Recombinant norovirus-specific scFv inhibit virus-like particle binding to cellular ligands
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Abstract

Background: Noroviruses cause epidemic outbreaks of gastrointestinal illness in all age-groups. The rapid onset and ease of person-to-person transmission suggest that inhibitors of the initial steps of virus binding to susceptible cells have value in limiting spread and outbreak persistence. We previously generated a monoclonal antibody (mAb) 54.6 that blocks binding of recombinant norovirus-like particles (VLP) to Caco-2 intestinal cells and inhibits VLP-mediated hemagglutination. In this study, we engineered the antigen binding domains of mAb 54.6 into a single chain variable fragment (scFv) and tested whether these scFv could function as cell binding inhibitors, similar to the parent mAb.

Results: The scFv54.6 construct was engineered to encode the light (VL) and heavy (VH) variable domains of mAb 54.6 separated by a flexible peptide linker, and this recombinant protein was expressed in Pichia pastoris. Purified scFv54.6 recognized native VLPs by immunoblot, inhibited VLP-mediated hemagglutination, and blocked VLP binding to H carbohydrate antigen expressed on the surface of a CHO cell line stably transfected to express α1,2-fucosyltransferase.

Conclusion: scFv54.6 retained the functional properties of the parent mAb with respect to inhibiting norovirus particle interactions with cells. With further engineering into a form deliverable to the gut mucosa, norovirus neutralizing antibodies represent a prophylactic strategy that would be valuable in outbreak settings.

Background

Noroviruses are non-enveloped positive strand RNA viruses that cause foodborne illness worldwide [1]. They are classified as NIAID Category B priority pathogens because they are easily transmitted person-to-person and can cause persistent epidemics. Outbreaks generally occur in semi-closed community settings including day care centers, retirement facilities and nursing homes, hospitals, schools, and military training and operations facilities. Large outbreaks on commercial cruise-liners have been well publicized, and such outbreaks illustrate the rapid onset epidemic potential of noroviruses and a need for intervention measures that do not depend on pre-existing immunity. Recent data suggest the number of outbreaks attributable to noroviruses may be increasing [2].

The norovirus genome is a 7.7 kilobase RNA comprised of three open reading frames (ORF) [reviewed in [3]]. ORF1 codes for the nonstructural proteins that are processed co-and post-translationally by a single viral protease. ORF2 and ORF3 encode structural proteins VP1 and VP2, respectively, and form the icosahedral capsid. Ninety-dim-
ers of VP1 assemble into virus-like particles (VLPs) when expressed in insect cells infected with a recombinant bac-
ulovirus [4]. VP1 folds into two major domains termed
the shell (S) and protruding (P) domains [5,6]. The S
domain consists of the N-terminal 280 amino acids and
forms the icosahedron. The P domain is divided into sub-
domains P1 and P2 that participate in dimeric contacts
that increase the stability of the capsid. The P2 domain is
an insertion in the P1 domain and contains a hypervaria-
ble region implicated in receptor binding and immune
reactivity, as well as in interactions with histoblood group
antigens associated with norovirus infections [7-11].

Therapeutic antibodies have been used successfully in
treatment regimens for diseases including cancer and
rheumatoid arthritis, for transplant rejection, and against
respiratory syncytial virus infections in children [reviewed
in [12]]. Technological advances that include humaniza-
tion to avoid undesirable immunogenicity, and improve-
ments in stability and pharmacokinetics are strategies
employed to improve the clinical utility of antibodies.

Foremost among such strategies is the reduction of anti-
gen binding domains to minimal fragments that retain
reactivity with the targeted antigens [13]. Single chain variable
fragments (scFv) are ~27 kDa recombinant proteins
that consist of the light (\(V_L\)) and heavy (\(V_H\)) chain variable
regions of a monoclonal antibody (mAb) expressed in a
single construct where they are separated by a flexible pep-
tide linker [14]. Intramolecular folding of the recombi-
nant protein results in reconstitution of the antigen
binding domain. These small proteins are relatively easily
produced in high yield in recombinant bacterial or yeast
expression systems [15-17]. Further manipulation and
expression strategies have yielded forms of the scFv mon-
omer where valency is increased by assembly of mul-
timeric forms termed diabodies, triabodies and
tetraabodies [13]. These multimers have been shown to be
more stable and can be engineered to recognize more than
one antigenic target [18,19].

