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Exosome membrane bound TNF-receptor for the treatment of rheumatoid arthritis

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SANTA CLARA UNIVERSITY

Department of Bioengineering

I HEREBY RECOMMEND THAT THE THESIS PREPARED
UNDER MY SUPERVISION BY

Natalie Duong, Kevin Curley

ENTITLED

Exosome membrane bound TNF-receptor for the treatment of rheumatoid arthritis

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

BACHELOR OF SCIENCE
IN
BIOENGINEERING

June 15, 2017

Thesis Advisor(s) date

Zhiwen Zhang for Yuling Yan June 15, 2017

Department Chair(s) (use separate line for each chair) date

Exosome membrane bound TNF-receptor for the treatment of rheumatoid arthritis

By

Natalie Duong, Kevin Curley

SENIOR DESIGN PROJECT REPORT

Submitted to
the Department of Bioengineering

of

SANTA CLARA UNIVERSITY

in Partial Fulfillment of the Requirements
for the degree of
Bachelor of Science in Bioengineering

Santa Clara, California
2017

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease that causes painful inflammation of the synovium of the patient's joints. However, current treatments for RA have a variety of drawbacks. They often are ineffective, expensive, invasive, risky, cause an immune response, and/ or only provide short term relief. Thus, we developed a new treatment for preventing inflammation: TNF-receptors anchored onto exosome surfaces. Exosomes are nanovesicles that are naturally secreted by most of the cells in our bodies. The many benefits of using exosomes include non-immunogenicity, natural stability in the body, and non-invasiveness. We have demonstrated that exosome membrane bound TNF-receptors have the ability to prevent inflammation in mammalian cells. The success of this project could lead to a clinically effective treatment of rheumatoid arthritis as well as other inflammatory diseases by opening the doors to further research and development of exosomal therapies.

Keywords: exosome, rheumatoid arthritis, TNF-receptor

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We would like to acknowledge our advisor, Dr. Biao Lu, who offered his direction and advice regarding experimentation and presentation of data throughout the year. We would also like to acknowledge our reader, Dr. Riccomini, who guided proper documentation and presentation of our thesis. Lastly, we acknowledge Dr. Savas Tay from University of Chicago who donated one of the reporter cell lines we used.

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List of Abbreviations

Abbreviation	Definition
3T3 NF-κB reporter	3T3 NF-κB p65-RFP H2B-GFP reporter
ANOVA	Analysis of Variance
DI	Deionized
DMARDs	Disease Modifying Anti-rheumatic Drugs
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GPI	Glycosylphosphatidylinositol
HEK 293	Human Embryonic Kidney cell line
HEK NF-κB GFP/Luc reporter	HEK 293 NF-κB GFP/Luciferase dual reporter
LAB	Luciferase Assay Buffer
LAR	Luciferase Assay Reagent
LAS	Luciferase Assay Substrate
Luc	Luciferase
MFG-E8	Milk Fat Globule-EGF factor 8
MVB	Multi-vesicular body
NFkB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
PBS	Phosphate-buffered Saline
PLB	Passive Lysis Buffer
PS	Penicillin-Streptomycin
RA	Rheumatoid Arthritis
RFP	Red Fluorescent Protein
sTNFR	Soluble Tumor Necrosis Factor Receptor
TNFR	Tumor Necrosis Factor Receptor
TNFα	Tumor Necrosis Factor Alpha
TRE	Transcription Factor Response Element
VSV-G	Vesicular Stomatitis Virus Glycoprotein

1. Introduction

1.1. Background and Motivation: Developing a new treatment for rheumatoid arthritis

The problem we sought to address was inflammation in rheumatoid arthritis (RA). Currently, the options for RA treatment are limited and each has their own drawbacks. Exosomes, however, are a promising potential candidate for inflammation treatment with a variety of benefits.

Rheumatoid arthritis is a chronic autoimmune disorder where the host's immune system attacks its own synovium, the lining of the joint membranes. This causes inflammation of the synovium that not only causes pain, but also bone erosion and joint deformity. Approximately 35 - 70 million people are afflicted with RA worldwide and so it is an important focus for furthering treatment development.[1]

Current treatments such as surgery and disease modifying anti-rheumatic drugs (DMARDs) are not fully effective. Newer biologic therapies are generally quite expensive, demand frequent dosing, and pose some severe safety concerns including immune responses and high risk of infection. Our research sought to develop a treatment that would overcome these drawbacks and alleviate pain and damage in RA patients.

1.2. Literature Review: Rheumatoid arthritis and the therapeutic potential of exosomes

1.2.1. Rheumatoid arthritis

1.2.1.1. Role of TNFα in rheumatoid arthritis

Preventing inflammation, the primary cause of joint pain and damage in RA, is the primary focus of treating patients with RA. This swelling occurs as part of an inflammation pathway, which is often a signaled response to injury and a part of the body's natural healing process. The pro-inflammatory cytokines that are

[1] "8 Things People with Rheumatoid Arthritis Want You to Know." *Roche* -. F. Hoffmann-La Roche Ltd, 2016. Web.

directly involved in the inflammation pathway in RA can be rapidly generated and are done so in a specific order that dictates the effect each cytokine has on the inflammatory response. The most rapidly responding of these is tumor necrosis factor α (TNFα), which is produced and secreted in the first hour of stimulation, followed an hour later by interleukin 1 and 6 (IL-1 and IL-6)[2]. Within the rheumatoid synovium, or affected joint cavity of a patient with RA, there is a much higher concentration of these three cytokines than in non-pathogenic synovial fluid. Studies have found that by blocking these cytokines, a significant reduction of inflammation occurs. It was also shown that blocking TNFα inhibited the downstream upregulation of both IL-1 and IL-6 due to the specific order of cytokine release and was thus sufficient for inflammation reduction. [3]

1.2.1.2. Current treatments and their drawbacks

The following section further details the current treatments and their drawbacks which are summarized in the table below.

