Targeted Delivery of Human Exosomes via Bispecific Antibody Platform

Engineering Honors Program - Capstone Final Report

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Exosome Background

Exosomes are phospholipid membrane-bound nanovesicles that are produced by living cells. They have recently been identified as key mediators of intercellular communication to near-by cells, but also to far off cells via systemic circulation in the bloodstream.To facilitate this communication, they possess an innate ability to deliver macromolecular cargo across biological barriers and evade lysosomal degradation. This cargo can include proteins, mRNA, microRNA, and other biologics commonly adapted into therapeutics. For these reasons, exosomes have been investigated as potential drug nanocarriers.

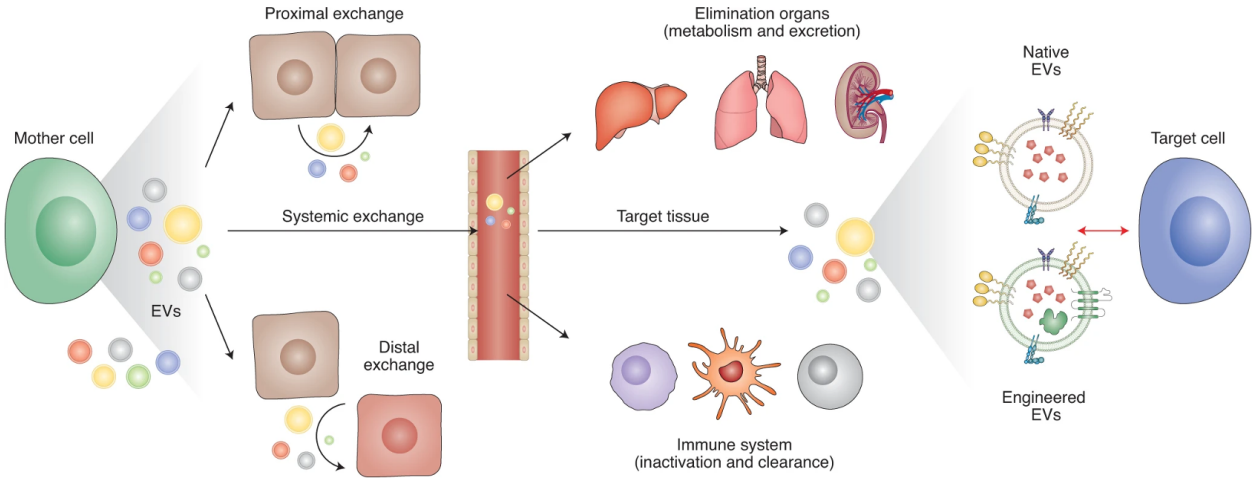

Figure 1. Exosome biogenesis, circulation, and elimination in the body [1]

Though some speculate that exosomes may exhibit a degree of natural tropism, there is little evidence that untargeted exosome drug carriers could prevent off-target delivery. Therefore, it would be beneficial to engineer them to display affinity ligands to direct delivery to target cell types. This can be achieved by genetically engineering fusions of affinity ligands to common exosome surface proteins; However, this method requires access to the parent cell line and

eliminates potential for stem-cell derived exosomes. Chemical conjugation to the exosome surface has also been used to target exosomes, but these methods have potential to alter the structure of the exosome and require highly reproducible chemistry to get consistent surface modification. To address these limitations, we designed bispecific antibodies that can direct exosomes to target tissues.

Exosome-Targeted Bispecific Antibody Structure and Characterization

Bispecific antibodies are antibodies that are engineered to display affinity at both ends. Traditional antibodies are Y-shaped made out of a light and heavy chain. The tops of the two arms of the antibody are made up of a variable light domain (VL) and a variable heavy domain (VH). These domains determine the unique affinity of the antibody to a specific antigen. Our exosome-targeted bispecific antibodies (ExTAbs) have bispecific affinity through the addition of single-chain variable fragments (scFv). scFvs are composed of a VH and VL region joined by a flexible peptide linker. Using this platform, we can treat antibodies as legos and take apart the VH and VL regions from one antibody and add them to the other end of another antibody.

For our proof of concept ExTAbs, we started off with an anti-CD63 immunoglobulin G (IgG) antibody as our foundation since the protein CD63 is a marker for exosomes. Then, we constructed scFvs from anti-PECAM-1 antibodies and anti-ICAM-1 antibodies, both of which target endothelial cells. Endothelial cells are the cells that line the vasculature, making it an accessible target from the bloodstream. It is important to note that this platform is versatile; we can choose scFvs from different antibodies to target various cell types and we can swap our foundation IgG to have affinities to other exosome markers such as CD9 and CD81.