We generated mAb to norovirus VLPs to characterize
domains of VP1 that function in virus binding to cellular
receptors [20]. One mAb (mAb 54.6) to the genogroup I
reference strain Norwalk (NV) blocks binding of recombi-
nant VLPs to CaCo-2 intestinal cells and inhibits VLP-
mediated hemagglutination. In the current study, we engi-
neered sequences encoding mAb 54.6 into an scFv to
determine whether functional activity was retained in the
isolated antigen binding domain. The data presented
show the scFv from mAb 54.6 (scFv\(_{54.6}\)) was expressed
successfully in \(P.\) \(pastoris\) and retained the antigen
binding and functional activity of the parent mAb. Engi-
neered antibody fragments that block norovirus binding
to cells have potential as an on-site prophylactic strategy
to prevent virus spread and contain epidemics.

Results

\(V_L\) and \(V_H\) domains of mAb 54.6 and design of scFv\(_{54.6}\)

Anti-rNV mAb 54.6 recognizes non-denatured VP1, inhib-
ts VLP-mediated hemagglutination, and blocks VLP bind-
ing to CaCo-2 cells. To determine whether functional
activity of the mAb could be reduced to a smaller antigen
binding domain, sequences encoding the \(V_L\) and \(V_H\) genes
of mAb 54.6 were cloned from the hybridoma cells (Fig-
ure 1). A database search for homologies to known murine V genes was performed with the IgBLAST protocol.
The \(V_L\) domain of mAb 54.6 is 98.9% identical to \(V_H\) genes
in the Vx-23-48 family [21], with the exception of a sub-
stitution of isoleucine for threonine at position 31 in the
Vx-CDR1. The \(V_H\) domain was amplified in a single form
highly homologous to the \(V_H\) 7183 gene family [22] and is
96% identical to the \(V_H\) 50.1 gene. \(V_H\) genes derived from
the \(V_H\) 7183 family are preferentially used by autoantibod-
ies of various specificities [23]. However, some antibodies
specific for foreign antigens such as galactan and the A/
PR8/34 strain of influenza virus hemagglutinin also are
encoded by \(V_H\) genes of this family [24,25].

The domain organization of the 245 amino acid scFv\(_{54.6}\)
consists of the \(V_L\) domain at the N terminus separated from
the \(V_H\) domain at the C terminus by a peptide linker
comprised of the sequence GGKGGSGKGTGGKGSGG-
KGS (Figure 1). The linker composition is a modification
of the peptide linker previously described [26]. C-terminal
myc and histidine tags are expressed in frame with scFv\(_{54.6}\)
to allow detection of recombinant protein by immunob-
lot and for downstream purification procedures.

Production of scFv\(_{54.6}\) in P. pastoris and reactivity with rNV

VP1

The pPICZ\(\alpha\)A vector contains the methanol-inducible
AOX1 promoter for high level expression in \(P.\) \(pastoris\) and
the \(\alpha\)-factor signal sequence from \(S.\) \(cerevisiae\) for secre-
tion of recombinant protein into the culture
supernatant [27,28]. During secretion, the signal peptide
is cleaved by the \(P.\) \(pastoris\) protease KEX2 (kexin), releas-
ing soluble scFv\(_{54.6}\) into the medium. Typical yields fol-
lowing induction of expression with methanol,
filtration and concentration, were approximately one
mg of purified scFv\(_{54.6}\) per liter of suspension culture.
scFv\(_{54.6}\) has a calculated molecular mass of approximately
29 kDa. Three closely migrating protein bands were
detected by silver stain (Figure 2A). The top two bands
strongly reacted with the anti-c-myc mAb under both
reducing and non-reducing conditions (Figure 2B). The
exact composition of the two bands is not clear, but they
likely are products of slightly different KEX protease cleav-
age sites at the N terminus of the protein, because the myc
epitope tag recognized by the antibody resides at the C terminus. In support of this interpretation, N-terminal amino acid sequence analysis of three forms of recombinant protein rhIFN-λ showed proteolytic processing adjacent to and outside of the KEX2 cleavage site [29].

