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Identification and Display of CRLF2 Ligands for Targeted Nanoparticle Delivery to Acute Lymphoblastic Leukemia

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Identification and Display of CRLF2 Ligands for Targeted Nanoparticle Delivery to Acute Lymphoblastic Leukemia

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Abstract

This is the final report for the Sandia Excellence in Engineering Fellowship awarded through the School of Engineering at the University of New Mexico for the 2011-2012 academic year.

Acute Lymphoblastic Leukemia (ALL) is the leading cause of cancer death in children. Almost 80% of the children diagnosed with ALL survive, but in order to reach that point, they must undergo intense regimens of systemic chemotherapy with short- and long- term side effects. Moreover, a significant percentage of cases (~30%) show high-risk features that result in relapse or failure to respond to treatment and account for most deaths. The long term goal of this project is to produce nanoparticles based on virus-like particles (VLPs) of the bacteriophage MS2 that specifically target ALL cells for delivery of cytotoxins. By taking advantage of the overexpression in high-risk pediatric ALL of the thymic stromal lymphopoietin (TSLP) receptor, CRLF2, it is possible to identify a peptide ligands specific for the receptor. We recently developed a peptide display and affinity selection system based on MS2 VLPs, thus integrating the targeting ligand identification and drug delivery functions into a single particle. This report summarizes work accomplished toward this goal.

ACKNOWLEDGMENTS

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FIGURES

NOMENCLATURE

ALL	Acute Lymphoblastic Leukemia
BaF3	A murine cell line used as a model to study cell surface receptors.
CRLF2	Cytokine receptor-like factor 2, a protein in humans encoded by the CRLF2 gene.
VLP	Virus-like particle
EGFR	Epidermal Growth Factor Receptor
GST-tag	Glutathione S-transferase protein sequence grafted onto a recombinant protein
MS2	A strain of bacteriophage that infects the bacterium *E. coli*
TAHASV	A peptide sequence that exhibited binding to CRLF2
FSYLPSH	A peptide sequence that exhibited binding to CRLF2
MTAAPVH	A peptide sequence that exhibited binding to CRLF2
PCR	Polymerase chain reaction, a molecular biology techniques used to amplify a single or a few copies of DNA across several orders of magnitude

1. INTRODUCTION

Targeted drug delivery has the potential to revolutionize the treatment of many cancers and various systemic diseases, such as autoimmune conditions or diabetes. The theory involves encapsulation of a drug in some manner and equipping it with a targeting mechanism so that the drug is only delivered to specific cell types. By targeting delivery to specific cell types, the side effects associated with systemic administration may be lessened or avoided altogether. Several different platforms have been employed in efforts to develop high-specificity targeted drug delivery systems, a list that includes liposomes, porous silica nanoparticles (Protocells) and VLPs. Liposomes can be readily produced and loaded with a desired cargo, but targeting is difficult – they seem to exhibit a high degree of non-specific binding. Protocells are porous silica nanoparticles with a fluid lipid bilayer on their surface. This lipid layer can be imbedded with targeting peptides, like those identified with phage display, but synthesis is a complicated, multi-step process. VLPs offer an alternative, and so far, under-exploited approach. VLPs of the RNA bacteriophage MS2 are monodisperse spheres, 28nm in diameter, with a hollow interior that can be loaded with a variety of molecular cargos. Moreover, MS2 VLPs have been adapted for peptide display and affinity selection and therefore offer the prospect that the same particle used for drug delivery can also be used for peptide ligand identification.

Filamentous phage display is a commonly used method for the identification of peptide ligands for specific cell-surface receptors. Filamentous phages provide an excellent platform for identifying targeting peptides, but are not suitable as drug delivery vehicles. Until now it has been necessary to chemically synthesize the targeting peptides and conjugate to an appropriate nanocarrier- a transition that results in an alteration of the local environment and therefore a significant decrease in selected affinity. But since MS2 VLPs have been recently adapted for peptide display and affinity selection, they can be used to integrate the ligand identification and drug delivery functions into a single particle and maintain the high affinity characteristics for with the ligands were originally selected.