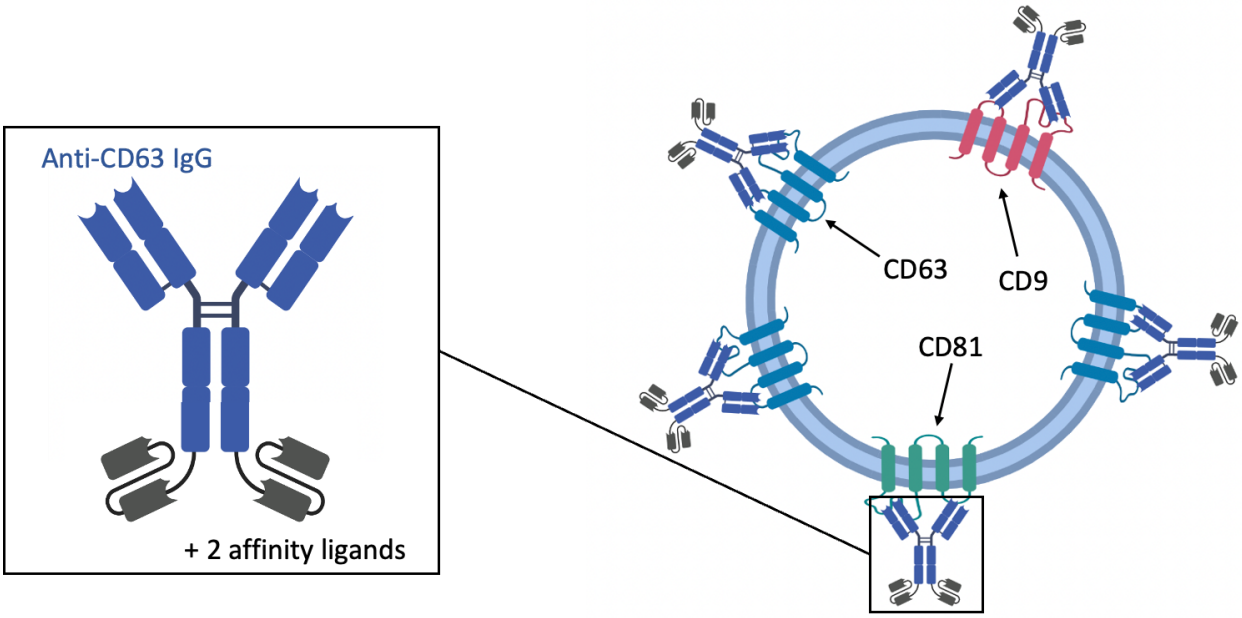

Figure 2. Exosome targeting bispecific antibody (ExTAb) platform uses an exosome targeted IgG and two scFv affinity ligands to deliver exosomes to target cells

To confirm the binding affinities on both ends of our ExTAbs, we performed an enzyme-linked immunosorbent assay or ELISA. First, we tested the IgG end of the bispecific was evaluated for affinity to the CD63 exosome marker. The ELISA showed that the dissociation constant, or Kd, of CD63 binding was 4.4 nM. This means that at a concentration of 4.4 nM CD63, half of the binding sites on the anti-CD63 end of the ExTAb are occupied. Therefore, a value in the low nanomolar range indicates tight binding. Next, the scFv end was evaluated for affinities to ICAM-1 and PECAM-1. These ELISAs yielded Kds of 1.1 and 2.1 nM respectively, which indicates even tighter binding.

