

Targeting Astroglisis:
Isolation and Characterization of Astrocyte Specific
Single Chain Antibody Fragments

by

William Marsh

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Graduate Supervisory Committee

Sarah Stabenfeldt, Chair
Michael Sierks
Michael Caplan

ARIZONA STATE UNIVERSITY

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ABSTRACT

Specificity and affinity towards a given ligand/epitope limit target-specific delivery. Companies can spend between \$500 million to \$2 billion attempting to discover a new drug or therapy; a significant portion of this expense funds high-throughput screening to find the most successful target-specific compound available. A more recent addition to discovering highly specific targets is the application of phage display utilizing single chain variable fragment antibodies (scFv). The aim of this research was to employ phage display to identify pathologies related to traumatic brain injury (TBI), particularly astrogliosis. A unique biopanning method against viable astrocyte cultures activated with TGF- β achieved this aim. Four scFv clones of interest showed varying relative affinities toward astrocytes. One of those four showed the ability to identify reactive astrocytes over basal astrocytes through max signal readings, while another showed a statistical significance in max signal reading toward basal astrocytes. Future studies will include further affinity characterization assays. This work contributes to the development of targeting therapeutics and diagnostics for TBI.

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INTRODUCTION AND BACKGROUND

Targeting Traumatic Brain Injury

Approximately 1.7 million Americans sustain a traumatic brain injury (TBI) annually. About 1.4 million are treated and released from emergency departments based upon behavioral analysis, while 275K of the 1.7 million TBI cases result in hospitalization due to the critical nature of the injury. Annually, 52,000 die in the United States each year due to TBI, contributing to almost one-third of all injury-related deaths in the United States. These values may be underestimates, considering the unknown number of unreported and hence untreated cases of TBI.[1] Treating the initial stages of traumatic TBI is a well-characterized and readily-identifiable medical procedure. However, there is still much room for understanding and developing medical technology for the recovery process of any TBI, as well as the detection and diagnosis of mild TBI.[2] A particular realm of interest in TBI is mild to severe TBI induced by pressure waves, such as those sustained by blast mechanics in veterans.[1]

Reactive astrocytes and astrogliosis can be strongly correlated to severe and mild TBI, and reactive astrocytes play diverse significant roles in the inception and aftermath of mild as well as severe TBI.[3] [4] Astrogliosis is defined as the process of Astrocytes becoming proliferative after severe trauma to neurons in particular neuronal death due to secondary chemical cues. Secondary chemical factors such as FGF-2 regulated by microglia, or TNF- α regulated by macrophages are upregulated during TBI.[5] There are many ways astrocytes may become reactive or undergo gliosis. Two umbrella categories

of induced gliosis are through the initial injury insult, and secondarily through chemotactic pathways.[6] [5] Our experimentation utilized the receptor mediated induction of astrogliosis using Transforming Growth Factor – β (TGF- β). Not only will this pathway mimic chemically induced astrogliosis, it also exists as a positive feedback loop when an astrocyte is in its reactive state.[7] We performed biopanning against this complex target in an attempt to discover a novel target scFv, and may even have discovered a novel epitope for reactive astrocytes.

Phage Display

Viruses are not seen in the most positive of light, but as with most good science, mimicking if not domesticating natural phenomena can lead to some very interesting developments in therapeutic techniques. Phage display utilizes the bacteriophage to express genetically designed proteins on the geneIII coat of an M13 phage particle. Historically, phage display has successfully created randomized peptide sequences.[8] Antibodies are nature's most specific binding proteins.[9][10][11][12] As such, we are investigating more recently-developed phage libraries that produce proteins derived from human antibody frameworks.

Phagemid and Phage-Format Systems

While the production of desired protein is one key aspect of phage display, finding a useful high-affinity product is necessary to make this technology viable. Phage expression of the scFv may differ depending on the method of phage display, two of which we will discuss: phagemid vs. phage-format.[13]

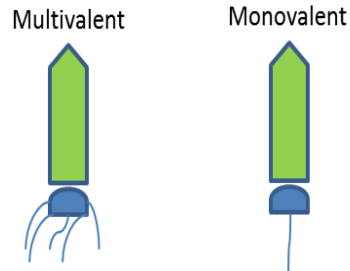


Figure 1: Multivalent Phage Format vs Monovalent Phagemid

The phagemid system is a method of producing phage with an scFv conjugate by utilizing two different phage that insert synthetic DNA plasmids within host bacterial cells. One part of the phagemid system inserts genetic coding for the scFv library and the geneIII protein, while the other “helper phage” (HP) inserts genetic material within the host bacteria, which codes for the production of the genevIII coating protein of the M13 phage (Fig.2) allowing for the monovalent display of the encoded protein sequence.[14][15][16][17]

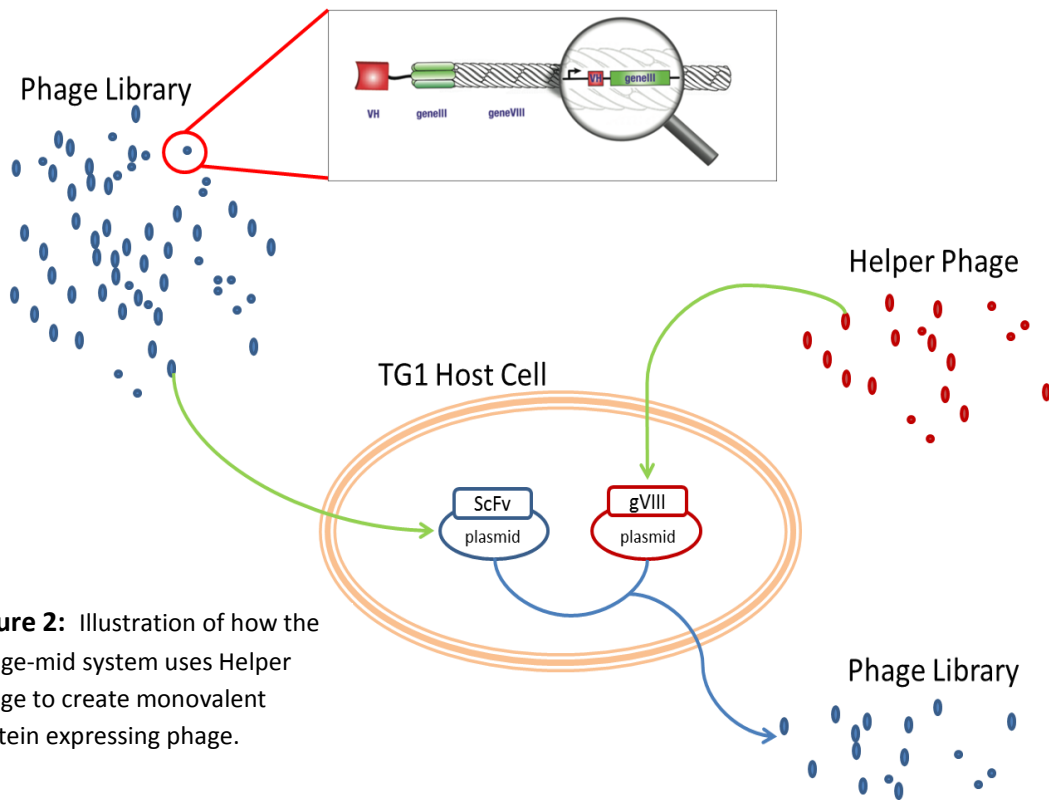
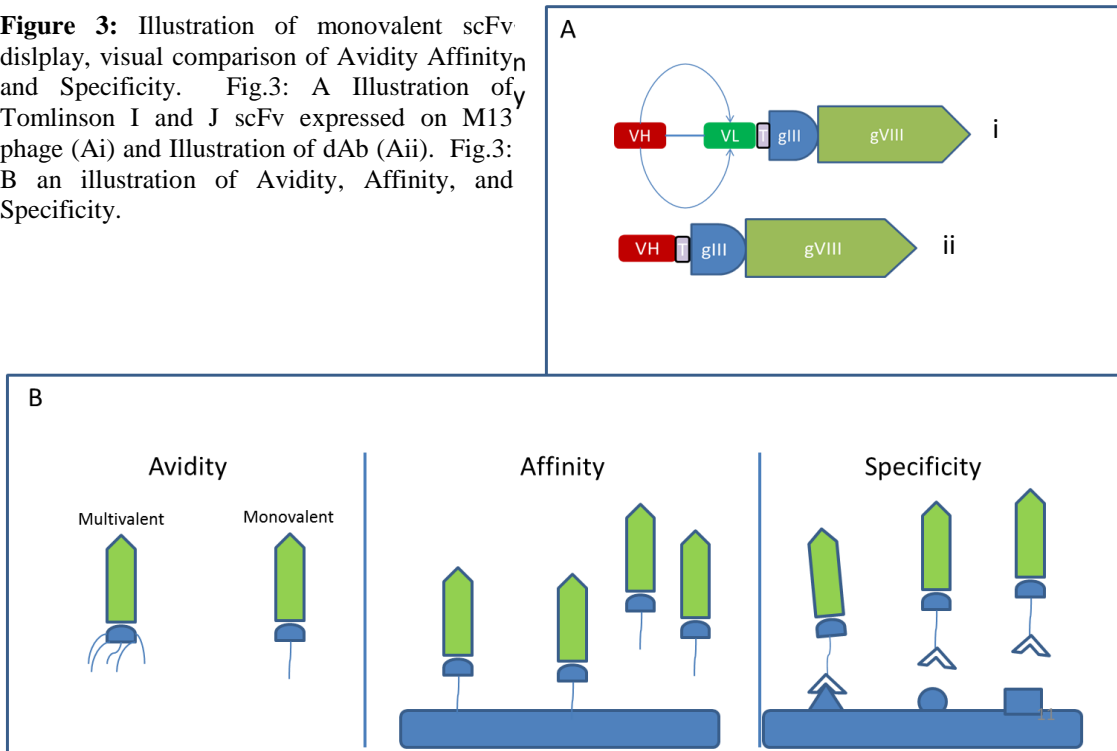


Figure 2: Illustration of how the phage-mid system uses Helper Phage to create monovalent protein expressing phage.

