SAND2024-12310C

Sandia National Laboratories

Characterization of novel BBB-penetrating nanobodies for delivery of therapeutic cargo to the CNS

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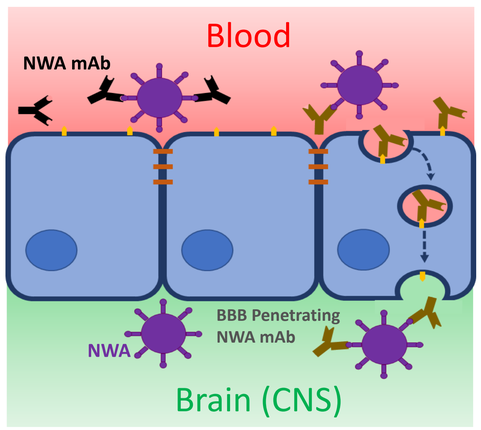
Biologic therapies such as monoclonal antibodies are increasingly popular as rapidly deployable therapeutics, especially targeting emerging viral pathogens. Like other drug classes, the blood brain barrier (BBB) prevents the penetration of monoclonal antibodies into the brain parenchyma at therapeutically relevant concentrations, limiting their utility against neurotropic pathogens and diseases of the central nervous system. For example, although there are multiple neutralizing antibodies showing promise prophylactically against encephalitic alphaviruses such as Venezuelan Equine Encephalitis virus (VEEV) or Eastern Equine Encephalitis virus (EEEV), once infection is established in the brain there are no effective treatment options available. Receptor mediated transcytosis (RMT) has been identified as a potential method of cargo delivery across the BBB, however approaches targeting previously known BBB RMT pathways, including antibodies binding transferrin receptor and insulin receptor, have demonstrated modest transport efficiency, requiring high peripheral concentration to achieve therapeutic levels within the CNS.

At Sandia, we have utilized a phage-display bio-panning approach to identify several novel nanobody candidates which can cross the BBB and are enriched in the brain after IP injection. These novel nanobodies, or BBB penetrating moieties (BBB PMs), present the opportunity to develop a brain delivery platform for biologic therapy. Given that our BBB PM candidates were selected agnostic of previously identified RMT pathways in a mouse in vivo model system, we are currently working to 1) validate that these BBB PMs are also functional in human endothelial systems and 2) characterize the cellular transport pathways utilized by top candidates to cross the BBB endothelial layer. Our findings will advance our understanding of the mechanisms utilized by these novel nanobodies to cross the BBB and will inform their development as shuttles for therapeutic antibodies targeting encephalitic new world alphaviruses as well as a platform which can be adapted to transport therapeutics targeting other diseases affecting the CNS.

Introduction:

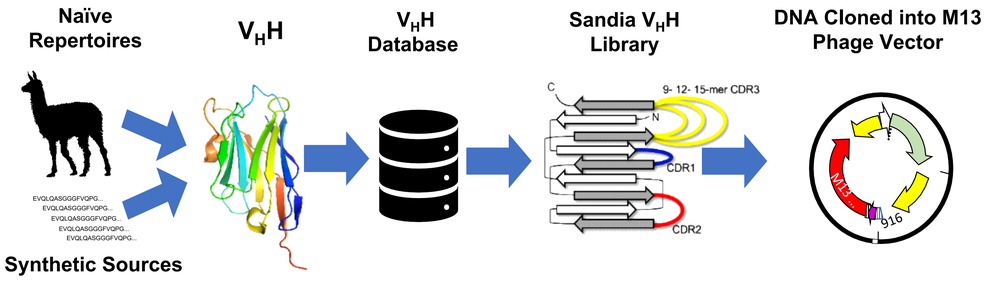
A major challenge in the treatment of encephalitic new world alphaviruses (NWA) such as VEEV or EEEV is that effective anti-viral therapeutics lack access to the brain parenchyma, where these viruses cause severe disease. Central nervous system (CNS) access is restricted by the blood brain barrier (BBB), which includes an endothelial layer which tightly controls the passage of ions, molecules, cells, and pathogens into the CNS. Similarly, the BBB restricts the entry of many drug products into the brain after systemic administration, including biologics such as monoclonal antibodies. Many metabolites are transported to the brain by receptor mediated transcytosis (RMT), and antibodies targeting these receptors have previously been demonstrated to co-opt these pathways to cross the BBB.

To expand the catalogue of BBB crossing shuttles, and to improve on previously characterized low-efficiency transport systems, we have isolated and characterized nanobody sequences which penetrate the BBB *in-vivo* for further development as BBB penetrating shuttles. These nanobodies, which are much smaller than Fab fragments, can be used to functionalize neutralizing antibodies such as those against encephalitic New World Alphaviruses (NWA) to allow them to cross the BBB, and improve their utility against established NWA infection.