Table 1-1: Drawbacks of current RA treatments.

Current Treatment	Drawbacks
Surgery: synovectomy or joint replacement	• Invasive • General surgical risks
Disease-modifying antirheumatic drugs (DMARDs)	• History of failure • Insufficient alone
Anti-TNFα antibody injections (Adalimumab)	• Immune response • Short term & expensive
Soluble TNF-receptor (sTNFR) injections (Etanercept)	• Risk of infection and tumorigenesis • Short term & expensive

As the table indicates, each option has significant drawbacks.

Surgery is invasive, expensive, and increases the risk for further damage. It is also ineffective at preventing further development of RA, and is solely useful for repairing damages already incurred by severe inflammation. DMARDs have significant side effects, must be taken multiple times a week, and are ineffective

[2] Feldmann, M., Brennan, F., Williams, R., & Maini, R. (2004). Definition of TNFα as a therapeutic target for rheumatoid arthritis. *TNF-Inhibition in the Treatment of Rheumatoid Arthritis,* 1-22.
[3] Feldmann, M., Brennan, F., Williams, R., & Maini, R. 1-22.

when given alone. They are often used in tandem with biologic TNFα-inhibiting drugs.

Adalimumab is an anti-TNFα chimeric antibody that triggers an immune response to remove TNFα by natural processes, but it has a risk of over stimulating your immune system.[4] Chimeric antibodies, built from both human and mouse genes, have been known to elicit unwanted immune responses due to their foreign nature. This immune response includes the development of naturally produced antibodies that target Adalimumab, thus reducing its long-term effect. Etanercept, upon which we have built the concept of our solution, is a soluble TNF-receptor (sTNFR1) that binds to TNFα and preventing it from initiating the inflammation pathway.[5]

Current TNFα inhibiting medications are released into the entire blood stream and put the patient at high risk of serious infection and increased risk of cancerous tumorigenesis. Both TNFα inhibiting medications have been shown to be successful and efficacious; however, they each cost over $3,000 per month and are often administered bi-weekly by subcutaneous injection. Because of these problems, many patients diagnosed with RA are still suffering or are at risk for more serious health concerns.

1.2.2. Exosomes as a potential solution

1.2.2.1. Definition and therapeutic potential of exosomes

Exosomes are extracellular nanovesicles that are naturally secreted by most of the cells in the body for transportation and communication between cells.[6] Using exosomes as a therapy is a novel strategy with a variety of benefits that address the issues of current RA treatments (Table 1-2).

[4] Putte, L. V., Salfeld, J., & Kaymakçalan, Z. (2004). Adalimumab. *TNF-Inhibition in the Treatment of Rheumatoid Arthritis*, 71-88.
[5] Sule, S., & Bathon, J. (2004). Etanercept. *TNF-Inhibition in the Treatment of Rheumatoid Arthritis*, 47-69.
[6] Stickney, Z., Losacco, J., McDevitt, S., Zhang, Z., & Lu, B. (2016). Development of Exosome Surface Display Technology in Living Human Cells. *Biochemical and Biophysical Research Communications*, 472(1), 53–59.

Table 1-2: How exosomes overcome the many drawbacks of current RA treatments.

Current Treatment	Drawbacks	Exosomal Solution
Surgery: synovectomy or joint replacement	• Invasive • General surgical risks	• Noninvasive, injection based administration • Permanent tissue damage would not occur
Disease-modifying antirheumatic drugs (DMARDs)	• History of failure • Insufficient alone	• Founded upon most robust current treatment option (sTNFR) • No combination therapy needed
Anti-TNFα antibody injections (Adalimumab)[3]	• Immune response • Short term & expensive	• Naturally produced- exosomes elicit no immune response • Natural stability in the body extends duration of efficacy
Soluble TNF-receptor (sTNFR) injections (Etanercept)[4]	• Risk of infection and tumorigenesis • Short term & expensive	• Local Short term & expensive administration would maintain normal levels of systemic TNF • Natural stability in the body extends duration of efficacy

As demonstrated in Table 1, exosomes have great therapeutic potential. However, these nanovesicles would require modification for a specific goal. For treating inflammation in rheumatoid arthritis, we modified the surface of the exosome with TNF-receptors to occupy the TNFα that initiates the inflammation pathway in RA. Considering the potential benefits above, this proposed solution would hopefully be able to navigate the affected synovial joint easily, provide long term relief, and would not cause any immune response, unlike current treatment options.

1.2.2.2. Exosome surface engineering

Exosomes are formed through an endocytic pathway[7,8,9] (Figure 1-1).