The two aforementioned phage allow for the production of the fully-functional phage particle with scFv conjugated protein to the geneIII lesser coat protein as illustrated in Fig.3.

Figure 3: Illustration of monovalent scFv display, visual comparison of Avidity Affinity_n and Specificity. Fig.3: A Illustration of Tomlinson I and J scFv expressed on M13 phage (Ai) and Illustration of dAb (Aii). Fig.3: B an illustration of Avidity, Affinity, and Specificity.



The phagemid system operates under the principle of inserting DNA plasmids within the intracellular space of a bacterial host through infection. Two phage are required for the production of a fully functional phage particle. One phage contains the genetic code for the scFv fragment and the geneIII protein, while the second phage, the “helper” phage, contains the necessary genetic code to great the geneVIII protein cap of the phage. Additional information on the synthetic vectors of the libraries of interest may be seen in Fig.4 below.[18]

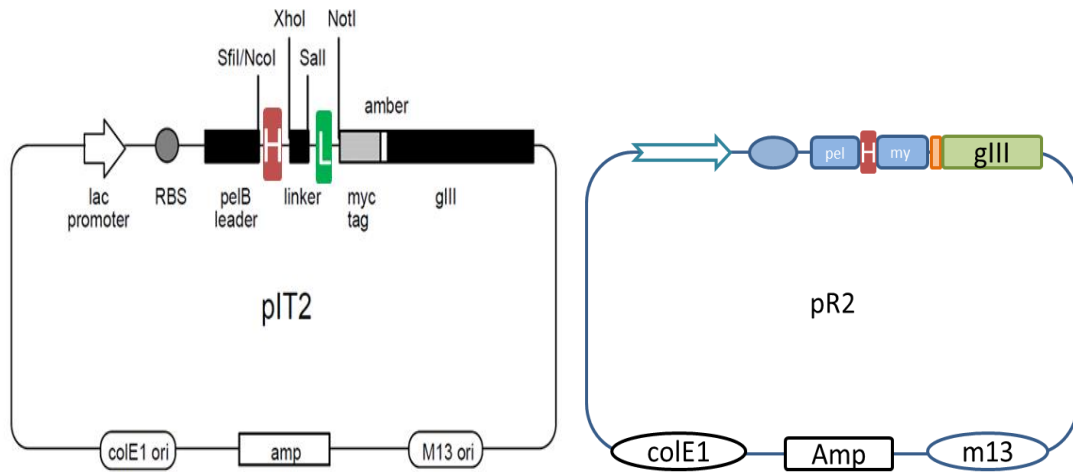


Figure 4: Illustrations of the phage library vector and genetic insert. An illustration of Tomlinson I and J synthetic vector on the left. An illustration of dAb synthetic vector on the right.

Another common method of phage production is the phage-format method, which inserts genetic material of the engineered phage genome. A key difference between phagemid and phage-format systems is the number of scFv fragments expressed for each phage particle.[19] Phagemid systems express one or fewer scFv chains on the geneIII protein cap per particle, whereas phage-format may express 3-5 chains on the geneIII protein cap of the M13 phage.[10] [20] Avidity is the overall measure of effective binding to target due to multiple binding interactions, such as polyclonal vs monoclonal antibodies. This can be seen most easily through the equilibrium affinity constant $K_D = \frac{[R][L]^n}{[RL]}$. In this equation [R] and [L] denote unbound receptor and ligand complexes while [RL] denotes bound receptor ligand complex. We could use n as the multivalent variable. Essentially, n would represent the number of connections between the scFv expressing phage. It would be satisfactory to represent n as a value of 3-5 with

multivalent phage display, and an n value of 1 would be appropriate for monovalent phagemid display. Clearly, having more binding connections can have a direct impact on the overall effective affinity of our target scFv. Affinity is a measure of the direct binding kinetics between a unique paratope and epitope (monovalence) and is quantified using K_D which has units of concentration. The lower the K_D the higher the antibody's affinity (Fig.3).[21] Specificity is the ability for an antibody to differentiate between epitopes and is usually measured in a difference of K_D values between epitopes and an antibody of interest. If avidity is a function of phage display, future characterization may be complicated due to the variability of phage expression per phage and hence require more detailed exploration for more complicated characteristics. Utilizing the phagemid system minimized the complicating variables within our experimental setup.[13] Previous researchers harnessed the power of the bacteriophage by altering the genetic coding that dictates the behavior of the bacteria host. The behavior of a bacteriophage is largely unaffected. It seeks out the natural TG1 host and implants genetic material. The significant difference is the genetic alteration made within the infected code implanted within the host cell. While the overall goal is for the bacterial host to produce scFv, each library we have experimented with produces a unique set of antibody fragments based upon each library's genetic material. Tomlinson I and J libraries are scFv fragments which contain a partial FAB fragment comprised both of a variable heavy (VH) and variable light (VL) domains. While similar Tomlinson I and J differ between what amino acid sequences are mutated among the complimentary determining regions (CDR) of each scFv DVT and NNK for I and J respectively[22], only the second and third CDR of

each VH or VL side contain mutations within the I and J libraries. The dAb library has been temperature treated to be more resistant to temperature fluctuations and is comprised of only the VH domain, which also has three complimentary determining regions (CDR), of which all three contain mutation sites as illustrated in Fig.5 below.[23]

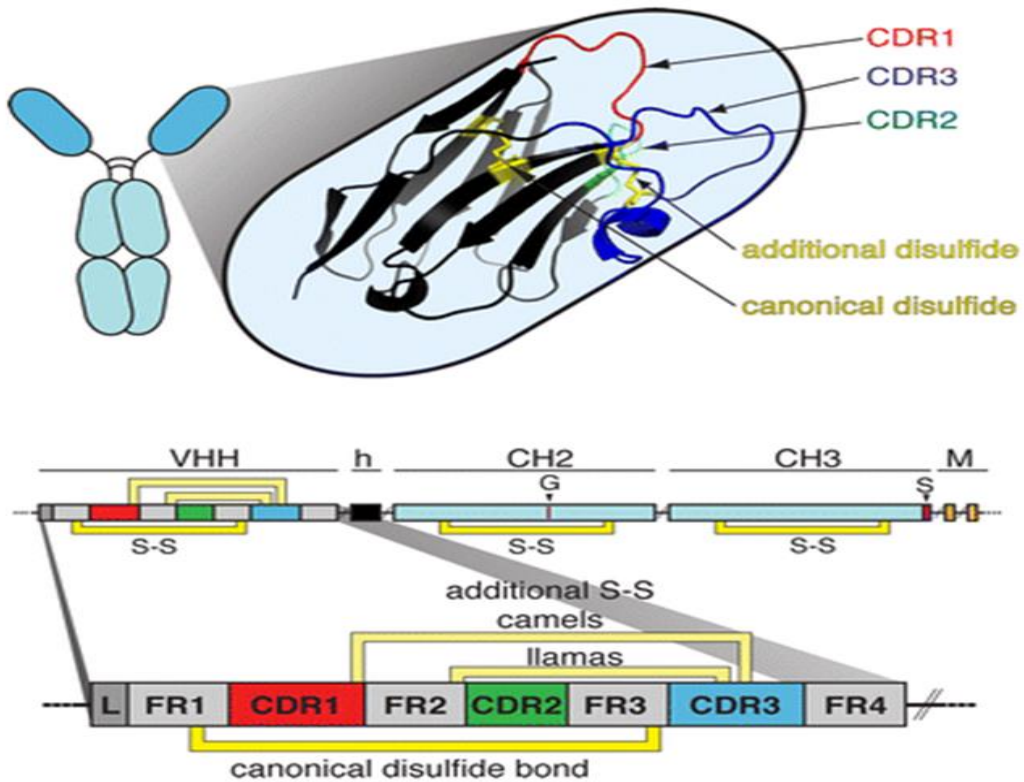


Figure 5: Illustration of CDR in a variable heavy antibody.