Recognition of rNV VP1 by mAb 54.6 is conformation dependent because this antibody reacts in immunoblots only when VP1 has not been denatured by boiling in SDS and β-mercaptoethanol [20]. scFv 54.6 was tested for the ability to bind rNV VP1 in immunoblots to determine whether the fragment retained reactivity of the parent mAb. Immunoblots were probed with scFv 54.6 and binding to rNV VP1 was detected with anti-c-myc mAb and goat anti-mouse secondary antibody. Similar to mAb 54.6, scFv54.6 recognized VP1 only under non-denaturing, non-reducing conditions (Figure 3). Neither the mAb 54.6 nor scFv54.6 recognized denatured VP1.

**scFv54.6 blocks rNV VLP-mediated hemagglutination**

Norovirus VLPs agglutinate red blood cells in a type-specific manner [30] and mAb 54.6 inhibits this activity for rNV VLPs [20]. scFv54.6 was tested for the ability to bind rNV VP1 in immunoblots to determine whether the fragment retained reactivity of the parent mAb. Immunoblots were probed with scFv54.6 and binding to rNV VP1 was detected with anti-c-myc mAb and goat anti-mouse secondary antibody. Similar to mAb 54.6, scFv54.6 recognized VP1 only under non-denaturing, non-reducing conditions (Figure 3). Neither the mAb 54.6 nor scFv54.6 recognized denatured VP1.

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and 4B), yielding an HI titer for scFv54.6 eight-fold lower than that of mAb 54.6. While it is possible that part of the difference in HI titer can be attributed to slight differences in concentration, it is likely that the lower HI titer of scFv54.6 reflects the monovalent binding activity of the fragment.

**scFv54.6 blocks binding of rNV VLPs to CHO-FTB<sub>KE</sub> cells**

CHO cells do not express ABH histoblood group antigens, but CHO cells stably transfected with the rat FTB gene encoding α,1,2-FT confers the ability to bind rNV VLPs to these cells [31]. scFv<sub>54.6</sub> was tested for the ability to block binding of rNV VLPs to CHO-FTB<sub>KE</sub> cells. In the absence of antibody, 48.2% of cells bound VLPs (Figure 5). When VLPs were pre-incubated with scFv<sub>54.6</sub>, the percentage of positive cells was reduced to 10.2%. No reduction in binding was observed with samples from non-expressing X33 culture subjected to the same purification procedure as the scFv<sub>54.6</sub> (see Figure 2A). These results suggest scFv<sub>54.6</sub> is able to block binding of rNV VLPs to H type antigen on the cell surface.

**Discussion**

Immunity to noroviruses is complex and the correlates of protection from re-infection are unclear. Short-term immunity can be induced by prior infection, but long-lasting immunity apparently is more difficult to achieve. Gastroenteritis caused by norovirus generally is a self-limiting disease. However, the ease in which noroviruses are transferred by person-to-person spread suggest inhibitors designed to interfere with the initial steps of virus infection could reduce the duration of illness, shedding of infectious virus, and the number of susceptible individuals in a population. This issue is particularly relevant in semi-closed communities including nursing homes and daycare centers, where age may affect the efficacy of vaccines. We envision that inhibitors will be easily administered on-site as necessary and will by design, be independent of an adaptive immune response.

*P. pastoris* was chosen for scFv<sub>54.6</sub> expression because of reported high protein yield, low levels of glycosylation, simplicity of the culture medium and protein solubility. Numerous scFvs have been produced in *P. pastoris* with variable yields ranging from 0.4 to 20 mg/L [26,32,33]. We have shown that scFv<sub>54.6</sub> expressed in *P. pastoris* at yields of ~1.0 mg/L retain the inhibitory functions of the parent mAb, suggesting humanized derivatives of these fragments could prove useful for development of antinorovirus prophylactics.