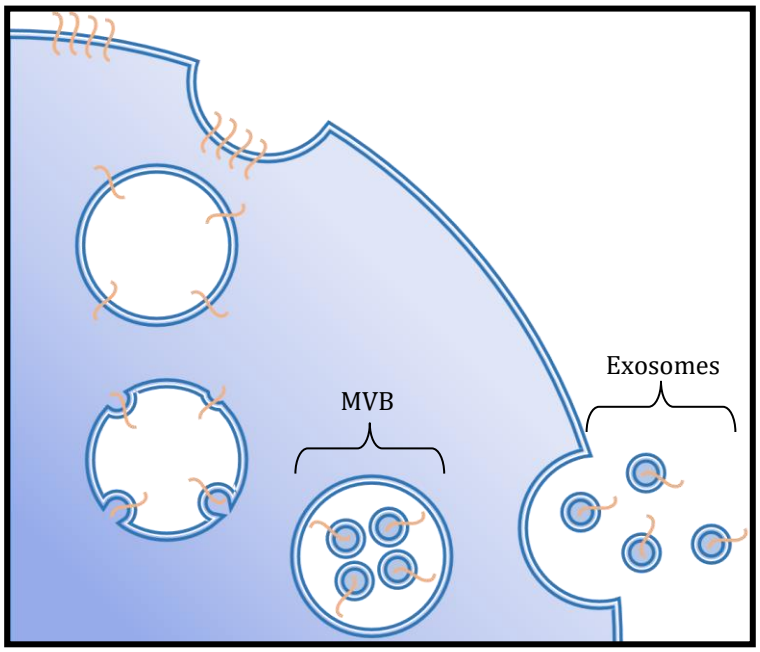

Figure 1-1: Exosome biogenesis.

As shown in the figure above, the proteins on the exosome membrane originate from the cellular membrane. Proteins destined for the exosome will self-organize within the cellular membrane, and this specialized domain invaginates to form an endosome with the same proteins. This endosome invaginates further, forming an endosome containing many smaller vesicles, otherwise known as a multivesicular body (MVB). Upon fusion of the MVB to the plasma membrane, exosomes are secreted out of the cell.[10]

The knowledge of this pathway allows engineers to manipulate surface expression by modifying the proteins that are known to localize onto exosome membranes. One method of modifying membrane proteins is by changing the DNA sequence that codes for the target membrane protein, so that the structure of

[7] Babst, M. (2011). MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. *Current Opinion in Cell Biology*, *23*(4), 452–457.
[8] Hurley, J. H., Boura, E., Carlson, L. A., & Rycki, B. (2010). Membrane budding. *Cell*, *143*(6), 875–887.
[9] Théry, C., Zitvogel, L., & Amigorena, S. (2002). Exosomes: composition, biogenesis and function. *Nature Reviews. Immunology*, *2*(8), 569–579.
[10] Théry, C., Zitvogel, L., & Amigorena, S. 569–579.

the protein can be altered to produce a desired function. Engineering the surface of exosomes requires targeting of proteins that are commonly found in exosomes. Primary molecular scaffolds for exosome surface engineering include glycosylphosphatidylinositol (GPI) anchored proteins and the milk fat globule-EGF factor 8 (MFG-E8) protein found on the outer membrane, as well as transmembrane proteins such as tetraspanins and vesicular stomatitis virus glycoprotein (VSV-G).

1.2.2.2.1. Tetraspanin CD63 as an exosome engineering scaffold

Tetraspanins proved to be a great candidate for the display of molecules on the surface of exosomes because of their prevalence in exosomes. Tetraspanins are a membrane-bound protein, with an intrinsic membrane localization signal and play a role in the endocytic budding from the cellular membrane through the formation of tetraspanin enriched microdomains (TEMs), which explains their abundance on the surface of exosomes.[11] Fluorescent reporters have been successfully fused with tetraspanin CD63 and used to track the biogenesis, secretion, and uptake of exosomes.[12] Cells with the engineered tetraspanins secrete the modified exosomes into the culture medium, which can be isolated and introduced to and taken up by recipient cells.[13] This indicates that tetraspanins can be fused with other desired proteins, such as a TNF-receptor, for potential therapeutic applications. Thus, we used this scaffold to modify the exosome surfaces with TNF-receptors for the potential treatment of inflammation in RA.

1.2.3. Inflammation reporters

TNFα's effects on the inflammation pathway have been tested at the cellular level in various past experiments. Biosignals have been developed to measure the presence of an inflammatory response in a cell culture model. They target the NF-κB pathway (Figure 1-2) as a means of identifying this response.

[11] Hassuna, N., Monk, P. N., Moseley, G. W., & Partridge, L. J. (2009). Strategies for targeting tetraspanin proteins: Potential therapeutic applications in microbial infections. BioDrugs, 23(6), 341–359.
[12] Stickney, Z., Losacco, J., McDevitt, S., Zhang, Z., & Lu, B. (2016). Development of Exosome Surface Display Technology in Living Human Cells. Biochemical and Biophysical Research Communications, 472(1), 53–59.
[13] Stickney, Z., Losacco, J., McDevitt, S., Zhang, Z., & Lu, B. 53–59.