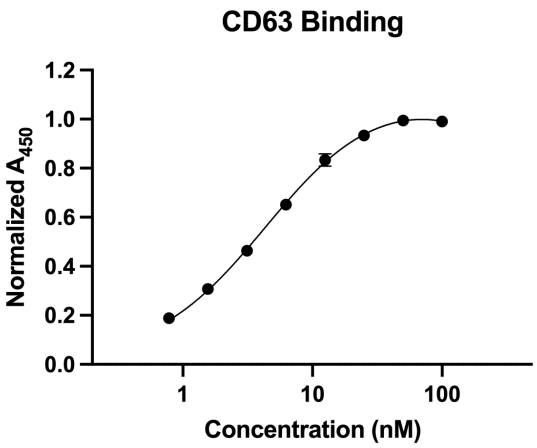

Figure 3. ExTAb binding affinity to exosome marker CD63, Kd = 4.4 nM

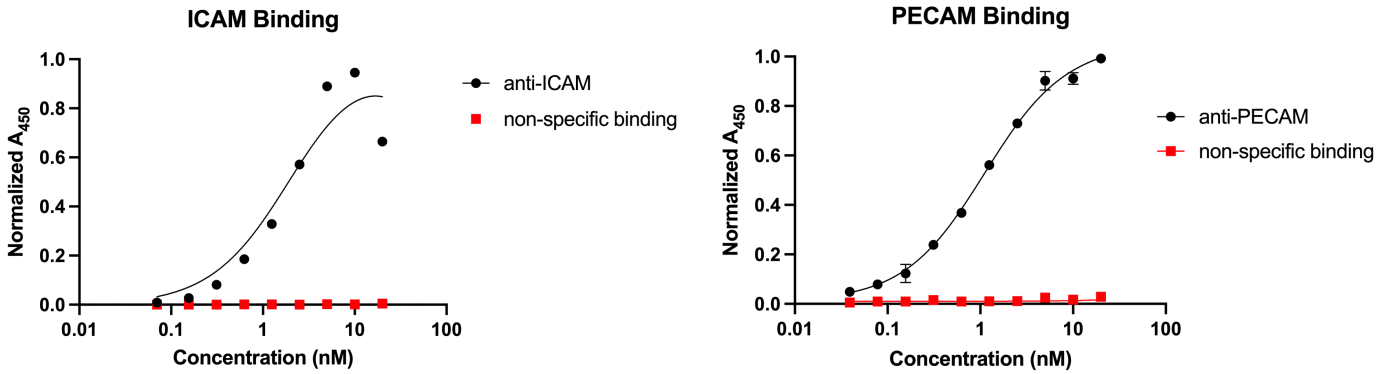

Figure 4. ExTAb binding affinity to target cell maker ICAM-1, Kd = 1.1 nM (left) and PECAM-1, Kd = 2.1 nM (right)

Exosome Purification and Decoration

To facilitate the exosome purification and decoration process, we derived our exosomes from a parent cell line that was genetically engineered to express green fluorescent protein (GFP) fused to the end of the CD63 protein. This way, we could use GFP as a visual green fluorescent marker for the exosomes. They were purified from cell culture through ultracentrifugation and size exclusion chromatography, which separates the exosomes from other cellular debris. The

fractions eluted off of the size exclusion column fluoresced green if they contained exosomes due to the GFP marker.

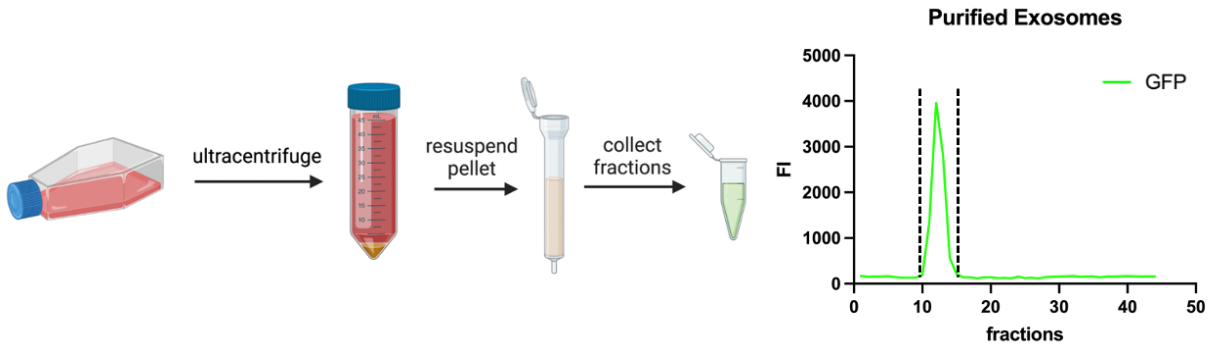

Figure 5. Purification protocol for GFP-labeled exosomes (left); GFP fluorescence in fractions from size exclusion column (right)

Decoration of the exosome surface was achieved by incubating the exosomes with AF647 labeled ExTAbs for 1 hour at room temperature. During this step, the anti-CD63 portion of the ExTAb would bind CD63 on the surface of the exosomes. Then, the solution would undergo size exclusion chromatography to remove the unbound antibody. The efficiency of antibody decoration is highly dependent on the degree of CD63 expression on the exosome surface; therefore, excess free antibody is to be expected after decoration.