2. EXPERIMENTS AND RESULTS

2.1 Initial Peptide Idenitication (Phage Display)

The original peptides used in these experiments were identified using the process of phage display. In this process, complex library of phage displaying random peptide sequences is allowed to bind to a cell-based selection target. Unbound phage are washed away and bound phage are eluted and used to infect bacteria for amplification. This process can be carried out iteratively until a population of phages that tightly bind the target is obtained (Figure 1). The selections were carried out on BaF3 cells that had been induced to create the cell receptor CRLF2 and express it on their surface. Negative selections were carried out on the parental cell line (no CRLF2) in order to assure that the ligand identified was in fact binding to the desired target and not to other features on the surface of the cells.

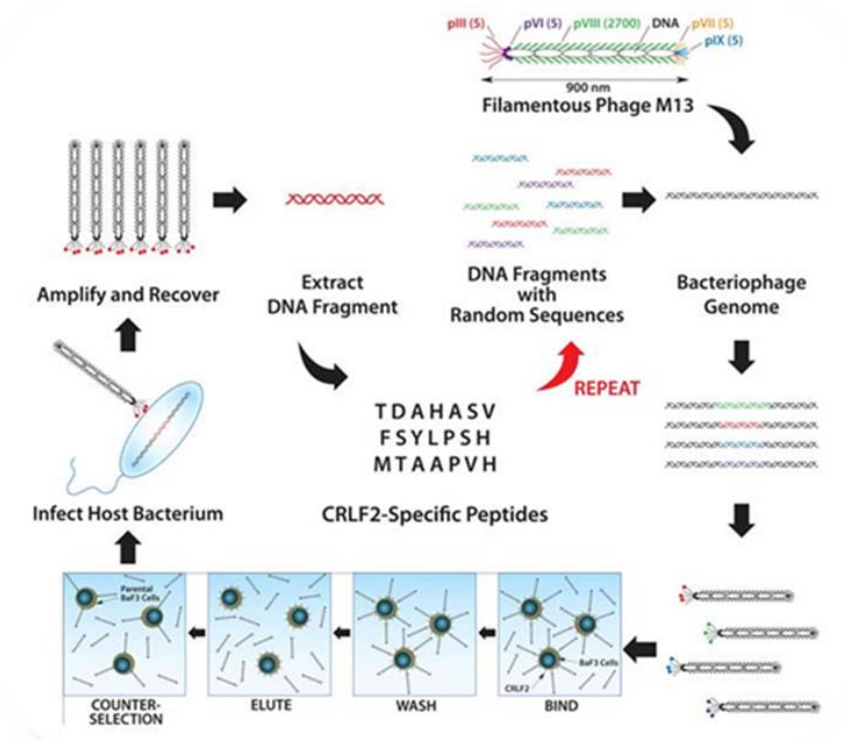

Figure 1: Affinity selection via phage display. A library of filamentous phage displaying a complex random library of peptides is created and allowed to bind to cells displaying the target surface marker. The sample is washed and the bound phage are eluted and subjected to negative selection against a parental cell line lacking the target surface marker. Bacterial cells are then infected with the reduced library for amplification and the process is repeated iteratively until an enriched population of binding phage is acquired.

Three peptide sequences were pulled from the enriched population of peptides that exhibited binding to CRLF2: TDAHASV, FSYLPSH, and MTAAPVH (best binder).