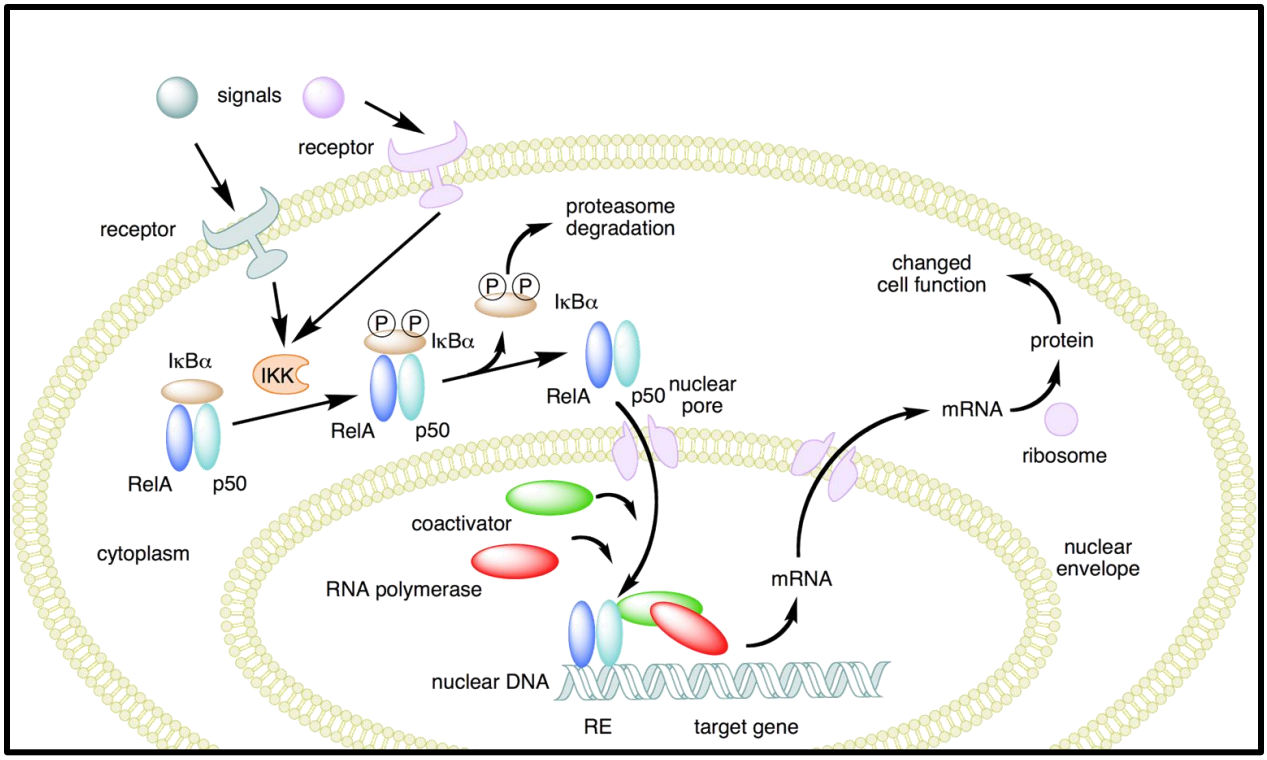

Figure 1-2: NF-κB pathway. [14]

The pathway begins with the binding of TNFα to the TNF-receptors on the exterior of the cell membrane. This activates an enzyme that activates NF-κB, a transcription factor consisting of p65 (RelA) and p50, which is then translocated into the nucleus. Once in the nucleus, NF-κB binds to the response elements just upstream of the gene encoding for the inflammatory response. Once bound, it induces the expression of this gene, yielding inflammation.

1.2.3.1. 3T3 NF-κB p65-RFP H2B-GFP reporter

One such reporter cell line uses a p65 knockout line of 3T3 mouse fibroblast cells with an added p65-RFP complex.[15] The modified NF-κB pathway is displayed in Figure 1-3, below.

[14] Rock Creek Pharmaceuticals Inc. NF-kB Signaling Pathway. Digital image. Securities and Exchange Commission. June 2015.
[15] Tay, Savas. (2011). Single-cell NF-κB dynamics reveal digital activation and analog information processing in cells. *Nature*. *466*(7303), 267–271.