For this study, a completed screen and statistical performance analysis of Tomlinson I and J libraries was completed. Thus, our focus shall be considering these libraries. In addition, dAb is currently being pursued because its temperature stability and smaller design make exploration of this diverse antibody library quite interesting. Tomlinson I and J have a base diversity of 1.47×10^8 and 1.37×10^8 possible combinations of CDR mutations. This diversity makes biopanning and the possibility of novel binding proteins plausible. dAb has a mutation diversity of 3×10^9 varieties. The base library has not been targeted to any possible epitope, which greatly broadens the diversity of applications to which these libraries may be attributed. We aim to use the diversity of these libraries to target astrogliosis. Successful targeting of a biological complex target would open the doorway to novel therapy, diagnostic, and characterization modalities.

MATERIALS AND METHODS

The overall goal was to attempt finding a novel antibody fragment which has a high affinity to its target, a reactive astrocyte, by binding to surface protein that is unique to or highly upregulated during astrogliosis. Reactive astrocytes can be induced by chemotactic as well as mechanical measures. We chose to screen against astrogliosis induced by transforming growth factor- β (TGF- β), a chemokine that induces astrogliosis. This growth factor is also shown to upregulate the ECM, extracellular matrix, protein Neurocan.[24] To achieve a specific high affinity scFv, a modified biopanning protocol was created to screen against adherent living astrocytes. This modified biopanning protocol based on Lee et al. consisted of one round of negative screening and three rounds of active screens as described below.[18]

ScFv Production and Purification

Expansion of phage library stocks began by thawing an aliquot of frozen antibody library on ice. The dAb aliquot was then diluted with 500mL 2xTYE medium supplemented with 4% (wt/vol) glucose and 100µg/mL of ampicillin, while the I and J libraries were both diluted into 200ml of 2xTYE medium supplemented with 4% glucose (wt/vol) and 100ug/mL of ampicillin. The presence of 4% glucose allowed the effective suppression of antibody expression during bacterial growth by bypassing the LacO operon in the synthetic plasmid insert. The ampicillin was used to kill off any bacterium which did not contain the synthetic plasmid vector that had the insert as well as an ampicillin-resistant region. The libraries were allowed to grow until they reached an OD measurement of 0.5 at 600nm in a UV-visible spectrophotometer. The initial measurement was at approximately 0.1, when the aliquot was first diluted. Growth of the culture to OD₆₀₀ of 0.5 typically took about 1.5-2hrs at 37C and 250 RPM. The dAB library was grown in a minimum of 1L Erlenmeyer flask, but it was found to be preferable to grow the dAb library in a 2L flask. Similarly, the I and J libraries were grown in a minimum of 500ml flasks, but were preferably grown in 1L flasks. When taking OD measurements, nanodrop wells were inconsistent in readings, so OD₆₀₀ readings were taken using a Cuvette with 4mL capacity. As cuvettes are typically read one at a time in available UV scanners, a baseline measurement was taken using 2xTYE media (around .9) and then compared to OD₆₀₀ reading of the grown library.

Once an OD₆₀₀ of .5 was obtained, 1×10^{12} KM13 helper phages were added to 250mL of dAb culture and incubated in a water bath at 37C for 30-45 min. 2×10^{11}

helper phages were incubated within 50mL of I and J libraries also at 37C for 30-45 mins. Incubation occurred within a water bath or within an incubator set to 30-50rpm at 37C. After the incubation period, cultures were spun at 3,200g for 10 min at 4C in 250mL autoclaved centrifuge bottles. This allowed a dense pellet to form without damaging the TG1 host cells. Discarding the supernatant, and the dAb pellets were re-suspended in 500mL of 2xTY medium supplemented with 0.1% (wt/vol) glucose, 100µg/mL of ampicillin and 50µg/mL of kanamycin. The .1% glucose provided a food source for the overnight growth of bacteria without arresting phage expansion.

I and J libraries were then re-suspended within 100ml of 2xTYE supplemented with .1% glucose 100ug/ml of ampicillin and 50ug/ml of kanamycin. The helper phage provided a kanamycin resistance and the library insert provided ampicillin resistance. Therefore, adding ampicillin and kanamycin killed any bacterium hosts that did not contain both the library insert and helper phage plasmid.

The libraries were then ready to undergo an overnight growth for 16–20 hours at 25C and 100 r.p.m. This overnight growth allowed for the mass production of phage, as both helper phage and library insert must coexist in the same TG1 infected cell to produce a full viral particle or library phage. During the overnight growth period, a 1-2L flask for dAb and a 500ml-1L flask for I and J libraries were used.

After a night of growth, a dense population of phage was typically produced from the infected TG1 hosts. To utilize the soluble phage, they were removed from solution using PEG precipitation. To begin PEG precipitation, the total of the overnight cultures

were spun down for 15 minutes at 10,800g at 4C. The supernatant was collected and the PEG/NaCL solution was added at 15% by volume. The 500ml dAb supernatant was placed into an autoclaved media bottle during the PEG precipitation process. I and J both typically had about 100ml of supernatant and could be placed into the 250ml autoclave bottles for the PEG precipitation process. After adding the PEG solution to the phage supernatant, the container was inverted approximately 50 times before setting the solution to incubate for a minimum of 2 hours at 4C.

After the PEG incubation, the solution was spun down at 6000g for 45mins. I and J could go into the centrifuge immediately because they were incubated in centrifuge tubes. However, dAb was divided into two 250ml centrifuge tubes before spin down. The supernatant was discarded, as it contains biological material and 2xTYE media. The phage pellet accumulated during spin down was re-suspended in 15ml of PBS pH of 7.4. To aid re-suspension, the phage solution was typically placed on a rocker at 4C for 30 minutes to 1 hour; if a rocker was unavailable, the phage pellet was incubated at 4C for 1 hour and then the solution was manually swirled to re-suspend the pellet at the end of incubation. Care was taken with the manual re-suspension so as to neither vortex nor aspirate with a sterile pipet. After re-suspending in 15mL of PBS, 15% PEG-NaCL solution by volume was added to the phage solution, and the container was inverted about 50 times.

The phage PEG solution was then incubated at 4C overnight, then spun down after overnight incubation for 45mins at 6000g and 4C. The supernatant was discarded and the phage pellet was re-suspended in 5ml of PBS/EDTA/BSA solution. The EDTA

acts as a chelating agent, most likely towards the salt ions in solution to help protect the phage protein as well as remove PEG binding to phage particles. The BSA also acts as a protein preservative/protectant. After the phage pellet had been re-suspended in PBS/EDTA/BSA solution, a clear spin at 10,800g was performed to remove any remaining biological material from solution. The supernatant was stored in polypropylene storage container and used within 7 days. While the phage particles remained stable for a week's time, the purified phage was used as quickly as possible to avoid complications.

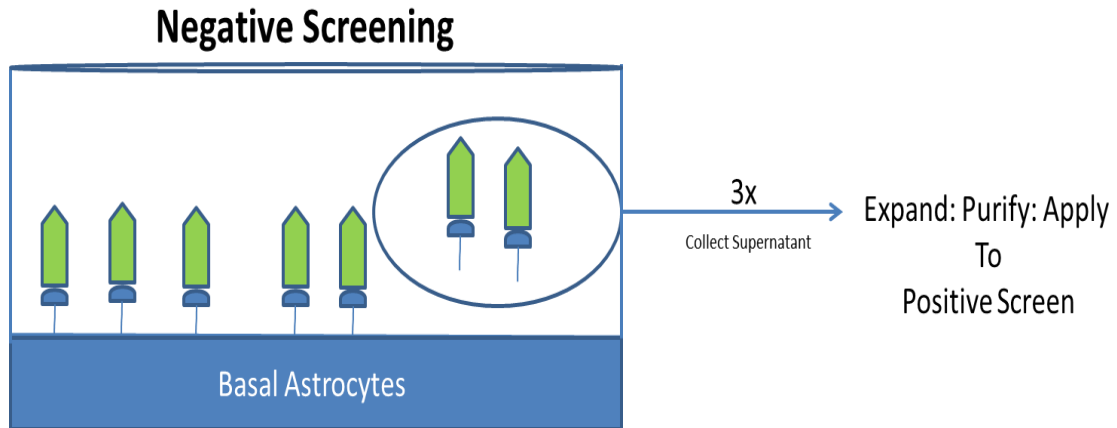
After the phage was purified, concentration of produced phage needed to be obtained for further experimentation, and the most effective method was through CFU/ml titring or colony forming units per mL. To achieve a cfu count, a 100uL sample of the purified phage stock was taken and a dilution series was created, of 10^{-5} , 10^{-7} , 10^{-9} , 10^{-11} in PBS. The most effective means of doing this was to create a 10^{-3} dilution in 1mL of PBS, followed by 100 fold dilutions in PBS. Once the PBS dilutions of the phage were created, four 90ul of overnight starved TG1 cells were also created, which were grown to an OD600 of .5. Overnight starved TG1 cells refers to preparing a 5ml solution of 2xTYE media inoculated with one colony of TG1 cells previously grown on a TYE agar plate. 10uL of the 10^{-5} , 10^{-7} , 10^{-9} , 10^{-11} phage dilution were placed into respective 90uL of TG1 cells. This created a final dilution of 10^{-6} , 10^{-8} , 10^{-10} , 10^{-12} for the phage. The inoculated TG1 cells were incubated within a water bath set at 37C for 30mins. During this incubation, TAG plates were dried open faced for 15 minutes within the bacterial incubator at 37C. After the TAG plates dried, four distinct regions were created on a plate to pipet the incubated TG1 cells with their respective dilutions. Within these four

regions, three areas were created to pipet down a specific concentration, allowing for a n=3 for creating an average CFU count at a given concentration.