Antibodies have received significant interest as biopharmaceuticals. At least 18 mAb are in use in humans and > 100 are in clinical trials as therapeutics for treatment of cancer and chronic inflammatory diseases, and for prevention of transplant organ rejection [12]. Likewise, engineered smaller domains consisting of the functional antigen binding domains are viable alternatives to the costs associated with humanization and production of...
mAb in mammalian cell culture systems [13]. Monovalent scFv are easily produced in high yield in recombinant bacterial or yeast expression systems, and when secreted into the medium, are readily purified to homogeneity by scalable purification procedures. scFv also can be designed to be bispecific, or engineered to preferentially form multimers including diabodies, triabodies, and tetrabodies that increase valency [13]. These multivalent properties with consequent increased avidity are particularly relevant for prophylaxis of viral diseases because, similar to other cellular receptor-ligand interactions, viral attachment proteins engage multiple copies of receptors on the cell surface.

Several examples of antibody fragments capable of neutralizing virus infection both in vitro and in vivo have been reported. For example, fragments selected from a human scFv library for reactivity with the West Nile virus envelope protein protected mice against lethal virus challenge when administered both prior to or shortly after infection [34]. Recombinant virus-specific scFv efficiently neutralized human papillomavirus infection in culture [35]. Affinity-selected scFv against hepatitis B virus also neutralized infection in vitro [36], and purified scFv that recognize intercellular adhesion molecule ICAM-1 effectively blocked transmission of HIV across an epithelial cell monolayer both in vitro and in a small animal model [37]. Together, these data illustrate the utility of scFv as a viable way to provide passive immunity to viral infections.

Our studies were approached with the idea that engineered antibody fragments could provide a rapidly deliverable substance for protection against norovirus infection where the potential for epidemics is heightened in semi-closed community settings. However, successful delivery of soluble proteins, including scFv, to the gut is unlikely because of potential instability from exposure to gut proteases. Recently, pathogen-specific scFv expressed on the surface of probiotic lactobacilli provided protection in small animal models of enteric viral infection [38]. This or a similar system that is amenable to oral administration could provide scaffolding for stable presentation of norovirus neutralizing scFv to the gut.

The antigenic specificity in the norovirus capsid lies primarily in the hypervariable P2 domain of VP1, and ideally, antibody fragments would be reactive with multiple strains. Genogroup II.4 strains currently are the predominant strains circulating worldwide, but others also have been reported [2,39-42]. In the absence of broadly cross-reactive antibodies, the ability to manipulate scFv fragments into bispecific molecules suggests these neutralizing fragments can be engineered to cover a combination

**Figure 5**
sFcV54.6 blocks the binding of rNV VLPs to CHO-FRTBke cells. rNV VLPs were incubated with CHO-FRTBke cells in the presence or absence of fCfV54.6 and sorted by flow cytometry (A) VLP binding detected by incubation with scFv54.6 followed by anti-c-myc mAb and PE-conjugated goat anti-mouse antibody. (B) VLP binding detected by incubation with anti rNV MAb 72.1 PE-conjugated goat anti-mouse and C) scFv54.6 were pre-incubated with VLPs for one hour prior to addition to the cells. Bound VLPs were detected by MAb 72.1.
of antigenic types within a single delivery system. Construction of such bispecific fragments is underway.

**Material and methods**

**Cloning of variable domain genes**

Total RNA was isolated from hybridoma 54.6 [20] with the RNeasy Mini kit (Qiagen). Reverse transcription (RT) was conducted with 5 μg of total RNA using M-MLV RT (Invitrogen) and 3’ oligonucleotides MVK-R and MVH-R (Table 1) for first-strand synthesis of cDNA corresponding to the variable regions of the light and heavy chains, V_L and V_H, respectively. Two μl of the cDNA reaction were used in PCR reactions conducted with degenerate 5’ oligonucleotides for leader sequences (Table 1) and the 3’ oligonucleotides used in the RT reaction. A single PCR amplicon obtained in each reaction was cloned into the pCR2.1 TOPO vector (Invitrogen), and sequenced using M13 reverse and M13 forward (-20) primers. The V_L and V_H PCR products were digested with Kpn I and ligated with T4 DNA ligase. The ligation products were used as α restriction sites to yield pPICZ_A-scFv. The resulting PCR products then were cloned into the pTOPO clones rated by a 60 bp linker. The resulting cDNA products then were amplified from the pTOPO clones using the oligonucleotides described in Table 1. Two μl of the cDNA reaction were used in PCR reactions conducted with degenerate 5’ oligonucleotides for leader sequences (Table 1) and the 3’ oligonucleotides used in the RT reaction. A single PCR amplicon obtained in each reaction was cloned into the pCR2.1 TOPO vector (Invitrogen), and sequenced using M13 reverse and M13 forward (-20) primers. The V_L and V_H sequences then were amplified from the pTOPO clones using the oligonucleotides described in Table 2. Both V_L and V_H PCR products were digested with Kpn I and ligated with T4 DNA ligase. The ligation products were used as templates to amplify the joined V_L and V_H regions separated by a 60 bp linker. The resulting PCR products then were cloned into the *Pichia pastoris* expression vector pPICZaA (Invitrogen) utilizing the *EcoR I* and *Xba I* restriction sites to yield pPICZaA-scFv. Colonies were selected on zeocin-containing low salt agar plates containing 1% tryptone, 0.5% yeast extract, 0.5% sodium chloride and 25 μg/ml zeocin.