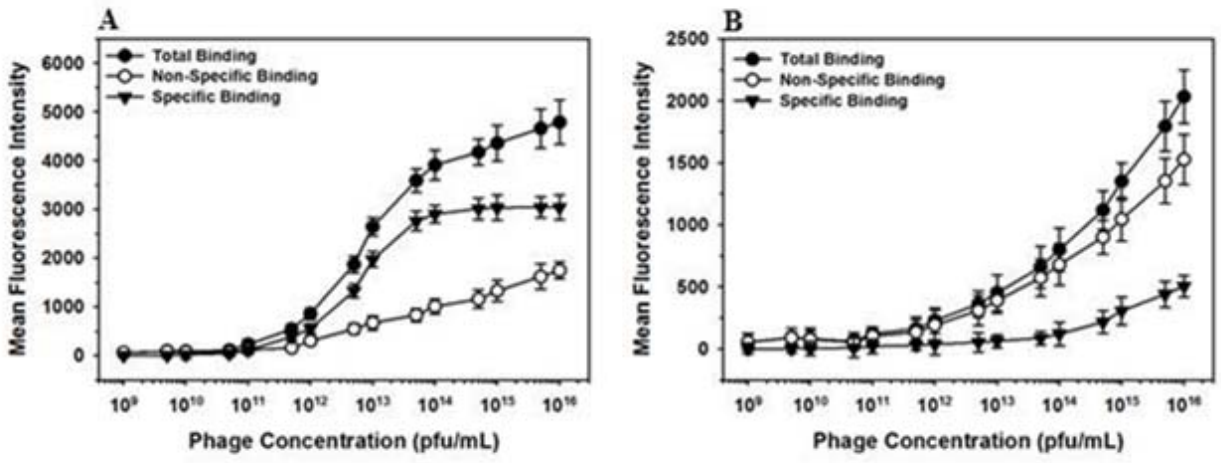

Figure 2: Binding saturation curves. Data shown for the MTAAPVH phage clone on BaF3/CRLF2 (A) and parental BaF3 cells. (B) Binding curves were constructed by titrating the amount of phage with constant cell concentrations in order to quantitatively describe binding. Non-specific binding was determined by incubating wild-type phage with the same cell lines. Specific binding to CRLF2 positive cells is significant; Specific binding to the parental cells is extremely minimal.

2.2 Virus-like Particles and Virus-like Particles as a Display Platform

A recently developed alternative to phage display involves the use of Virus-Like Particles or VLPS. VLPs of the bacteriophage MS2 are constructed of 90 fused dimers that self-assemble into an icosahedral shell 23nm in diameter. These perfectly monodisperse particles can be engineered to display a complex random library of peptides on their surfaces in either constrained (inserted into the A-B loop) or non-constrained (inserted at the N-terminus) conformation. Unlike the phage these particles are modeled after, VLPs are non-infectious. When they self-assemble, they encapsulate their own RNA. This not only allows for a mechanism of introducing cargo, but allows for particles with specific binding affinity to be isolated, the RNA extracted, reverse transcribed and amplified to allow for the production of more particles displaying that specific peptide (See Figure 3). VLPs are thought to be comparable to phage in their ability to conduct selections, and have successfully identified peptides that mapped directly onto the variable regions of specific antibodies. In addition, they are a suitable vehicle for delivery of cargo. Small molecule therapeutics and labels can be tagged with the RNA pac site that triggers self-assembly and thereby encapsulated within VLPs with relative ease.

