1
TPBP: Specificity #2+
Specificity #3 (TPBP or other)
IgG Fc
Linker

Figure 2A

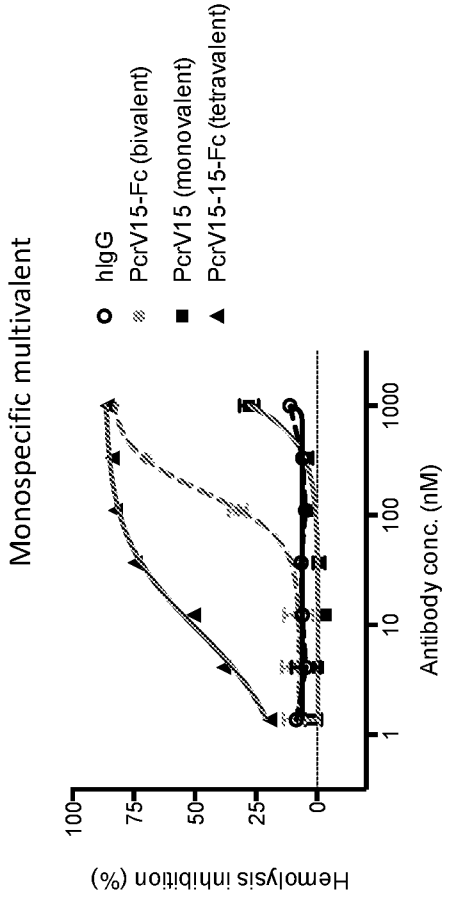


Figure 2B

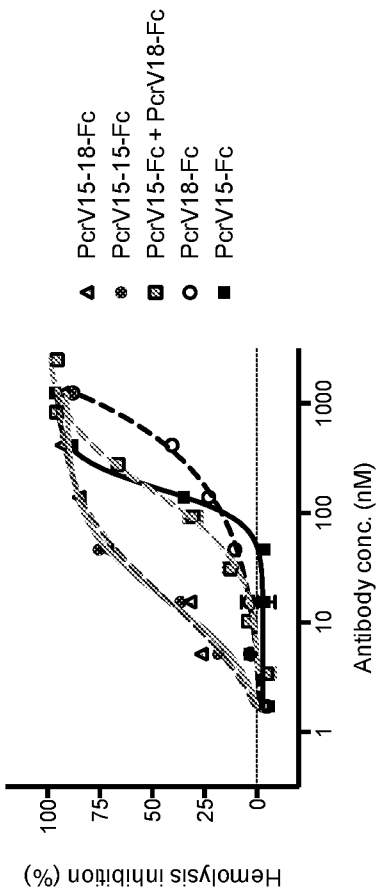


Figure 2C

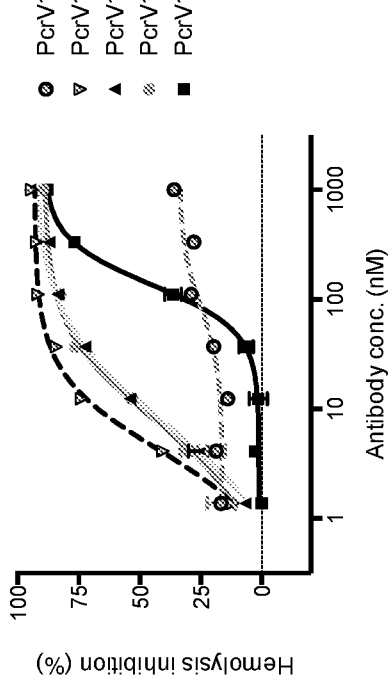


Figure 3A