Once the solution had absorbed into the TAG plates, the TAG plates were placed into the incubator at 37C for a minimum of 9 hours. Lower temperatures were utilized to achieve longer incubation periods. Setting the temperature to 25C typically created visible colonies at 16 hours. After colonies became visible, a physical count was taken of the colonies visible within the concentration regions on each TAG plate. Sometimes regions were covered with a bacterial lawn at higher concentrations; this was expected. In this case, the concentration at which one could obtain the highest visible colony counts for the CFU calculation was chosen. If a lawn formed at lower concentrations such as 10^{-12} , it was assumed that the culture had been allowed to grow too long or that there might have been an issue with the ampicillin content in the TAG plate. Once a count was possible, the following formula was used to help back-calculate the CFU/ml. Establishing a known concentration allowed the accurate application of phage to the biopanning stage of experimentation.

$$\frac{\text{Average Count}}{\text{Dilution} * \text{pipet volume (in ml)}} = \text{CFU/mL}$$

Biopanning



Positive Screen: Reactive Astrocytes

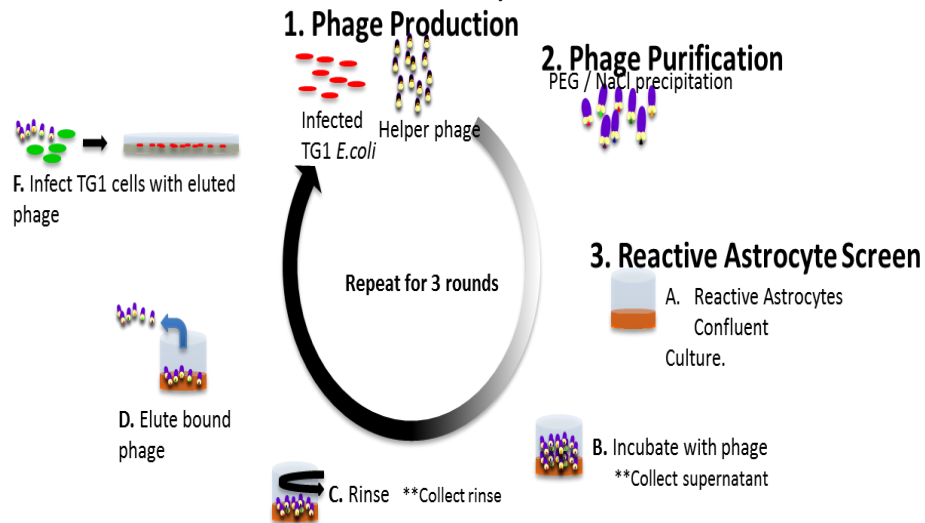


Figure 6: An illustration of Negative and positive screening for Biopanning. Biopanning method adapted from Lee et. Al.

Biopanning involves panning for a biological antibody fragment using a biological target. We are able to screen for functionality of the scFv of interest due to the surface expression of the scFv upon the phage particle. Typically, biopanning is utilized when targeting a specific peptide sequence, antigen, or other highly specific target.[25] This biopanning is typically performed using standard enzyme-linked immunosorbent assay (ELISA) protocols. The aim of this research was to biopan against a complex target, and in our case, that target was a living adherent astrocyte. Overall, the goal of complex biopanning is to find an epitope region unique to the living host that is not necessarily present when targeting specific ECM proteins. In order to biopan against living astrocytes, a new protocol had to be created to biopan against this complex target. There were two major selection rounds utilized in our biopanning protocol: negative screening and positive screening as illustrated in Fig.6 above.[18][26][27]

Astrocytes: In Vitro Culture

Primary astrocytes may be cultured and isolated from a mixed population of astrocytes through mechanical agitation and maturation through passaging. Tracking of the population purity may be done through ICC analysis of the astrocyte population. Also, due to the morphological difference in primary and secondary astrocytes, a quick bright field analysis and cell count will give decent information on the purity of primary to secondary astrocytes. Confluent astrocytes were passaged a minimum of 6 times in

order to achieve a primary astrocyte heavy culture and ensure that the astrocytes are fully developed and mature when harvested from rodent pups. Astrocytes were passaged when they reached 90% confluence and plated down at a seeding density of 20,000cells/cm². Excess cells were frozen down in liquid nitrogen for future use, if desired.

We also tested the astrocyte cultures for purity through Immunocytochemistry (ICC). A2B5 is an antibody unique to secondary astrocytes, so one can do a brief cell count utilizing ICC to gain a percentage of primary to secondary astrocytes. All experiments were conducted with primary astrocyte cultures exceeding 95% purity. In addition to a highly pure culture, one must induce astrogliosis through the use of TGF- β . To induce astrogliosis, astrocytes were starved for a period of 12 hours by removing regular media and supplementing the feeding schedule with Dulbecco's Modified Eagle Medium (DMEM) absent of Fetal Bovine Serum (FBS). After 12 hours of starvation, the astrocytes were treated with normal media containing 10ng/ml of TGF- β . Reactive astrocytes express an up regulation of an ECM protein Neurocan. This fact was used to identify reactive versus basal astrocytes through ICC imaging.

Selection Rounds

Negative screens

A negative screen against basal adherent astrocytes in vitro was performed to achieve an enriched population of phage that has low affinity to basal astrocytes. Instead of blocking the cells or target with a blocking protein, we recognized that the cell medium already contains 10% by volume Fetal Bovine Serum (FBS), which exceeds the

necessary volume percentage of typical blocking protocols. To conduct the negative screen, 5×10^{12} phage were incubated with 5 ml of standard astrocytes medium against adherent basal astrocytes for 1 hour. The supernatant was then removed and placed onto a fresh plate containing another one million basal astrocytes for a second round of negative screening. A complete set of three rounds of negative screening were performed to enrich the phage population towards non-specific binding to basal astrocytes. Each round of screening was conducted at ~50rpm and 30C to help maintain cell viability. Samples of supernatant from each round were collected to create Colony Forming Unit (CFU) concentration titers in order to track library enrichment between each round of screening. At the end of the negative screen, the phage in the final supernatant were used to inoculate TG1 bacterial cells for later expansion as detailed in the Phage Production and Purification section. After the negative screen was complete for each of the three libraries, the first round of positive screens began.

Positive screens

Positive screens were made against reactive astrocytes to enrich the phage population towards affinity with high affinity towards reactive astrocytes. After expanding, purifying, and doing a CFU quantification of the phage libraries from the negative screen, 5×10^{12} phage particles in 5ml of astrocyte media were applied to 1 million adherent reactive astrocytes. The phage were allowed to incubate with the reactive astrocytes for 1 hour at 30C and ~50rpm. After the hour-long incubation, the cells were rinsed with 5ml of PBS for 5mins at ~50rpm for 5mins three times. After the three rinses, a final elution step occurred by applying 2ml of Trypsin solution to the

phage bound astrocytes for 15 minutes. While the phage had a trypsin degradable Myc Tag between the phage body and scFv fragment, the adherent cells had mostly been removed from their tissue culture plate. The cells were observed during each rinse to ensure attachment was persistent throughout the process. The 2ml of elution was then used to inoculate 30mL of overnight starved TG1 cells for 45mins at 37C. This inoculation was spun down at 3200rpm for 15 minutes. After discarding the supernatant, the TG1 pellet was re-suspended in 1mL of 2xTYE media, and then this concentrated mix was plated onto a 254cm x 254cm TAG plate. The culture plates were then placed into an incubator at 37C overnight. After the overnight growth, about 20mL of 2xTYE media was applied to each plate in order to achieve about 10mL of concentrated cellular growth. 15% glycerol was added by volume to the concentrated liquid culture to be frozen down at -80C for long term storage. A sample of the original supernatant, each rinse, and the final elution step were taken to track the phage enrichment of each library.

The above process describes one round of positive screening for each explored library. As the binding of the phage is eliminated after trypsinization, only 1 round of positive screening needed to occur before the library had to be expanded again for another round of screening. Three rounds of positive screening were conducted to enrich the phage population for specific scFvs to reactive astrocytes.

ELISA Phage Clone Prep (96-well plate format)

After three rounds of selections, three plates of TG1 cells infected with the eluted phage from the third round screen were streaked out, using TYE + 4% glucose +

100µg/mL ampicillin plates. Each control group was also streaked out on a plate: library stock infected TGI cells. The plates were incubated overnight at ~28C. When the plates had developed distinct clones on the TAG plates, a 96-well round bottom plate was prepped with 200µL of 2xTY + 4% glucose +100µg/mL ampicillin in each well. Picking colonies using a sterile pipette tip, one colony was added per well of the 96-well plate. The plates were placed in 96-well plate racks and secured with autoclave tape. This was to help prevent evaporation of growing media during overnight growth. Growth was allowed overnight at 37C and 250 rpm to establish highly-concentrated liquid cultures of the TGI infected hosts. After the overnight growth, a fresh 96-well round bottom plate containing 200µL of 2xTY + 4% glucose +100µg/mL ampicillin in each well was inoculated with 5µL of the overnight cultures. The freshly-inoculated plates incubated at 37C and 250 rpm for 3 to 5 hours to create a highly-dense liquid culture. For the remainder of overnight cultures, 55µL of a 50% glycerol in 2xTY solution was added to each well, and store glycerol stock at -80C.