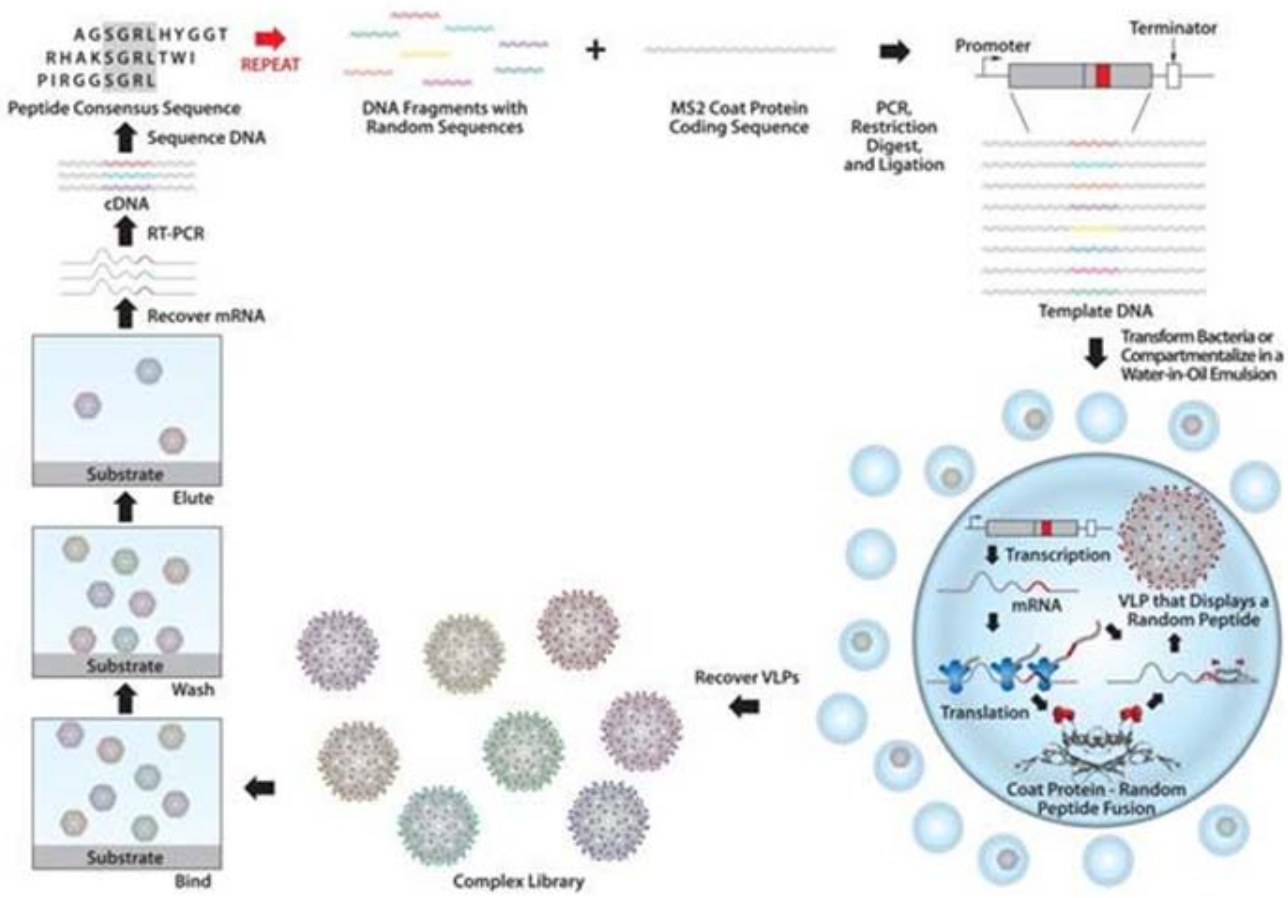

Figure 3: Virus-like particle based affinity selection. Similar to phage display described in Figure 1, although VLPs are non-infectious. The RNA must be isolated from eluted VLPs, reverse transcribed into DNA, amplified, re-inserted into a plasmid encoding for coat protein which was then transformed into bacteria for production of particles.

The progression of this project aimed to identify binding peptides via phage display, quantify their interactions with CRLF2 expressing cells while displayed on filamentous phage, genetically display these original peptides on the surface of VLPs, quantify the VLP interactions with CRLF2 expressing cells, and then identify peptides via the VLP based affinity selection process illustrated above.

2.3 Quantification of Identified Peptides on MS2 VLPs

In order to quantify the original peptides on MS2 VLPs, the peptides first had to be genetically inserted in the coat protein dimer. This is done without too much difficulty by

designing primers that anneal to the DNA in a desired location and contain an insert coding for the peptide sequence you would like to display. Through PCR, restriction digests, and ligations, a new DNA strand, or plasmid, is produced. This plasmid can then be transformed into *E. coli* and induced to produce coat protein at a large scale. These proteins self-assemble into VLPs that can then be isolated and used to conduct experiments.

For flow cytometry experiments, particles were labeled with Alexa-fluor-647 and incubated with various cell types for an hour before the samples were washed and immediately measured using a FACSCaliber flow cytometer (Data shown in Figure 4). Samples included both targeted VLPs (displaying the targeting peptides identified via phage display) and non-targeted VLPs (displaying a non-relevant peptide and particles not displaying any additional peptides). These particles were screened against both BaF3/CRLF-2 and parental BaF3 cells. As expected, none of the samples demonstrated significant binding other than the targeted VLPs incubated with target –expressing cells (lower right panel).