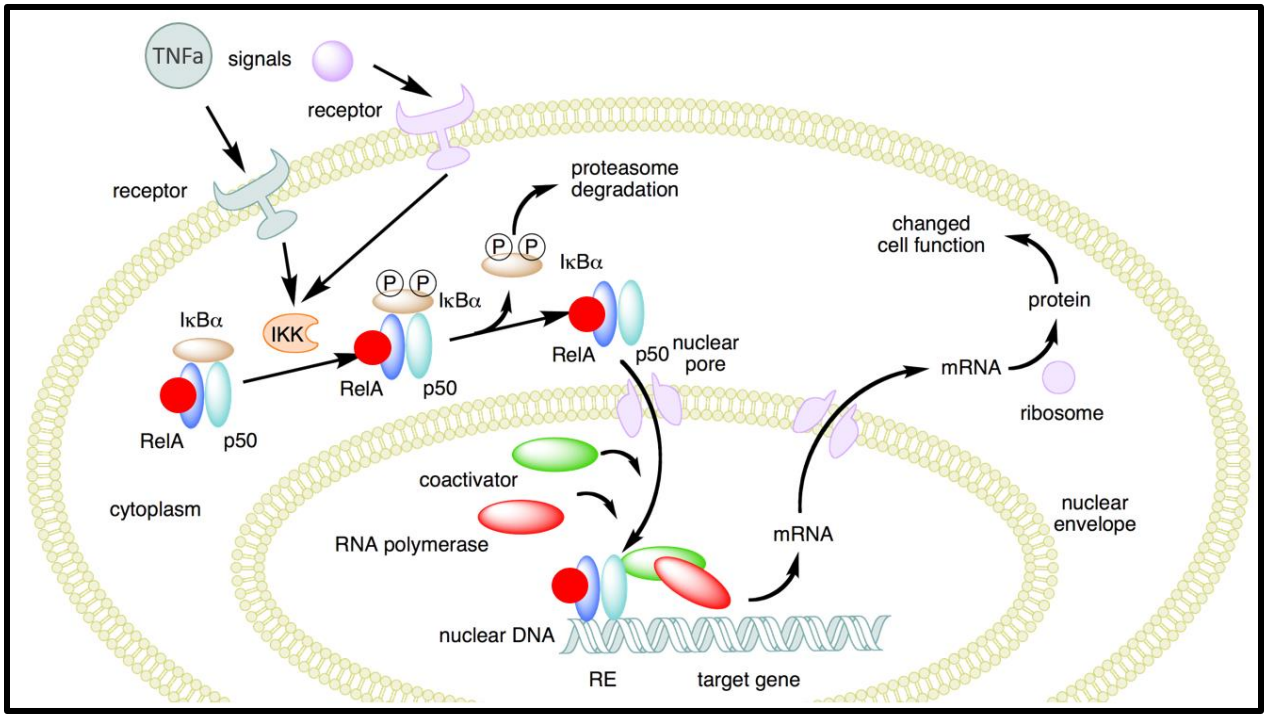

Figure 1-3: NF-κB pathway for 3T3 NF-κB reporter. The red circle represents red fluorescent protein (RFP).

In the presence of TNFα, this reporter should show p65-RFP complex migration from the cytosol into the nucleus upon the initiation of the inflammation pathway as NF-κB is activated to promote the expression of inflammatory response. The same cell line includes a H2B-GFP nuclear marker (not shown) to assist with nuclear identification in imaging. (This reporter was donated to us by Dr. Savas Tay from University of Chicago.)

1.2.3.2. HEK 293 NF-κB GFP/Luciferase reporter

We contributed to the development of another reporter in a previous project in our lab. This reporter was designed to express both GFP and firefly luciferase in the presence of TNFα[16] not by modifying the proteins in the NF-κB pathway, but rather by adding DNA coded to be a target of NF-κB. This modification is shown in Figure 1-4, below.

[16] Zhang, Z., Stickney, Z., Duong, N., Curley, K., & Lu, B. (2017). AAV-based dual-reporter circuit for monitoring cell signaling in living human cells. Journal of Biological Engineering, 11(1), 18.

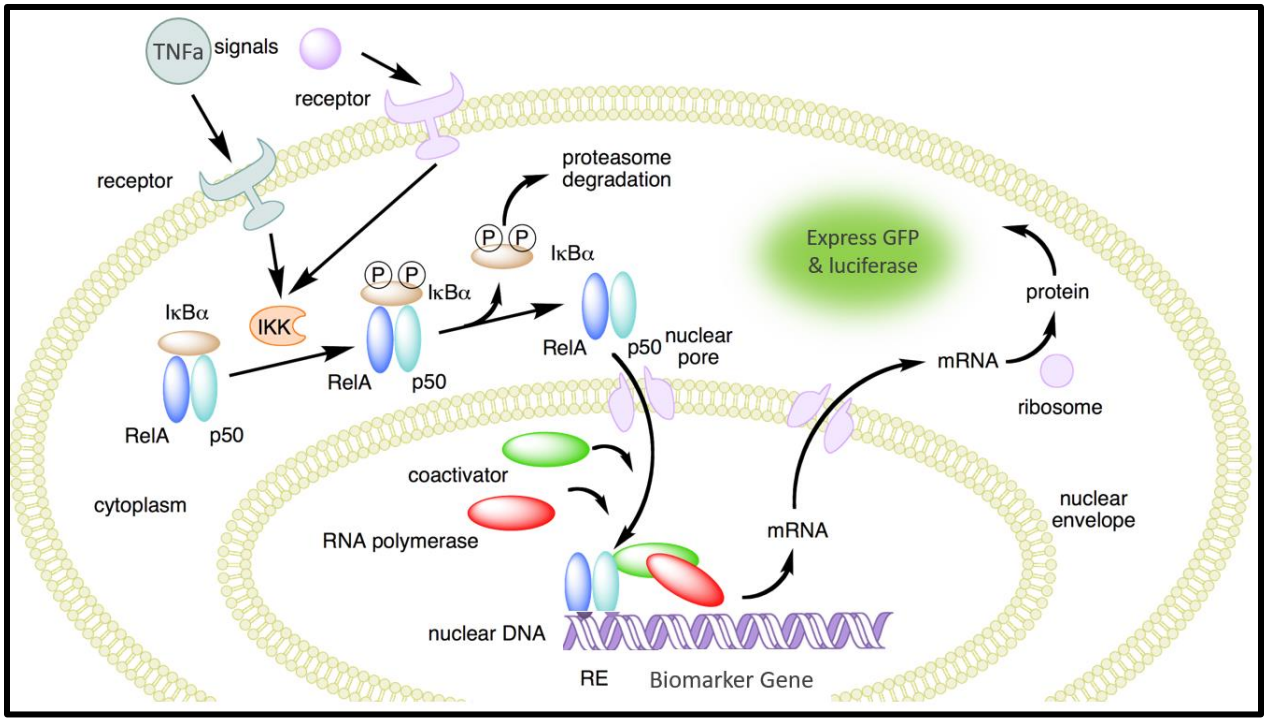

Figure 1-4: NF-κB pathway for HEK NF-κB GFP/Luc reporter.