**Transformation of *P. pastoris* X33 and selection of recombinants**

*P. pastoris* strain X33 was transformed with 10 μg of *Pme I*-linearized pPICZaA-scFv following the Easyselect *Pichia* expression protocol (Invitrogen). In brief, electrocompetent cells were prepared from 200 ml of YPD medium inoculated with a fresh culture of *P. pastoris* X33 and incubated at 29°C, shaking at 250 rpm until the OD_600 nm_ reached 1.3. The cells then were harvested by centrifugation for five minutes at 1,500 × g, washed twice with sterile ice-cold water and once with 10 ml of ice-cold 1 M sorbitol, and then suspended in 0.5 ml of 1 M sorbitol. Electroporation was conducted by mixing 80 μl of this cell suspension with 10 μg of linearized pPICZaA-scFv in a 0.2-cm cuvette. The cells were pulsed with 1.5 kV at a resistance setting of 129 ohms using a BTX ECM 630 electroporator. One ml of 1.0 M sorbitol was immediately added to the pulsed cells which then were transferred to a 15 ml tube and incubated for two hours at 29°C. Cells then were plated onto YPD agar supplemented with 1.0 M sorbitol and containing 100 μg/ml of zeocin. Colonies were screened for multi-copy recombinants by patching onto YPD agar plates containing concentrations of zeocin ranging from 100 to 2,000 μg/ml. Recombinants selected on 2,000 μg/ml zeocin were screened for Met^+ phenotype by streaking onto minimal methanol agar medium and inserts were confirmed by PCR.

**scFv expression and purification**

scFv expression was conducted by growing recombinants in 50 ml buffered complex glycerol medium (BMMY, pH 6.0) for 18 hrs at 29°C. Cells cultured to log phase were harvested by centrifugation at 1,500 × g, diluted to an OD_600 nm_ of 1.0 x 10^6 to an OD_600 nm_ of 1.0 and added to 1 ml of 1 M sorbitol, and then incubated for 72 hrs at 25°C in a shaking incubator at 250 rpm. At each 24 hour time point, 100% methanol was added to a final concentration of 1.0%. Casamino acids were added to BMMY to a final concentration of 1.0% to reduce proteolysis. One ml samples were taken at selected time points and supernatants were analyzed for scFv expression and purification by SDS-PAGE and immunoblotting using anti-c-myc (Clontech) as the primary antibody.

The pre-cleared supernatant from induced cultures was precipitated by addition of ammonium sulfate to 45% saturation and incubated overnight at 4°C. Proteins were harvested by centrifugation for 25 minutes at 6,700 × g and then dialyzed against sodium phosphate saline buffer