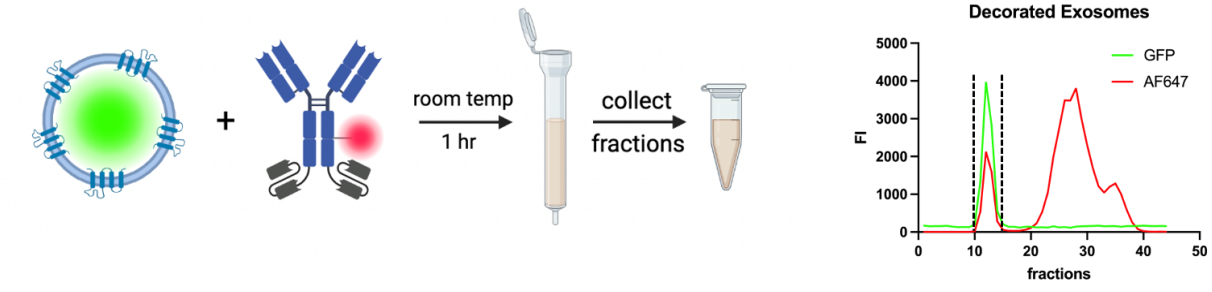

Figure 5. Alexa Fluor 647 (AF647) ExTAb labeling protocol for GFP-labeled exosomes (left); GFP and AF647 fluorescence in fractions from size exclusion column (right), free antibody is shown in the rightmost AF647 peak without GFP colocalization

In Vitro Exosome Delivery

After confirming successful exosome surface decoration, we wanted to determine if this strategy could increase exosome delivery to target cells in vitro. To do this, we incubated untargeted and targeted exosomes with model ICAM-1 positive and ICAM-1 negative cells to evaluate which combinations would lead to increased delivery. When the change in GFP signal on the cells was measured using flow cytometry, it was observed that 1) untargeted cells incubated with ICAM-1 positive cells and 2) targeted exosomes incubated with ICAM negative cells both showed no difference in delivery when compared to cells alone. When ICAM-1-targeted exosomes were incubated with ICAM-1 positive cells, however, a significant increase in GFP fluorescence can be seen in the model cells. It is important to note that this model cell line was engineered to express ICAM-1, so it may not reflect natural ICAM-1 expression.

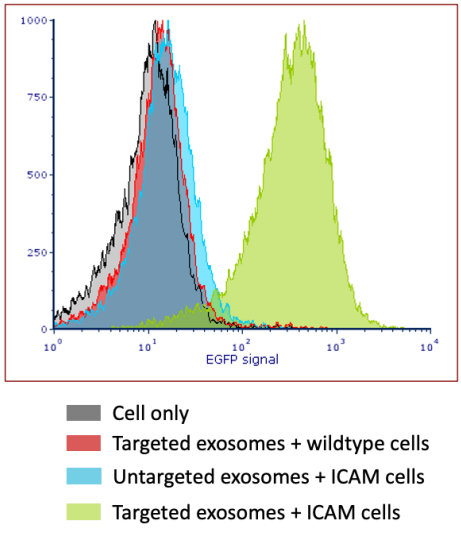

Figure 7. Increase in GFP signal when ICAM-1-targeted exosomes are incubated with ICAM expressing cells compared to negative controls

To address this caveat, we next verified exosome delivery to actual mouse endothelial cells (bEND3 cell line). These cells express PECAM-1 naturally and therefore, we tested our PECAM targeted exosomes. This flow cytometry experiment showed similar results, with our negative control of untargeted exosomes leading to no change in GFP signal, while the targeted exosome led to an increase in GFP. The shift in GFP fluorescence with targeted cells is not as dramatic as it was in the ICAM-1 experiment, but this could potentially be due to overexpression of ICAM-1 in the engineered cell line compared to normal levels of endothelial marker expression.