Phage Preparation for ELISA

After 3 hours, 50µL of 2xTY containing 4×10^8 KM13 helper phage (8×10^9 phage/mL) was added to each well. Incubation occurred at 37C without agitation for one hour. The plate was spun at 3200g for 10 minutes. The supernatant was discarded by quickly inverting the plate. The result was re-suspended in 2xTY + 0.1% glucose + 100µg/mL ampicillin + 50µg/mL kanamycin, then grown overnight at 25C and 250 rpm for 16 to 24 hours. After that, the plate was spun at 3200g for 10 minutes. The

supernatant was transferred to a new 96-well round bottom plate and stored at 4C for up to one week.

Initial Interest ELISA Protocol

Plate Prep

Two 96 well black-walled polystyrene plates were seeded with 40,000cells/cm² and allowed to grow for a minimum of 48 hours in 10% BSA DMEM media. At the end of the 48 hour growth cycle, one plate was starved (DMEM without BSA) in preparation for treating the astrocytes with 10ng/ml of TGF- β . After the 12 hour starvation period, starvation media was substituted with working astrocyte media supplemented with 10ng/ml of TGF- β for a minimum of 48 hours. This growth time of both the basal astrocytes and TGF- β induced reactive astrocytes produced highly confluent cell densities to ensure a lawn of targets for the phage of interest.

Concentration Independent Phage ELISA

In a new 96-well round bottom plate, 62.5 μ L of phage supernatant was diluted in 185.5 μ L of working astrocyte media (10%BSA DMEM). This was followed by a transfer of 100 μ L of diluted phage into basal astrocyte ELISA plate (prepped above) and another 100 μ L from the same dilution to the TGF- β ELISA plate (prepped above). These were incubated at 30C for 1 hour at ~50rpm in an incubator. After the hour-long incubation, the wells were washed three times with PBS. 100 μ L of 1:2000 HRP-anti-M13 conjugate (diluted in working astrocyte media) was added to each well of both ELISA plates, and these were incubated at 30C and ~50rpm. The wells were washed three times

again with PBS. 65 μ L of 1-Step Ultra-TMB-ELISA were added to each well. The solution was allowed to develop for approximately 30min at about 50rpm at room temperature. A deep blue color developed in the positive wells. The reaction was stopped with 40 μ L of 2N sulphuric acid, and the solution turned yellow. The absorbance was read at 450nm in the plate reader.

Characterization ELISA Protocol

After the initial interest ELISA, we created a comparative ratio of OD readings for TGF- β treated astrocytes and basal astrocytes. Four clones of interest from the OD readings and one stock clone of the investigated library were selected to explore during the characterization ELISA. After expanding and purifying phage for each of the five characterization clones (as described above), the concentration of the phage was normalized to 1×10^{11} phage/ml. A dilution series of the phage was created to final concentrations of 10^{11} , 10^9 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^1 in working astrocyte medium. 200ul of dilutions were added to 3 astrocyte ELISA prepped plates as described above to create a minimum of n=3 for each dilution of each of the five clones (described above) on TGF- β treated cells and basal cells. The same incubation as described in the HRP procedure was then applied for the initial interest ELISA.

Additional Experimental Methods

ICC immunocytochemistry

After following the astrocyte growth cycle as mentioned above onto circular coverslips in a 24 well polystyrene plate, the cells were fixed for 5 minutes with 4%

paraformaldehyde, then rinsed three times with PBS. After fixing, they were permeabilized using 8% goat serum (GS) and .2% TritonX solution for 15 minutes. The permeabilized cells were then incubated with primary antibodies for GFAP, A2B5, and anti-Neurocan in .1% TritonX to detect astrocytes, Type II astrocytes, and reactive astrocytes respectively. This primary incubation occurred for two hours at room temperature. After the primary incubation, the cells were rinsed three times with PBS. They were then incubated with secondary Alexa Fluor antibodies for two hours at room temperature. After another three times rinse in PBS, the coverslips were mounted onto microscope slides.

PCR Amplified Electrophoresis

Unique clones were picked off streaked TG1 plates for the library of interest, n=4. These were placed into prepped PCR solution containing nucleotide mix, dyed polymerase solution, forward and reverse primers, and nuclease free water. After PCR amplification, 10ul of solution in 2% agarose gel treated with 1X TAE buffer was placed on a horizontal electrophoresis device, and the gel was allowed to run for 1 hour 15 minutes at 75V.

Plasmid Purification Sequencing

To identify sequences of the clones, we used a Plasmid Prep Kit to purify plasmids from the overnight culture growths of libraries of interest. We then submitted the purified plasmids to DNASU sequencing core for sequencing of the specific clones.

Reagent Setup

Helper phage: Production of HP followed the phage production and purification protocol outlined above. Instead of using ampicillin in the growth media and agar plates, kanamycin was used. In addition, a trypsin titer was created to test the trypsin sensitivity of the HP. The trypsin titer was conducted on TKG plates (TAG plates substituting kanamycin for ampicillin) and revealed treated phage at a concentration of 10^5 - 10^8 lower concentration than non-treated phage.

PBS/BSA/EDTA: (0.005M EDTA, 0.1 mg/mL BSA in PBS)

TYE ampicillin glucose agar plates (TAG): Plates were dried before use. 15g of agar, 8g of NaCl, 10g of bacto-tryptone and 5g of yeast extract were dissolved in 800ml of deionized water and autoclaved. The plates were cooled to 50C. 1 ml of ampicillin solution and 200 ml of glucose solution were added and the plates were poured. The plates were stored at 4C for up to 4 weeks.

TYE agar plates: Plates were dried before use. 15g of agar, 8g of NaCl, 10g of bacto-tryptone and 5g of yeast extract were dissolved in 1000ml of deionized water and autoclaved. The plates were cooled to 50C and then poured. The plates were stored at 4C for up to 4 weeks.

2xTYmedium: 16g of bacto-tryptone, 10g of yeast extract and 5g of NaCl were dissolved in 1 liter of deionized water and autoclaved. The plates were cooled to room temperature (25C). The plates were stored at RT or 4C. Antibiotic solutions and glucose solution were added as required and needed.

Kanamycin solution: Kanamycin powder was dissolved at 50mg/mL in deionized water. The solution was filtered through a 0.2µm filter and divided into 1ml aliquots.

These were stored at -20C indefinitely. Thawed aliquots were freshly diluted 1,000-fold into medium or agar.

Ampicillin solution: Ampicillin powder was dissolved at 100 mg/mL in deionized water. The solution was filtered through a 0.2µm filter and divided into 1ml aliquots. These were stored at -20C indefinitely. Thawed aliquots were freshly diluted 1,000-fold into medium or agar.

PBS buffer (1x phosphate buffer, pH 7.4): 3.6g of Na₂HPO₄, 0.2g of KCl, 0.24g of KH₂PO₄ and 8g of NaCl were dissolved in 1 liter of deionized water. The pH was adjusted to 7.4, and then the solution was autoclaved.

PEG solution: 25% PEG 8000, 2.5M NaCl, 125g of PEG-8000 and 73g of NaCl were dissolved in 500ml of deionized water. This solution was autoclaved, then stirred continuously while cooling. The solution could be stored at room temperature for up to a year.

Trypsin solution: Trypsin powder was dissolved at 10mg/mL in TBS (trypsin stock). This solution was filtered through a 0.2µm filter and frozen in 100µL aliquots. This could be stored at -20C for several months. When used, 100µL of trypsin stock was dissolved in 10ml of TBS (trypsin solution).

Glucose solution: 20% glucose solution. 200g of glucose was dissolved in 1 liter of deionized water and filtered through a 0.2µm filter. This solution could be stored at 4C for several months.