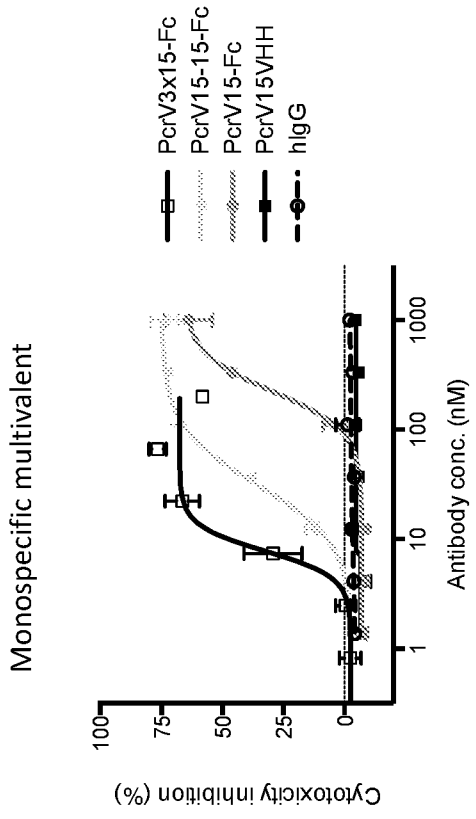


Figure 3B

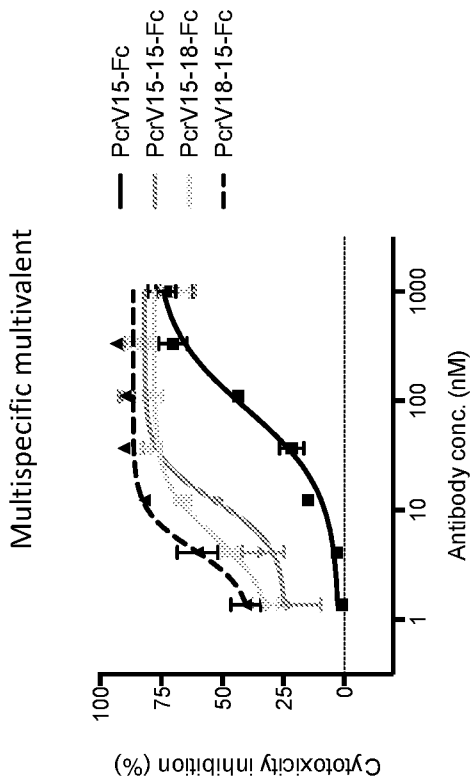


Figure 4A

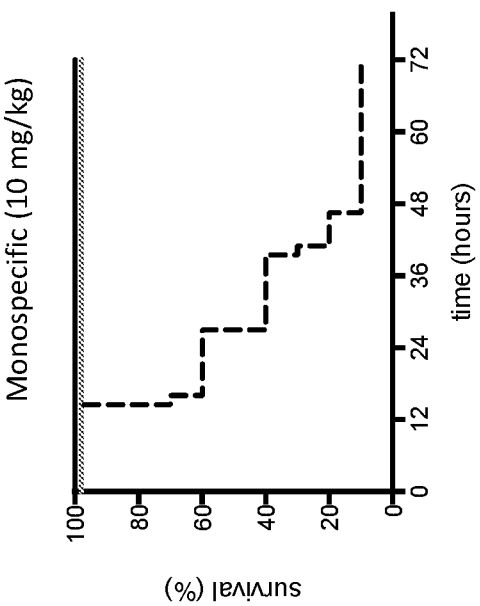


Figure 4B

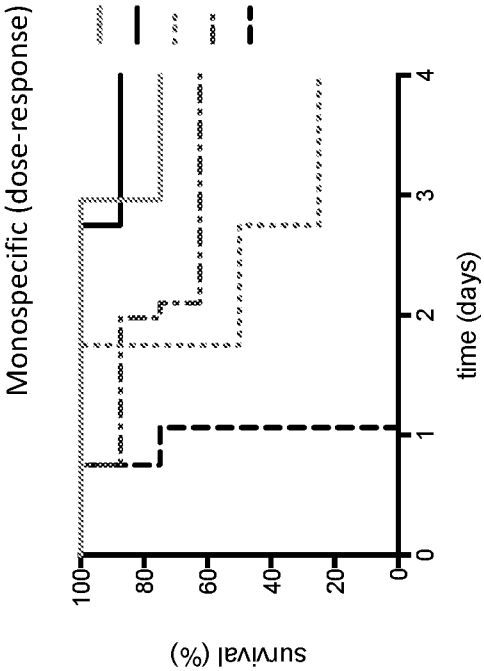
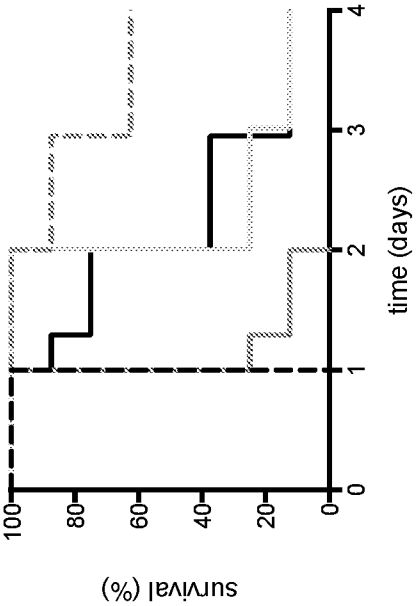