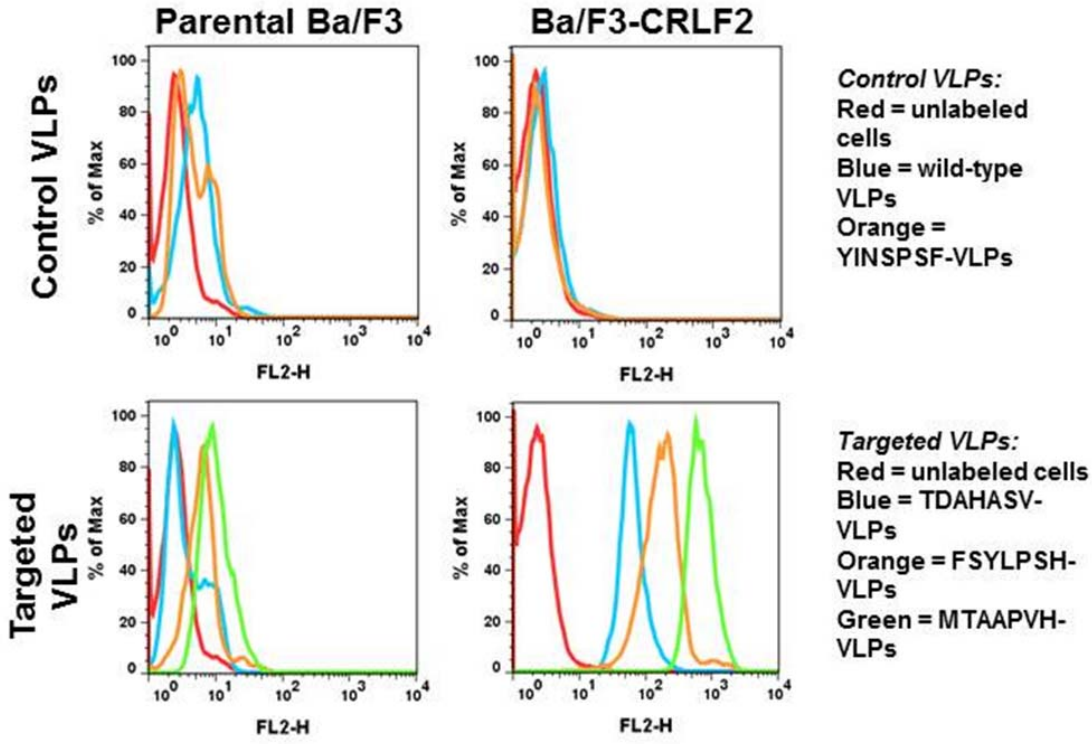

Figure 4: Flow cytometry data of targeted and non-targeted VLPs to Baf3/CRLF2 and BaF3 parental cell lines. Particles were labeled with Alexa-fluor-647 and incubated with various cell types for an hour before the samples were washed and immediately measured using a FACSCaliber Flow Cytometer.

To confirm binding, confocal microscopy images were taken of these samples as well, as shown in Fig. 5.