RESULTS AND DISCUSSION

Characterization of reactive astrocytes

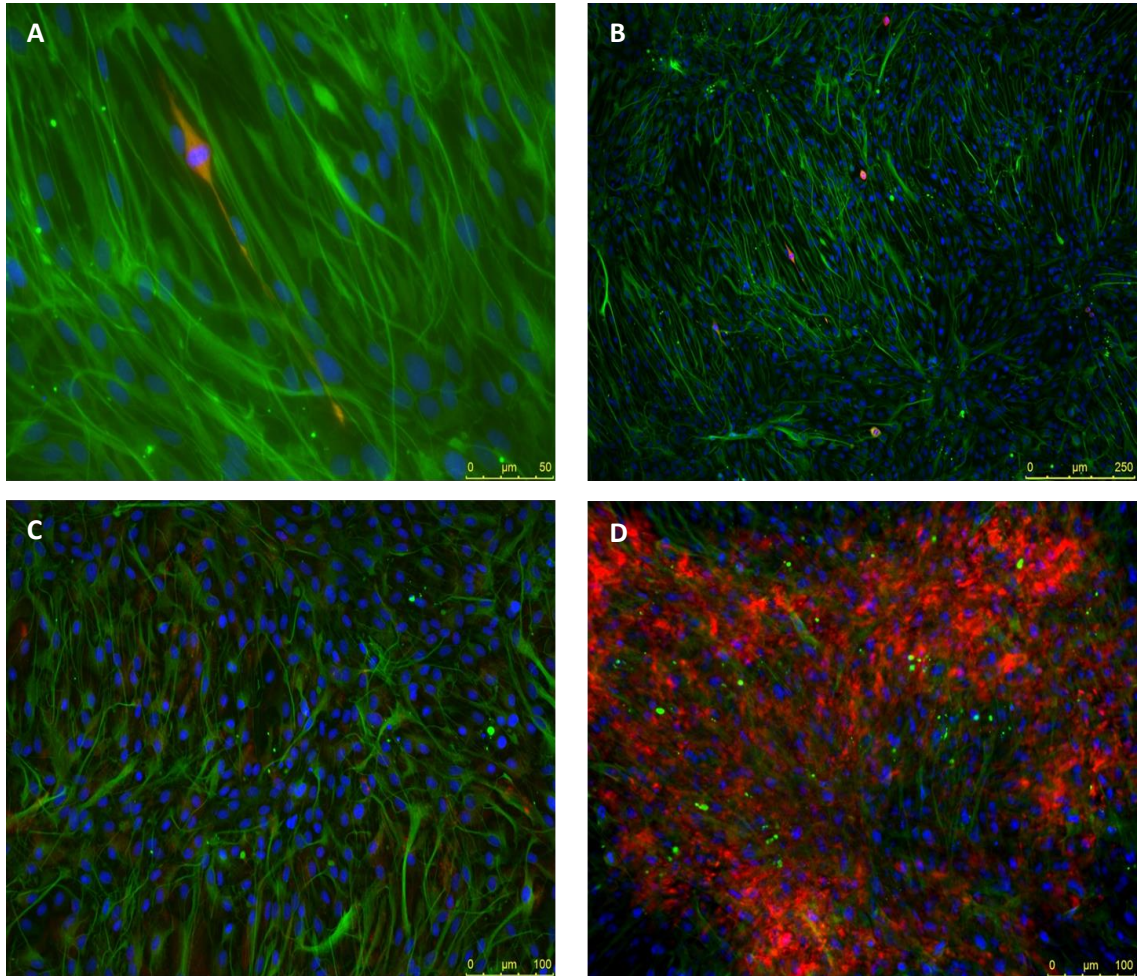


Figure 7: Immunocytochemistry Images for primary and secondary astrocytes as well as basal and reactive astrocytes. Image A shows the ICC image highlight Type II astrocytes (red) in a population of Type I astrocytes (blue stained nuclei) at 40x magnification. Image B is the same source image as A, showing a magnification of 10x. B shows the basal astrocytes (GFAP green, nuclei blue) treated with anti-Neurocan (red) at 20x magnification. D shows TFG-B treated astrocytes. (GFAP green, nuclei blue, anti-Neurocan red)

ICC

Results indicate a highly enriched Type I astrocyte population (>98% Type 1 to Type II ratio) as shown in Figure 7A and B of the ICC results. Also, as indicated, the difference between reactive astrocytes and basal astrocytes may be seen in the upregulation of Neurocan by the reactive astrocytes (Figure 7D) compared to basal astrocytes (Figure 7C).

PCR Amplified Electrophoresis

ScFv inserts may be lost from the phage vector during screening. Tracking the clones' stability over each screen is vital to ensure that the working scFv insert remains while enriching the target specific scFv population. The stock J library and negative screen contained 100% of inserts, while we began to see 25% loss of inserts starting at J1, or the first round of positive screens for the Tomlinson J library. Ultimately, we see how after the third positive screen, 50% of viable inserts survived (**Fig.8**). Tomlinson I only conserved 25% of inserts by the third screen, and sequencing of I3 showed many frame shifts and mutations within the working insert range. While tracking the inserts using PCR amplified electrophoresis is a viable approach for an initial analysis, sequencing of each screen is necessary to ensure the functional viability of remaining insert regions within the phage vector. The PCR may indicate the presence of the insert, but only sequencing of the insert can reveal whether frame shifts, mutations, or amber stop codons

have cropped up during the screening procedures. The aforementioned alterations to the insert can greatly influence the functionality of the scFv.

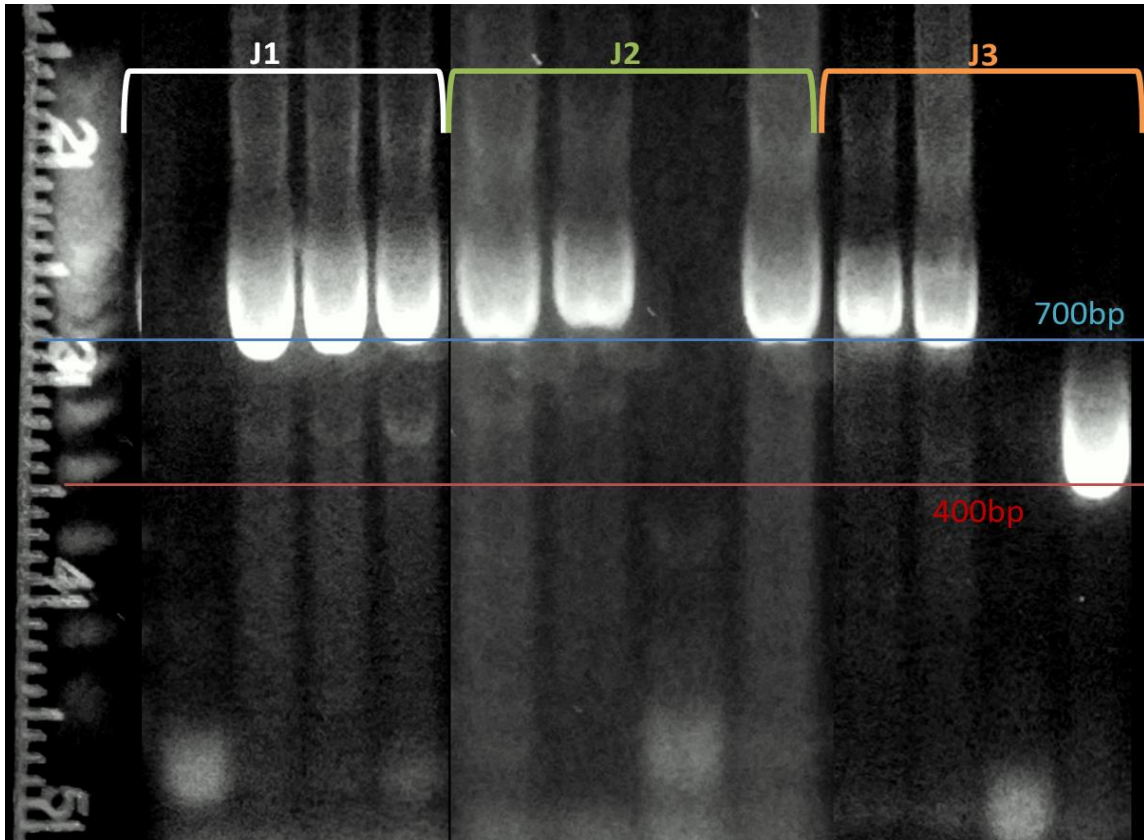


Figure 8: PCR amplified electrophoresis of the positive screens for Tomlinson J library. 700bp is a good indicator of the presence of viable scfv inserts. 400bp or lower is a strong indicator of insert loss. Note: this is a composite image of electrophoresis taken at different times and should be viewed more as an illustration than as exact measurements.

Initial Screen Elisa

Eighty clones of the Tomlinson J library were compared for significant differences in OD readings for TGF- β treated astrocytes compared to basal astrocytes. Of those 80, 10 were chosen for interesting characteristics. Nine were selected due to the

high specificity of TGF- β compared to basal astrocytes. One was chosen due to a high specificity of basal astrocytes over TGF- β treated astrocytes. All 80 clones of interest were compared to the average OD reading of control (**Fig.9**). The controls in this case were OD readings of stock J clones against TGF- β treated astrocytes. Controls against basal astrocytes were also taken. No statistical significance existed between the TGF- β treated astrocytes and basal astrocytes. (**Fig.10**)