The reporter consists of a viral vector cassette containing transcription factor response elements (TREs) and a minimal CMV promoter followed by the gene encoding for GFP and luciferase. In our reporter cells containing this biomarker DNA, in the presence of TNFα, NF-κB is activated and binds to the TREs, leading to the expression of GFP and luciferase.

1.3.Project Overview: 3 phases' goals and expected results

Our expected results for each phase of experimentation are outlined below. The actual results and discussion are in each phases' respective Results and Discussion sections.

1.3.1. Phase 1: Design Production

The goal of this stage was to manufacture and harvest the modified exosomes. We expected that live mammalian cells could be manipulated to secrete the modified exosomes that could be harvested and stored for later testing of efficacy in preventing inflammation.

1.3.2. Phase 2: Verification of Inflammation Reporter

In order to determine whether our design is effective in preventing inflammation, we needed a reliable method of detecting inflammation. Thus, the goal of this stage was to determine the best method in reporting inflammation. We predicted that the best reporter for our purposes would be one that is quantifiable, reliable, and high throughput.

1.3.3. Phase 3: Testing Efficacy of Design

The goal of this stage was to determine whether our modified exosomes are capable of preventing inflammation. Using the inflammation reporter verified in Phase 2, we measured the effect of the modified exosomes stored from Phase 1 on the inflammatory response of live mammalian cells. We expected our design to significantly decrease the level of inflammation by at least 50%.

1.4. Timeline

The phases described above were completed according to the following timeline.

Figure 1-5: Timeline of project phases.

1.5. Risk Managements: Potential setbacks and their work-arounds

In order to optimize our success in this project, we have analyzed the potential risks that could contribute to the project's failure. We had back-ups and work-arounds in place in order to avoid or be able to quickly recover from most of these potential risks moving forward.

Table 1-3: Analysis of project risks.

Risk	*Severity*	*Chance*	Solution	*Ease*	***Score****
• Failure of current design in preventing inflammation - This is an novel method of treating inflammation that may not work	10	5	• Use a different scaffold for anchoring TNFR onto exosome surface - VSV-G, GPI, or MFG-E8	10	**500**
• Contamination - Shared incubator in the tissue engineering lab has a recent history of contamination	9	3	• Isolate our cells to one corner • More thoroughly decontaminate going in and out of incubator • Limit outside use of incubator	5	**135**
• Resource depletion - Certain solutions may need to be replenished - Dependent on the need for repetition	7	2	• Coordinate with other groups in our lab • Mutually applicable resources should be shared to reduce waste	4	**56**
• Team member absence - Kevin was abroad from 12/10/16 - 1/9/17	2	10	• Freeze down unnecessary cell lines • Divide labor such that Kevin is responsible for remote work	2	**40**

*Score is calculated as the product of the three ranked columns. Higher scores are areas of greater concern.

1.6. Significance

Our project serves as a basis for further development and testing of a novel treatment for RA. Using TNF-receptors on exosomes would allow for a more efficient treatment for RA without the side effects of current treatment options. This will directly affect the well-being of the 35-70 million suffering with RA worldwide. Although we have not yet established a clinically ready product, our research lays the groundwork for animal and clinical trials of our modified exosomes. In addition, our success should inspire further attempts to use exosome surface engineering for various other medical therapies including immunotherapies, gene therapies, and/or targeted drug delivery.

1.7.Team management

With only two group members, we worked together equally in designing, executing, and summarizing experiments. Dr. Lu was our advisor, and as a team, all three of us met once per week to discuss results and upcoming experiments.

1.8.Budget

Outlined below is our project budget.

Table 1-4: Budget.

Flasks and plates	$250.00
Fetal bovine serum	$250.00
Serum free media	$120.00
Gene synthesis	$650.00
TNFα	$210.00
Culture Media	$500.00
Transfection Reagent	$470.00
Luciferase Assay	$870
Total Cost	**$3,320**

2. Design Production

Figure 2-1: Timeline of project phases. This chapter focuses on Phase 1.

The goal of this stage was to manufacture and harvest the modified exosomes.

2.1.Key constraints

The key constraints for this portion of the project include the method by which we manufactured exosomes and the imaging technology we had access to to demonstrate exosome production. At the time of experimentation, our lab only had access to a fluorescent microscope, not a confocal microscope that would have made imaging exosomes more clear and accurate. Also, the method by which we harvested exosomes had a low yield and mandated many repetitions to gather enough exosomes for one trial of our third phase of the project.

2.2.Design description

Because of their prevalence on the exosome surface, we used tetraspanin CD63 as a scaffold to anchor TNFRs onto the exosome surface (Figure 2-2).