Figure 4C



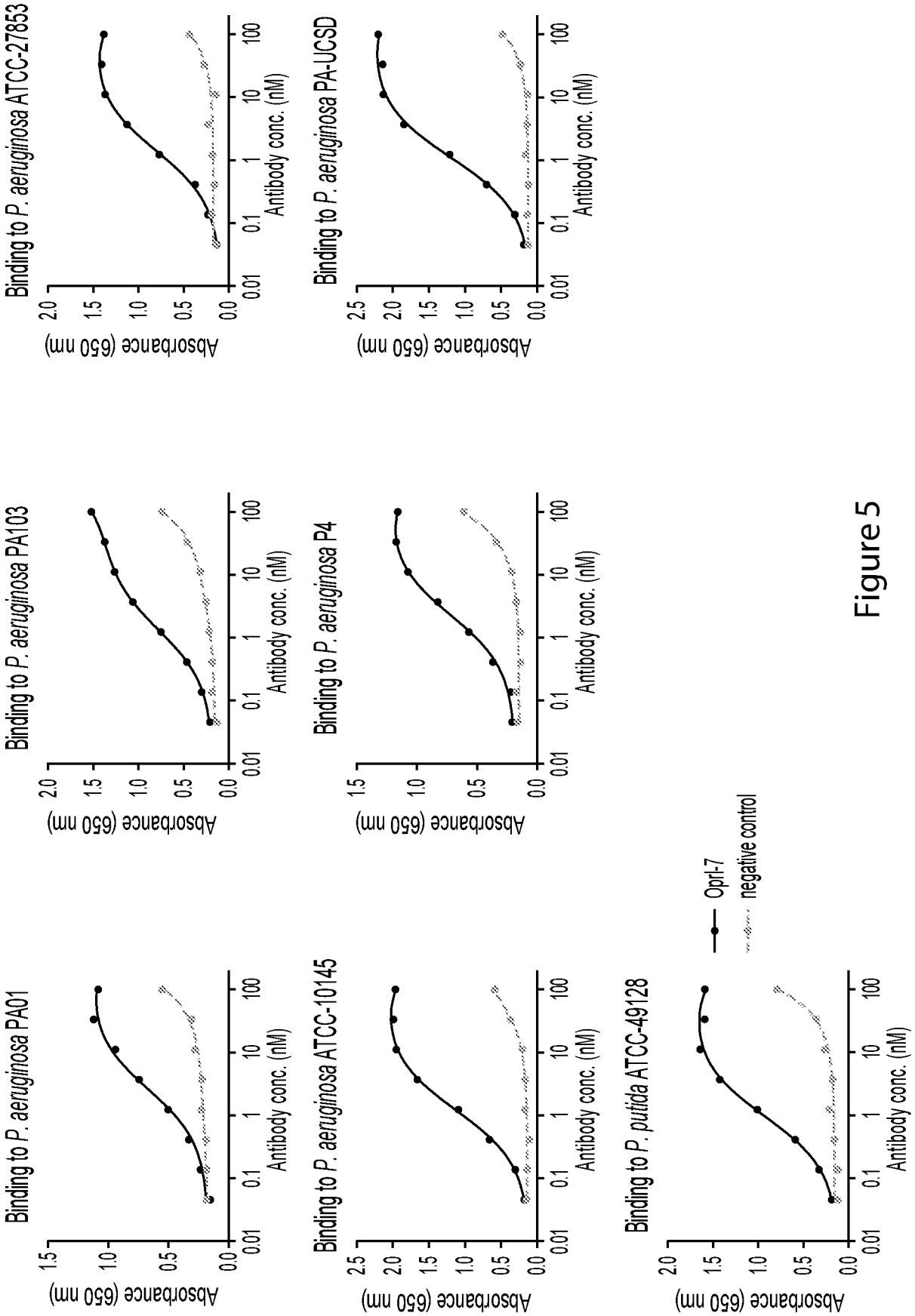

Binding to P. aeruginosa ATCC-27853
Binding to P. aeruginosa PA-UCSD
Binding to P. aeruginosa PA103
Binding to P. aeruginosa P4
Binding to P. aeruginosa PA01
Binding to P. aeruginosa ATCC-10145
Binding to P. putida ATCC-49128
Absorbance (650 nm)
Antibody conc. (nM)
Oprl-7
negative control