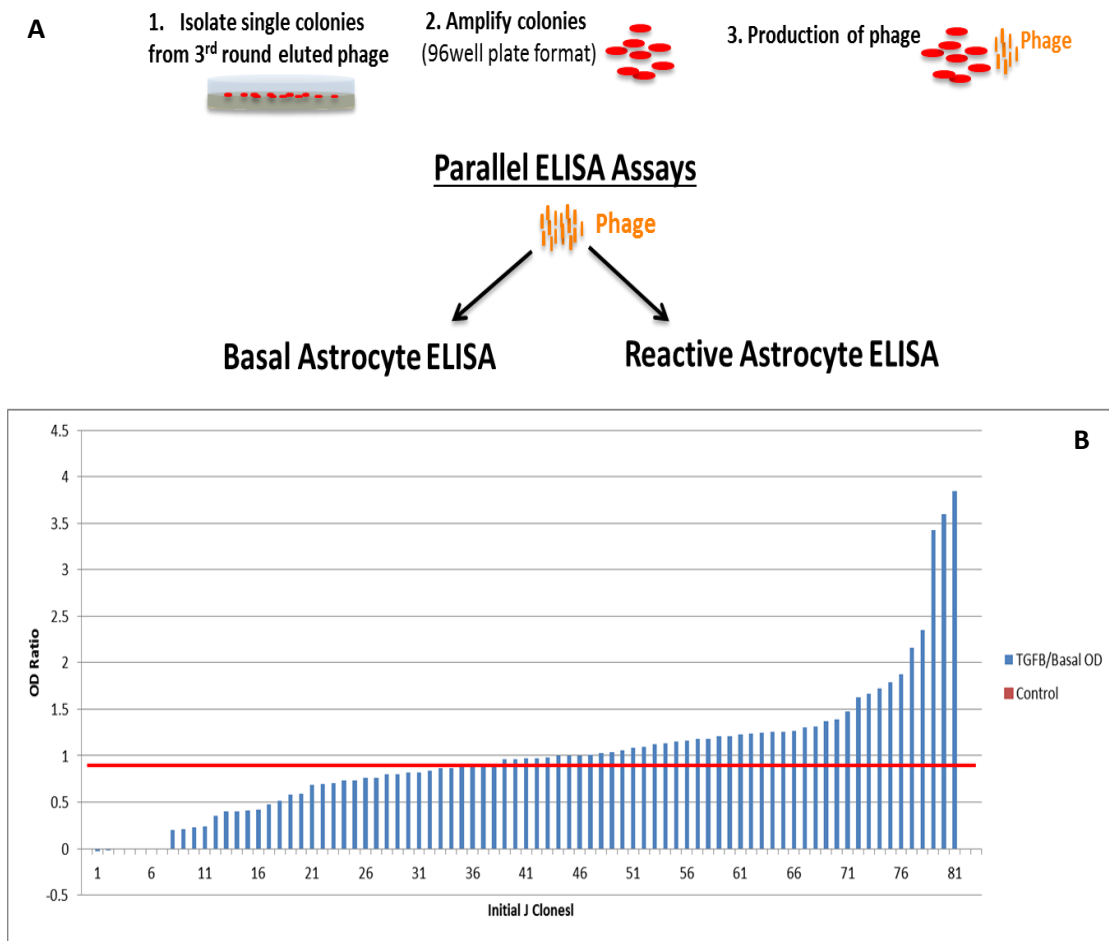
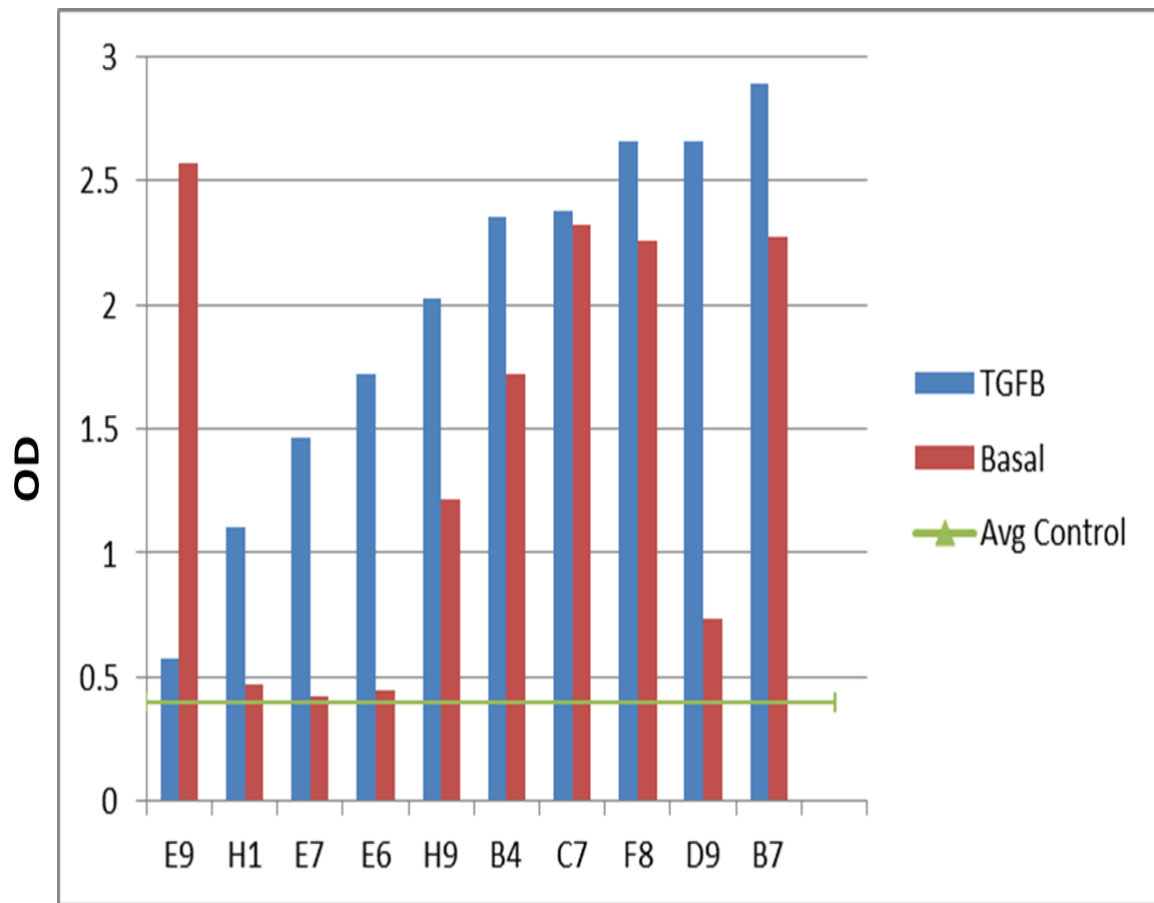


Figure 9: Initial Screen ELISA. Illustration of concentration independent ELISA Fig.9A and resulting data as a function of raw OD ratio Fig.9B



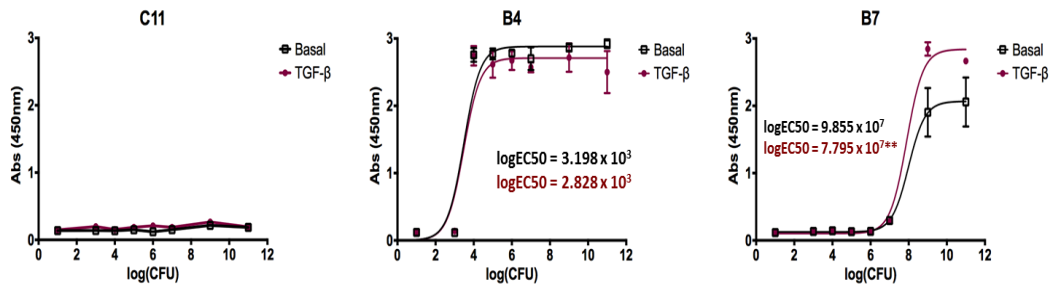
Clones of Interest

Figure 10: The final 10 clones of interest from the concentration independent initial interest ELISA. A comparison of raw OD readings between basal and TGF- β treated astrocytes for initial screen of phage clones. The ten clones listed above are the 10 selected to be of interest out of 80 clones developed for the initial screen

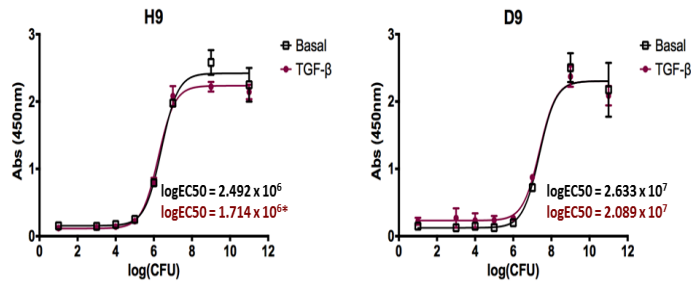
Klotz Plot Characterization of Final J Clones

The final four clones, B4, D9, B7, and H9, were compared to a control clone of the J stock library, C11. An OD comparison of a horseradish peroxidase colorimetric assay was taken between the clones' adherence to basal astrocytes as well as TGF- β treated reactive astrocytes. There was no significant difference between the OD readings of reactive and basal astrocytes in clones D9 or B4. However, we did achieve significant results in B7 as well as H9 when comparing the max OD readings. B7 showed a significant difference between reactive and inactive astrocytes showing a preference for reactive astrocytes. H9 showed partial significance between basal and reactive astrocytes. There was no significance between the EC50 readings of basal and reactive astrocytes for any of the four clones explored. Each data point is representative of an n=3 for each final clone of interest. The clones were unable to show specificity through Significance was determined utilizing non-linear regression curves to establish significance of the overall curve. One wave ANOVAs were used to compare individual data points. **(Fig.11)**

Non-linear Regression (3 parameter nonlinear fit)



* $p < 0.05$, ** $p < 0.001$



B4: High Affinity no specificity

B7: Lowest Affinity, **but** exhibiting specificity to reactive astrocytes

H9: Exhibiting moderate specificity to basal astrocytes

Figure 11: Klotz Plots showing EC50 calculations as well as if the graphs are significantly different. * and ** only denote if the linear regression showed significant difference.