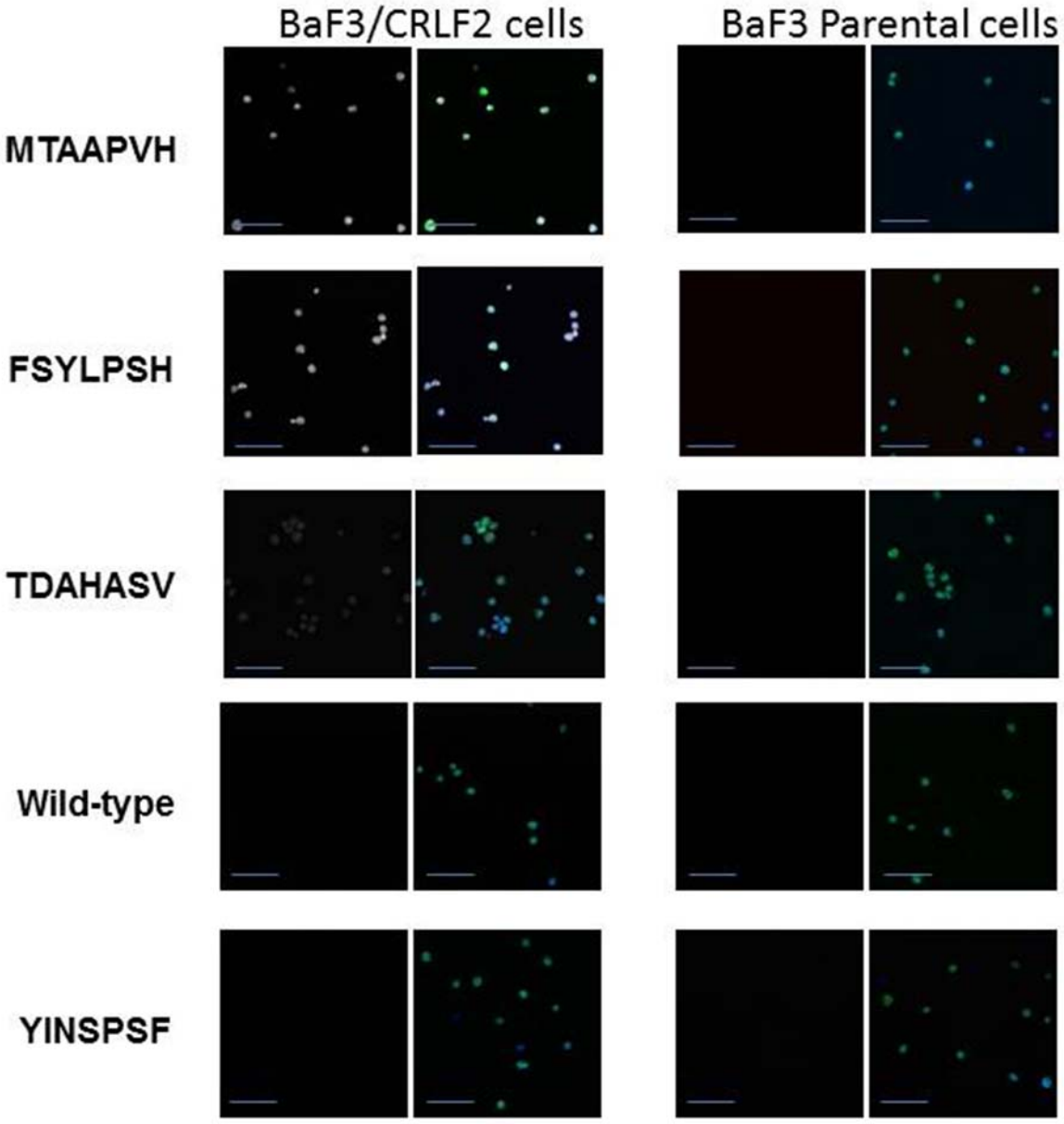

Figure 5: Confocal images of targeted and non-targeted VLPs with BaF3/CRLF2 and BaF3 parental cell lines. VLPs were labeled with Alexa-fluor 647; cells were stained with Invitrogen CellTracker Green, fixed in a 4% formaldehyde solution and mounted with SlowFade Gold with Dapi nuclear stain. Cells were incubated with VLPs for an hour at 37 degrees and then immediately stained, fixed, and mounted. Of each pair of images, the left side is the far red image, showing particles bound to cells, and the right side is a merge of all three channels. Scale bar = 20 microns.

2.4 Validation of Display Platform by Targeting EGFR

Issues arose with trying to conduct VLP-based affinity selection on the cell expressing CRLF-2. Due to differences between the way that CRLF2 is displayed on the surface of naturally expressing cells and the way it is presented on the BaF3/CRLF2 cell line, as well as an incomplete knowledge of the receptor, it was difficult to determine if obstacles in the course of the research were due to flaws in the protocol itself, or in the presentation of the target. To this end, it was decided to proceed with VLP-based affinity selection on a better understood target: Epidermal Growth Factor Receptor (EGFR). Not only is EGFR well understood, it is clinically relevant. Anti-EGFR antibodies are currently being used to treat several varieties of cancer. Also, a new approach is required because treatments with these anti-bodies are beginning to lose effectiveness, and some studies suggest might activate the receptor leading to increased tumor motility.

Selections are currently being conducted against EGFR protein using a mixed library of VLPs displaying peptides of 6, 7, 8, and 10 amino acids in length. Prior to selection, the EGFR was affinity captured onto the surface of a microcell plate via a GST-tag. This increases not only the amount of protein adsorbed to the well, but also orients the proteins in such a manner as to increase the statistical likelihood of selecting for peptides that bind in the receptor binding pocket. Selections are currently in the middle of the third iteration of positive selection (selection against EGFR) and have undergone one round of negative selection (to reduce the number of VLPs in the propagated library that are binding to the glutathione on the surface of the wells). Comparison run during the negative selections confirm that the enriched library does include VLPs that selectively bind to EGFR. A selection of these peptides have been sent out for sequencing, but no data has been received as of yet.

Once this protocol has been perfected, work will return to identifying a targeting ligand for CRLF2.

3. SUMMARY

In summary, there are several advantages to combining the affinity selection process and target delivery into the same platform, not the least of which is the preservation of the affinity characteristic for which a targeting ligand was originally selected. Targeting ligands that bind to CRLF2 were successfully identified via traditional phage display, and those peptides were then displayed on virus-like particles of the bacteriophage MS2. The binding characteristics of these particles were then confirmed using both flow cytometry and confocal microscopy. In order to perfect a protocol for targeting cellular receptors, work is currently being carried out to identify a targeting ligand to the well understood and clinically relevant cell surface receptor EGFR. Once these selections are complete and the resulting peptides are characterized, selections against CRLF2 will be revisited.

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