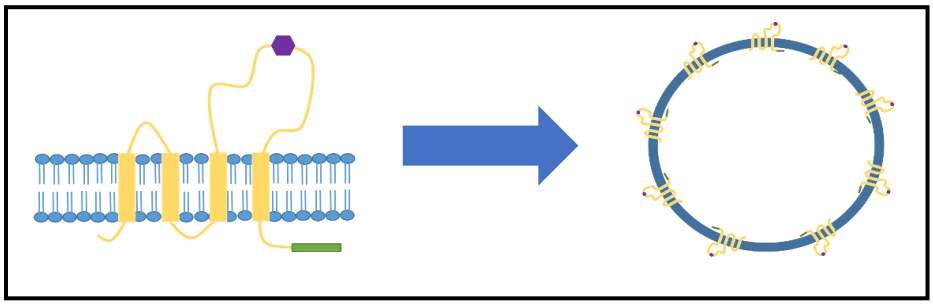

Figure 2-2: Design for anchoring TNF-receptor onto exosome surface. The purple hexagon represents a TNF-receptor and the green bar represents GFP anchored onto tetraspanin CD63 (left). This modified protein will be produced onto the exosome surface (right).

The TNFR functions as a sponge to soak up excess TNFα to prevent it from initiating the inflammation pathway in cells. The GFP enables visualization under a fluorescent microscope throughout the experiment. Cells with the engineered tetraspanins will secrete functional exosomes with the engineered tetraspanin into the culture medium, which can be isolated and introduced to recipient cells.

We also constructed a tetraspanin CD63 with a red fluorescent protein (RFP) in place of the TNF-receptor. This was to produce non-treatment exosomes as a control for experimentation.

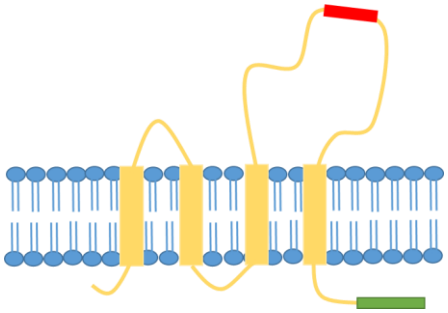

Figure 2-3: CD63-RFP-GFP construct. The bars represent red and green fluorescent proteins.

Human embryonic kidney cells (HEK 293) were used to produce both the CD63-RFP-GFP and CD63-TNFR-GFP exosomes.

2.3.Expected results

We expected to see smaller green dots in the cells with the engineered tetraspanins (as opposed to the whole cell being green) when examining them under the GFP filter of a fluorescent microscope. This would confirm that our modified tetraspanins are localizing onto exosomes which can be harvested from the cells.

2.4.Backup plan

If this design fails to produce exosomes with the CD63-TNFR-GFP, we planned to pursue a different scaffold for anchoring the TNFR onto the exosome surface. Other possible scaffolds include VSV-G, GPI, and MFG-E8.

2.5.Materials and Methods

2.5.1. Materials

Table 2-1: Materials – Phase 1

Material	**Company/ Brand**	**Model #**
Passaging Mammalian Cells Materials (Appendix A2-A)		
Dish 145mmx20mm	Sigma-Aldrich/ Greiner	639160
DMEM + 10% FBS + PS media*	Created in lab	N/A
Transfection Materials (Appendix A3-A)		
CD63-TNFR-GFP plasmid	GenScript	N/A
CD63-RFP-GFP plasmid	GenScript	N/A
Serum-Free Medium without L-Glutamine	BioWhittaker/ UltraCULTURE	12-725F
50 mL conical-bottom centrifuge tubes	VWR	89039-658
Syringe, 5 mL	BD Biosciences/ BD Luer-Lok	309646
Hydrophobic filter, 0.2 micron	BD Biosciences/ BD Influx	645270
Exosome precipitation solution	SBI/ ExoQuick	EXOQ5A-1
Phosphate-Buffered Saline pH 7.4 (PBS)	Thermo Fisher Scientific	AM9625
Cryogenic vials	Sigma-Aldrich/ Nalgene	V4757

*Refer to Appendix A1: Making Media - DMEM + 10% FBS + PS

2.5.2. Special notices

- Proper laboratory attire and practice (further explained in the SCU EHS guidelines) is required.
- Safe and proper aseptic technique is required for this procedure.
- All reagent and cell handling is to be done in the fume hood.
- No lids or caps are to be left open unless presently in use.
- When not in use, live cells are stored in an incubator (5% CO_2, 37°C).
- Do *NOT* attempt to perform this procedure unless the above requirements are met.

2.5.3. Overview of procedure

Table 2-2: Overview of procedure – Phase 1

	Step	**Section**
Day 1	Seed cells	1
Day 2	Transfect cells	2
Day 3	Image transfected cells	3
	Change media to serum-free medium	4
Day 4	N/A	N/A
Day 5	Exosome Harvest Part I	5
Day 6	Exosome Harvest Part II	6

2.5.4. Procedure

Day 1

1. Seed Cells
    1.1. Using a passaging mammalian cells protocol (Appendix A2-B), seed HEK 293 cells onto 2 145mmx20mm dishes at a density of 1-5x10^5 cells/mL with DMEM + 10% FBS + PS media.
    1.2. Incubate at 37°C for 24 hours.