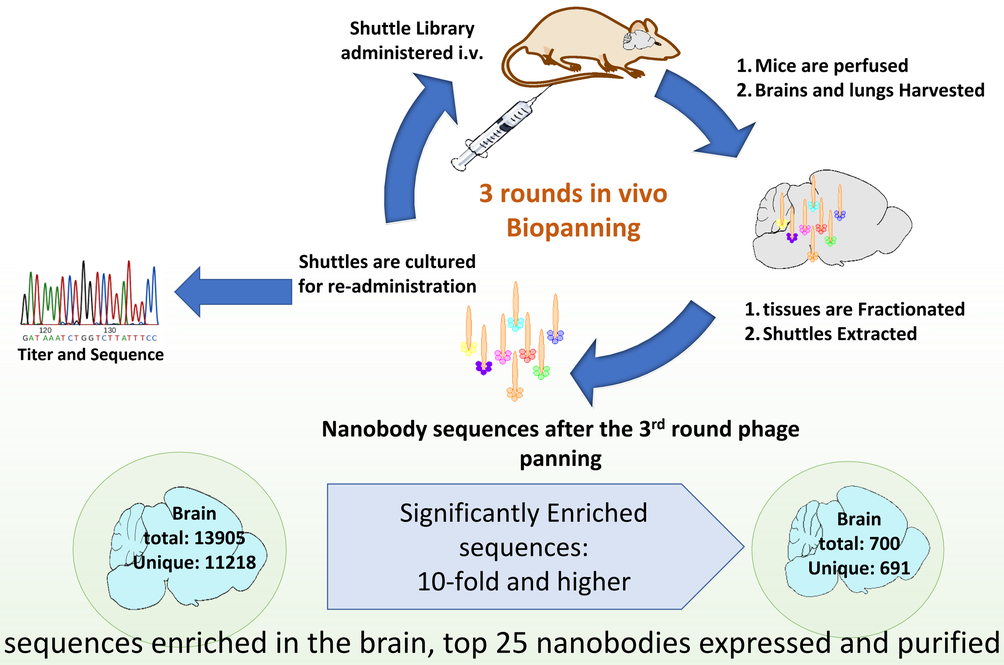
BBB endothelial layer and NWA therpy. NWA mAbs (Black) are excluded from the CNS, and can only bind peripheral viral particles. Bi-specific antibodies targeting both NWA and a BBB RMT pathway (Gold) can cross the BBB to neutralize virus both peripherally and in the CNS.

Nanobody Library Construction:



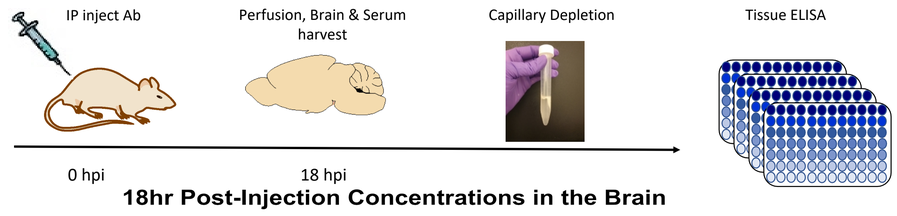
- Nanobody phage library profiled by next generation sequencing
  - High diversity library - 3.24×10^{10} sequences
  - Distribution of CDR3 length

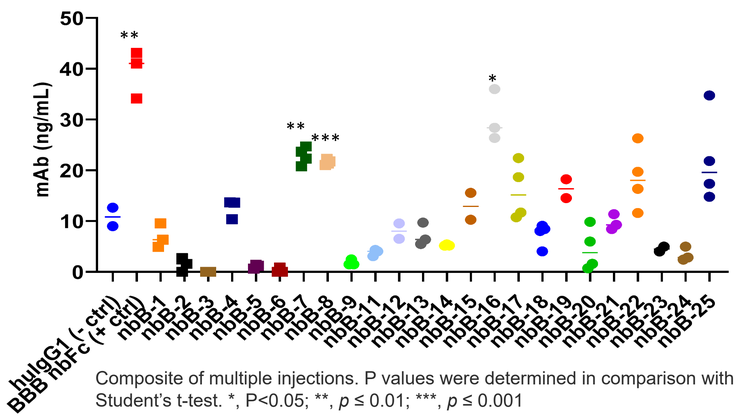
Screening for Functional Tissue Targeting Nanobodies *in vivo:*