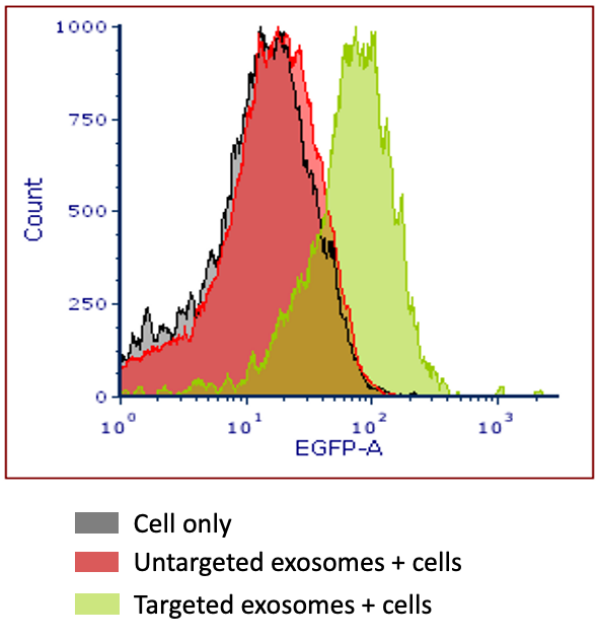

Figure 8. Increase in GFP signal when PECAM-1-targeted exosomes are incubated with mouse endothelial cells compared to negative controls

In Vivo Specificity for Vasculature

After verifying increased delivery *in vitro*, we tested if our method could increase delivery in vivo as well. For *in vivo* study, we created labeled untargeted, ICAM-1-targeted, and PECAM-1-targeted exosomes with a radioactive iodine-125 isotope so that we could measure the

radioactive counts to determine where the exosomes were delivered *in vivo*. Mice received approximately 0.12 million counts as the injected dose for each type of exosome. Blood was drawn at 5, 30, and 60 minutes and it was observed that all three types of exosomes cleared very quickly from the bloodstream. This follows with expectations for exosome biodistribution. The mouse organs were harvested at the 60-minute time point as well, and the percent of injected dose in each organ was measured by radioactive scintillation counting. These results revealed that the exosomes had significantly increased uptake in the lung, a highly vascularized organ, when compared to exosomes in the blood. ICAM targeting resulted in slightly higher uptake than PECAM-1 in the lung. In addition, we see an increase in exosome delivery in the kidney and heart, which are also endothelial-rich organs. PECAM-1 and ICAM-1 show different degrees of exosome targeting in different organs, but a marked increase in uptake is observed with both targets.

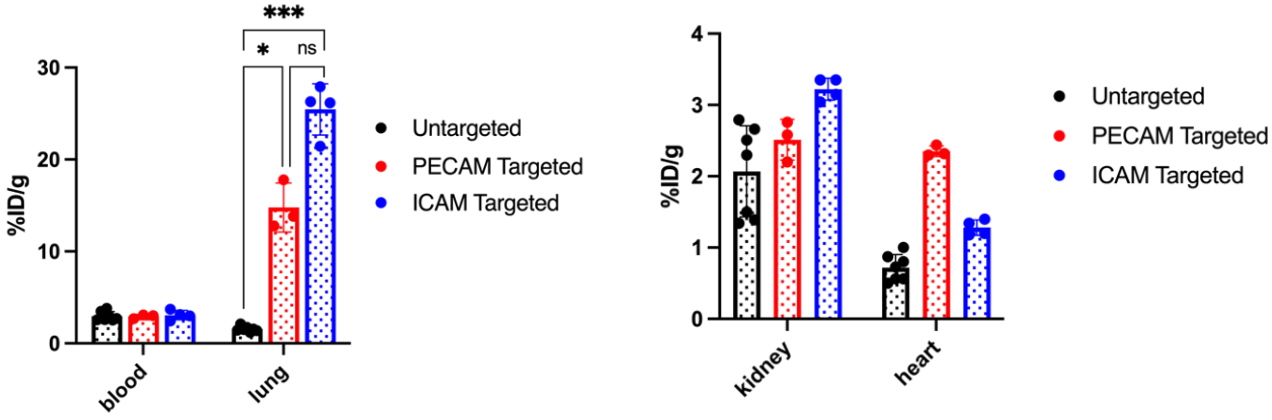

Figure 9. Percent injected dose (ID) per gram of tissue in mouse blood, lung, kidney, and heart after dosage of untargeted, PECAM-1, and ICAM-1-targeted exosomes

Future directions

Going forward, we intend on verifying specific delivery on a cellular level instead of the organ level to ensure that the exosomes are delivered to the vasculature through active targeting as opposed to passive accumulation. We are also in the process of evaluating if our ExTAb coated exosomes can deliver functional proteins to target cells once they are delivered. Finally, we look forward to exploring other target cells and organs for this versatile delivery platform.

Acknowledgements

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