| Table 1: Degenerate oligonucleotides for PCR amplification of V_L and V_H of mAb 54.6 |
|----------------------------------|----------------------------------|
| **VH** | **VH** |
| MVH-R | MVH-R |
| 5’- GAC HGA TGG GGS TGT YGT GCT AGC TGN RGA GAC DGT GA -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| MVK-R | MVK-R |
| 5’- GAA GT TAC CAT GGA GAC AGA CAC ACT CCT GCT AT -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VK1-F | VK1-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GAC AGA CAC ACT CCT GCT AT -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VK2-F | VK2-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA TTT TCA AGT GCA GAT TTT CAG -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VK3-F | VK3-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GWC ACA KWC TCA GGT CT TTT TR T -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VK4-F | VK4-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GWC ACA KWC TCA GGT CT TTT TR T -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VK5-F | VK5-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GWC ACA KWC TCA GGT CT TTT TR T -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VHI-F | VHI-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GAC AGA CAC ACT CCT GCT AT -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VH2-F | VH2-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GAC AGA CAC ACT CCT GCT AT -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VH3-F | VH3-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GAC AGA CAC ACT CCT GCT AT -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
| VH4-F | VH4-F |
| 5’- GGG GAT ATC CAT GAC CAT GGA GAC AGA CAC ACT CCT GCT AT -3’ |
| 5’- GGA TAC AGT TGG TGC AGT CGA CT TTT TTT KAT TAT CTT CAR CTT -3’ |
Table 2: Primers for cloning VL and VH of mAb 54.6 into P. pastoris expression vector pPICZαA

| Primer  | Sequence                                      |
|---------|-----------------------------------------------|
| VKF-54.6 | 5'-CAG ATC TTC GAC ATC TTG CTG ACT CAG TCT CCA GCC -3' |
| VKL-R-54.6 | 5'-CTA CGG TAC CCT TAC CTC CAG ATC CCT TAC CTT TGA TTT CCA ACT TGG TGC C -3' |
| VHLF-54.6 | 5'-CAC GGG TAC CGG AGG TAA GGG ATC TGG AGG TAA GGG ATC TGA AGT GAA ATT GGT GGA GTC -3' |
| VH-R-54.6 | 5'-GAC ACT CTA GAG AGG AGA CAG TGA GAG TGG TGC C -3' |

(50 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4). The dialysate was diluted with an equal volume of Ni-NTA buffer (50 mM monobasic sodium phosphate, 300 mM NaCl, pH 8.0) containing 25 mM imidazole and then incubated with Ni-NTA beads for 20 minutes at 4°C with end-over-end rotation. Beads were collected by centrifugation for two hours at 800 × g and then washed five times with wash buffer (50 mM monobasic sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0). His-tagged proteins were eluted with Ni-NTA buffer containing 250 mM sodium imidazole, dialyzed against sodium phosphate buffer, pH 6.5, and concentrated by ultrafiltration through a Centricon Plus-20 column (Millipore).

Hemagglutination inhibition assay (HI)
The HI assay was performed as described previously [20]. In brief, 50 μl serial dilutions of purified scFv54.6 or mAb 54.6 in sterile PBS-H (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 5.5) were added to the wells of a vinyl flexible V-bottom 96-well plate (Costar), followed by addition of 50 μl of a 0.5 % suspension of type O red blood cells. Ten μl of VLPs (5 ng/ml in PBS-H) or PBS-H were added to the appropriate wells indicated in the figure legend. The plate was gently agitated and incubated for four hours at 4°C.

Construction of CHO-FTBKE cells and VLP blocking assays
A stable Chinese Hamster Ovary (CHO) cell line that expresses α 1,2-fucosyltransferase (α 1,2-FT) was constructed as previously described [31]. The rat FTB gene encoding α 1,2-FT was amplified by RT-PCR from total RNA isolated from rat colon using oligonucleotides FTB-F (5’ CAGGATCCATGCGCCAGCGGCT-3’) and FTB-R (5’ CACCTCGAGTTAGCCTAAAGGAGTGGGGAC-3’). The PCR amplicon was cloned in BamHI and Xhol sites of pcDNA3.1/Myc-His(+)A vector (Clontech). CHO-K1 cells were transfected with the FTB cDNA and stably maintained in RPMI containing 0.4 mg/ml G418. Expression of α 1,2-FT was confirmed by binding of FITC-conjugated UEA-lectin in the plate. The plate was gently agitated and incubated for two hours at 4°C.

Acknowledgements
This work was sponsored in part by a subcontract to MEH from LigoCyte Pharmaceuticals, Bozeman, MT, through US Army Medical Research and Materiel Command Contract Number DAM17-01-C-0040. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation. Additional support was provided by the Montana Ag Experiment Station and PHS grant P20 RR020185.

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