Figure 5

Figure 6

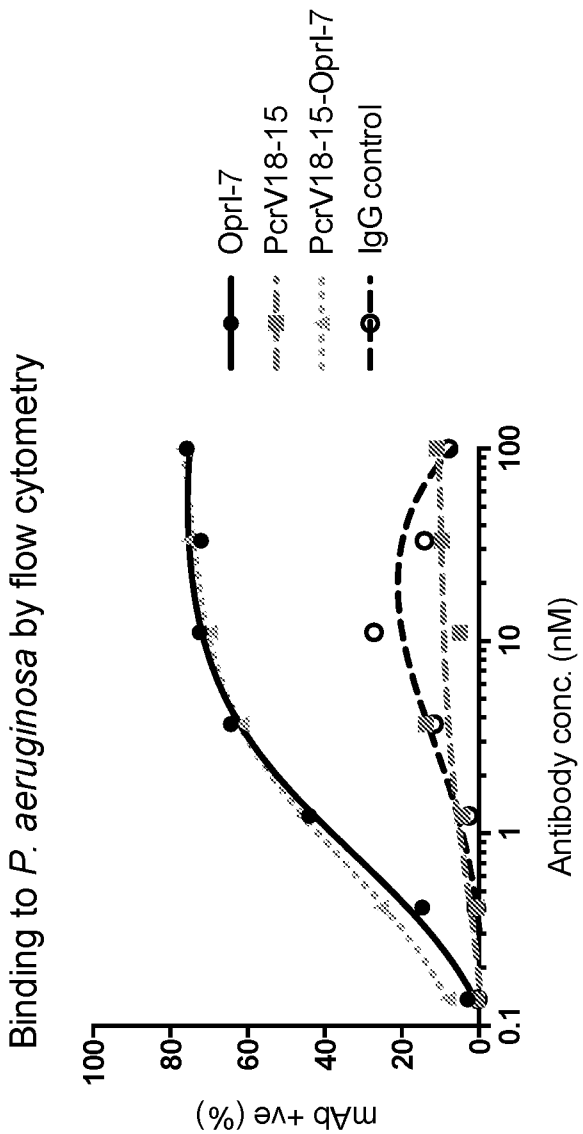
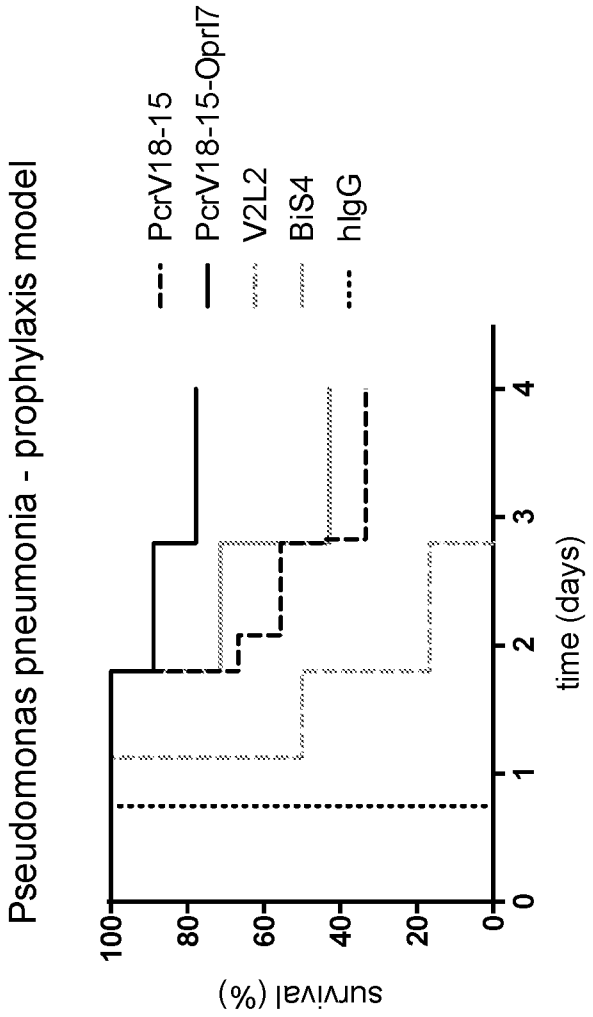
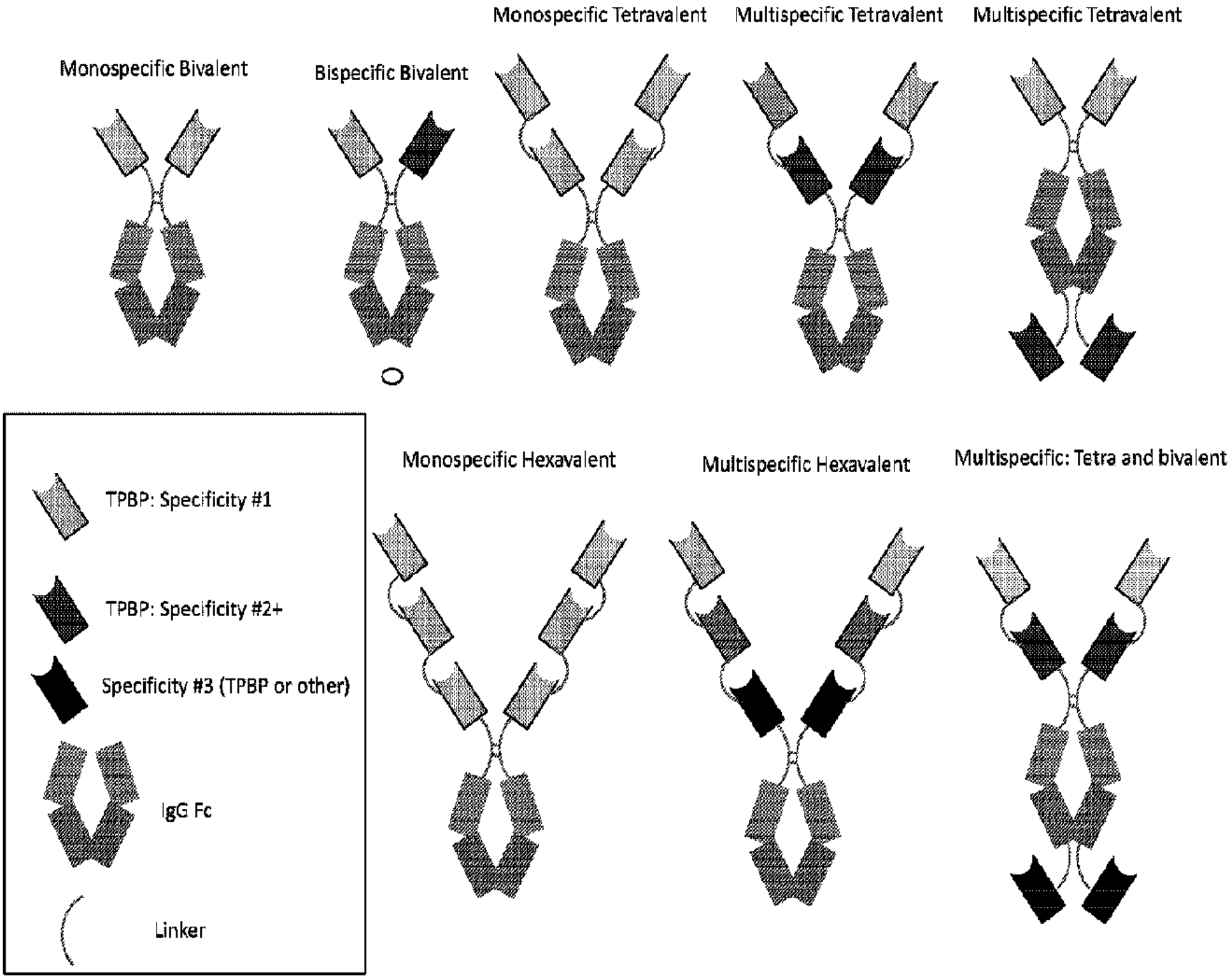

Binding to P. aeruginosa by flow cytometry
mAb +ve (%)
100
80
60
40
20
0
0.1
1
10
100
Antibody conc. (nM)
OprI-7
PcrV18-15
PcrV18-15-OprI-7
IgG control

Figure 7


Pseudomonas pneumonia - prophylaxis model
PcrV18-15
PcrV18-15-OprI7
V2L2
BiS4
hIgG
survival (%)
100
80
60
40
20
0
0
1
2
3
4
time (days)


Monospecific Bivalent
Bispecific Bivalent
Monospecific Tetravalent
Multispecific Tetravalent
Multispecific Tetravalent
TPBP: Specificity #1
TPBP: Specificity #2+
Specificity #3 (TPBP or other)
IgG Fc
Linker
Monospecific Hexavalent
Multispecific Hexavalent
Multispecific: Tetra and bivalent