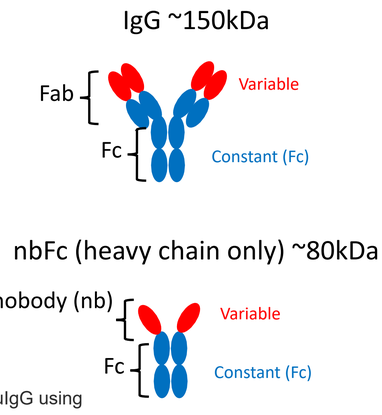
- Of sequences enriched in the brain, top 25 nanobodies expressed and purified as heavy chain only antibody with human Fc region (nbB-1 to nbB-25)

Brain Nanobody *in vivo* Screen Identifies Novel, BBB-Penetrating Hits

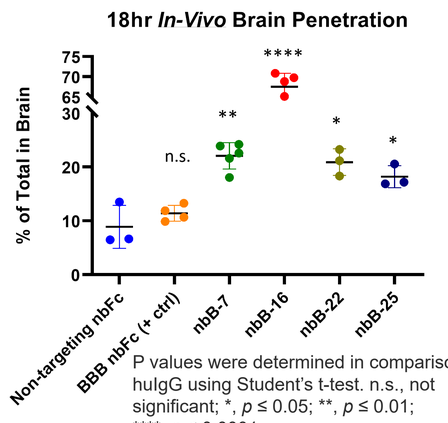




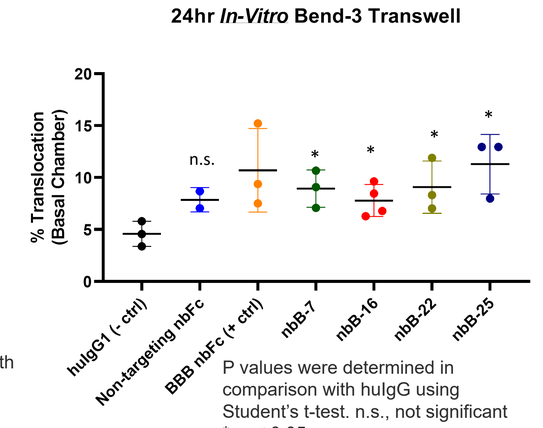
Composite of multiple injections. P values were determined in comparison with either huIgG using Student's t-test. *, P<0.05; **, $p \le 0.01$; ***, $p \le 0.001$



Validation of top BBB penetrating candidates *In-Vivo* and *In-Vitro*



P values were determined in comparison with huIgG using Student's t-test. n.s., not significant; *, $p \le 0.05$; **, $p \le 0.01$; ****, $p \le 0.0001$

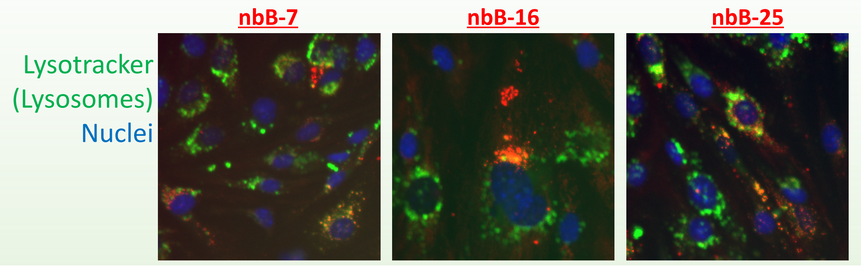


P values were determined in comparison with huIgG using Student's t-test. n.s., not significant

- SNL novel nanobodies are highly enriched in the brain *In-Vivo* *, $p \le 0.05$
- *In vitro* Bend-3 (mouse) trans well model does not fully replicate *In-Vivo* brain penetration phenotypes

Conclusions and Future Directions

- *In-vivo* bio panning used to identify BBB penetrating nanobody sequences enriched in the brain
- Selected nanobody sequences demonstrate BBB penetration in the nbFc format *In vivo,* suggesting specific RMT transport into the brain
  - *In-vitro* model must be improved to accurately reflect *In-vivo* BBB penetration phenotypes
  - Human endothelial model being developed for validation of top candidates.
- Currently optimizing live cell imaging of BBB penetrating nbFc intracellular trafficking as well as co-localization with cellular markers in order to characterize the mechanisms of RMT transport in both Bend-3 and human hCMEC-D3 endothelial monolayer systems



Intracellular nbB distribution (Bend-3). 6 hours after 30 min incubation with nbB.

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SAND No. SAND2022-15240 C

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