Sequencing Results for Final J Clones

Ten clones of interest were chosen from the initial interest ELISA to pursue further. In order to identify differences among the initial interest clones, all ten were sequenced using plasmid purification. Three of the sequenced clones did not contain functional inserts. The remaining seven showed three unique sequences and four identical clones. The four identical clones suggest a convergence of the sequenced insert for H9 in the final screen for the J library. The four final clones of interest for the J library proved fairly positive at a pH of 7.4, as all clones have an Isoelectric Point (IP) above 9. The four clones all have a higher IPs than the stock insert C11.

Clone	IP	(+/-)	HCDR-2	HCDR-3	LCDR-2	LCDR-3
C11 (control)	8.57	+	KIGDRG**TA	SEPG	GASYL	VEHFPL
B4	9.76	+	LISAQGARTV	GRPK	HASRL	SRLTPL
B7	9.28	+	TIGPSGPKTM	DLAR	SASML	PRTLPM
D9	9.75	+	TIAGTGLKTR	RPGM	RASNL	TAKFPL
H9 (*)	9.73	+	LISTQGKKT	GTAK	SASRL	EKRLPM

Figure 12: Final sequencing results for primary clones of interest in the Tomlinson J library. IP (IsoElectric Point) and overall charge (+/-) indicated in table as well along with the regions of the mutated CDR. **BOLD** denotes non-mutating sites within the CDR. **BLUE** refers to positively charged. **RED** denotes negatively charged. **GREEN** denotes hydrophobic. (*) clone which represents a %40 convergence of the 10 sequenced clones from our Initial ELISA.

Discussion

Reactive astrocytes which have undergone astrogliosis due to mechanical or secondary chemical means are a strong indicator of TBI. This research set out to find a novel, highly-specific target scFv. The hypothesis that a novel epitope on reactive astrocytes could be used to create antibody selectivity is not supported. However, this research has shown that phage display may be used to specify between basal and active astrocytes. The work conducted has shed insight into altering future protocols to create more effective biopanning. While a high-affinity, highly-specific antibody was not discovered, we found clones with varying affinities toward astrocytes; found one clone, B7, which did show a significant preference to reactive astrocytes over basal astrocytes; and obtained data which illustrated some flaws in our current protocol and ways to improve the effectiveness of complex target biopanning.

Analyzing the saturation regions of the Klotz plots (fig 10) of the concentration dependent phage ELISA, we can see a significant difference between basal and reactive astrocytes. This difference seems to be more a factor of increased epitope binding domains and max OD readings than specificity. When referring to antibodies, specificity is defined as the ability for a paratope to differentiate between binding domains by having a high preference to, or by solely binding to, a given epitope. Specificity can be quantifiably measured in a difference of K_D . There was not a significant difference in affinities for any of our clones, so the difference in max OD readings at the saturation point has to be due to something other than specificity.

There is a possibility that a difference in cell population among the wells would lead to an overall difference in the max OD signal at the saturation stage of the concentration dependent ELISA. This experiment attempted to correct for difference in cell population by plating at a density of confluence, 40,000cells/cm², and letting the plates incubate for 48 hours to ensure confluence with the knowledge that TGF- β instigates astrogliosis. As astrocytes undergo contact inhibition, their rate of proliferation should be dramatically downregulated at confluence.

To also verify that cell growth does not represent this difference in max OD reading, we can analyze the data on clones B4 and D9. Clones B4 and D9 show no significant difference within the saturation region of their respective Klotz plots in Fig.10. As there is no significant difference in the EC50 point of the Klotz plot for any clone, it is safe to assume that none of the four clones of interest, including B4 and D9, had specificity between basal and reactive astrocytes. If our clones targeted the same epitope between basal and reactive astrocytes, we would see an increase in OD signal like with B7 in B4 and D9 due to an increased cell count and, therefore, increased epitopes. However, we do not see a significant difference between the saturation points of the Klotz plots for clones B4 and D9. Thus, it is safe to assume that the increase in max OD reading for clone B7 was not due to a difference in cell population.

The data provided suggests that clone B7 had no specificity between basal and reactive astrocytes, but rather a shared affinity towards both complex targets. Protein upregulation and increased expression occur during astrogliosis, such as with Neurocan. The data and known facts of astrogliosis suggest that clone B7 had bound to an

astrogliosis upregulated epitope present in both basal and reactive astrocytes. The increase in max OD readings could therefore be correlated to the increased presence of bound phage due to an overexpression of epitope in activated astrocytes for clone B7.

There is also a statistical significance suggesting clone H9 was able to select basal astrocytes over reactive astrocytes, possibly indicating a downregulation of epitope. However, the findings are less clear for H9. The data isolated from the series and analyzed at a concentration of 10^{11} through one-way ANOVA shows no statistical significance, yet the overall non-linear regression does suggest a significant difference between basal and reactive astrocytes.

The immunocytochemistry images in Fig.7 C and D show basal expression of Neurocan and upregulation of Neurocan during TGF- β induced astrogliosis respectively. This again highlights the idea that upregulation of epitope was providing clone B7 the means to identify between basal and reactive astrocytes through OD max readings. If we were to run negative screens against known upregulated proteins such as Neurocan, glial fibrillary acidic protein (GFAP), and so forth, the phage repertoire could be enriched towards novel epitopes and diminish the likelihood of creating a clone such as B7 that would bind to an upregulated epitope instead of an epitope unique to astrogliosis.

Within the four clones of interest, only B7 could identify basal astrocytes from reactive astrocytes, but this occurred through max OD readings, not through means of specificity. Therefore, amino acids for clone B7 found within the CDRs of its sequence seen in Fig.11 should be compared to the amino acids found within the other clones of

interest. Particularly of interest was how the third complimentary region within the heavy variable chain of clone B7 showed high expression of hydrophobic amino acids compared to the HCDR-3 of the other clones. Clone B4 showed the highest affinity towards astrocytes of the four clones of interest but no specificity. The mutations of B4 in the second complimentary determining region of the variable heavy chain had the highest number of hydrophobic amino acids within that region compared to the other clones. At this point, it seems coincidental that clone B7 has the lowest affinity compared to the other clones as well as the fewest hydrophobic groups in HCDR-2.

The Klotz plots from the concentration dependent phage ELISA were analyzed to determine the relative EC50 point for each clone of interest. Statistical significance was determined for the overall Klotz plot, the EC50 point, and the max OD readings through non-linear regression and ANOVA analysis. Previous statistical significance was mentioned when discussing clone B7 and H9. The fact that clones B4 and D9 showed no statistical significance between MC50s or max OD suggests that a more robust negative screen against basal astrocytes should be performed. We could increase the robustness of the negative screen by implementing a round of negative screening against basal astrocytes after a positive round of screening is completed. Implementing a more robust negative screen may help eliminate the unwanted enrichment of non-specific scFvs.

Establishing significant differences between phage bound to basal and reactive astrocytes is the first step towards creating a highly specific and high affinity target protein. We successfully created a novel scFv bound phage that can identify between basal and reactive astrocytes through max OD reading. However, the identification did

not succeed by creating a highly specific antibody. As it stands, the clone B7 from the Tomlinson J library does not have an effective enough means of differentiating between basal and reactive astrocytes to pursue for clinical studies, and it is known to be from a phage library prone to produce toxic aggregating proteins, which would make further exploration of this particular Tomlinson J clone difficult. To complete the conditions of J, the scFv will have to be produced, purified and characterized to see if functionality is maintained when not expressed on the geneIII coat protein of the M13 phage.

Finding viable target scFvs for application of specifically differentiating between reactive and basal astrocytes will require modifying the existing biopanning protocol and exploring more stringent negative screening. Creating a specific scFv to a novel epitope of interest still seems plausible, yet the initial data shown here illustrates scFv conjugated phage which discern between reactive and basal astrocytes through a max signal reading instead of specific binding mechanics.

Two major considerations present themselves for future exploration of screened libraries for Tomlinson J and dAb. First, the scFv fragment will have to be produced and purified as a standalone target protein to see if specific function is maintained when not conjugated to a phage particle. Second, dAb libraries will be investigated and compared to the viability of the Tomlinson J clones, as dAb may prove to be a more effective target protein due to its thermal stability, smaller size, and higher library diversity. Comparisons of the Vh chains will be completed through sequencing, EC50 data, and max OD signal.

This work contributes to the development of targeting therapeutics and diagnostics for TBI. Synthesizing a standalone specific scFv target protein could be a powerful tool in immunology, therapy, and diagnostics. For therapy and diagnostic functions, the scFv could be conjugated to liposome micelles for targeted drug delivery or even delivery of contrast agents for specific imaging.[28][29]. Reactive astrocyte-specific scFvs conjugated to liposomes may provide an excellent tool for quantifiably measuring mild TBI as well as quantifiably tracking biological recovery of damaged neural tissue, even when the blood-brain barrier is not compromised.[30] Despite the many challenges when targeting a complex target, the rewards may far outweigh the difficult nature of this experimentation.

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