Day 2

2. Transfect Cells
    2.1. Using a transfection protocol (Appendix A3-B), transfect the cells on 1 145mmx20mm dish from Day 1 with CD63-TNFR-GFP plasmid.
    2.2. Repeat step 2.1 for the other 145mmx20mm dish from Day 1 with CD63-RFP-GFP plasmid.

Day 3

3. Image transfected cells (for cells on each dish)
    3.1. Using a fluorescent microscope take phase, GFP, and RFP images of the cells at 20x.
4. Change media to serum-free medium (for each dish)
    4.1. Carefully aspirate the media off of the dish.
    4.2. Carefully add 20 mL of serum-free medium to the dish.
    4.3. Incubate at 37°C for 48 hours.

Day 5:

5. Exosome Harvest Part I (for each dish)
    5.1. Collect the serum-free medium into a 50mL centrifuge tube.
    5.2. Centrifuge the 50mL centrifuge tube at 1500xg for 10 minutes.
    5.3. Filter the supernatant through a 0.2-micron filter with a 5mL syringe into a new 50mL centrifuge tube.
    5.4. Add ¼ of the supernatant volume of exosome precipitation solution
    5.5. Incubate at 4°C for 24 hours.

Day 6

6. Exosome Harvest Part II (for each centrifuge tube from Day 5)
    6.1. Centrifuge the 50mL centrifuge tube from Day 5 at 3000xg for 30-45 minutes.
    6.2. Carefully aspirate off the supernatant.
    6.3. Resuspend the pellet in 50µl of PBS.
    6.4. Store in a cryogenic vial at -80°C until needed.

2.6.Results

One day after the transfection, the cells were imaged to examine the localization of the modified protein. These images are displayed below.

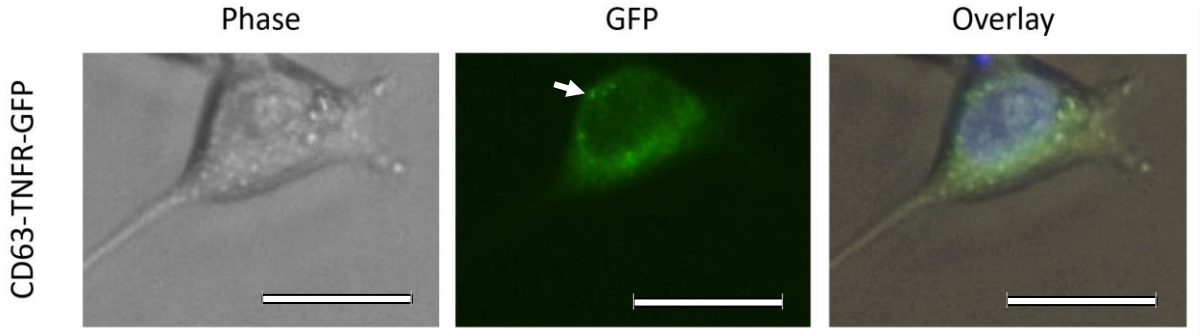

Figure 2-4: Exosome production of CD63-TNFR-GFP in HEK 293 cells. The nucleus is marked with a Hoescht stain for visualization in the last photo.

2.7.Discussion

The smaller green dots in the GFP images taken after the transfection demonstrate the successful localization of CD63-TNFR-GFP into exosomes. We were then able to harvest these exosomes and store them for usage in Phase 3.

3. Verification of Inflammation Reporter

Figure 3-1: Timeline of project phases. This chapter focuses on Phase 2.

In order to determine whether our design is effective in preventing inflammation, we needed a reliable method of detecting inflammation. Thus, the goal of this stage was to determine the best method in reporting inflammation.

3.1.Key constraints

The key constraints for this portion of our project were the availability, sensitivity, and quantifiability of methods to measure the biosignals given off by our inflammation reporters. Due to the nature of our various reporters, different measurement metrics were used: confocal imaging, flow cytometry, and a luminescence assay. We eventually selected the reporting mechanism that would be measured by a luciferase assay because of its highly sensitive and quantifiable nature and our access to the necessary equipment.

3.2.Design description

We tested three different reporting mechanisms for our project to get a more comprehensive look into where in the inflammation pathway the inflammatory response would be being inhibited by our therapy.

3.2.1. 3T3 NF-κB reporter

The 3T3 NF-κB p65-RFP H2B-GFP reporter cell line is an established reporter that can measure inflammatory response in a cell culture model. As discussed in section 1.2.3.1, the

initiation of the inflammation pathway is indicated by the migration of p65-bound RFP to the nucleus from the cytosol. We were able to roughly estimate this inflammation response visually using a confocal microscope. Cells that expressed cytosolic RFP were considered uninflamed and cells with distinct nuclear RFP were considered inflamed. The cells also contained the gene for H2B-GFP, which encodes for a green fluorescing nucleus. This allowed for more distinct visual identification of the nucleus, and helped in the process of roughly quantifying inflammation in a population of cells. As depicted in Figure 3-2, the nucleus will appear yellow in an overlay image of the green nucleus when the RFP has migrated into it.

We designed an experiment to visually estimate the percentage of cells with nuclear RFP after treatment with TNFα. This visual change is displayed in the figure below.

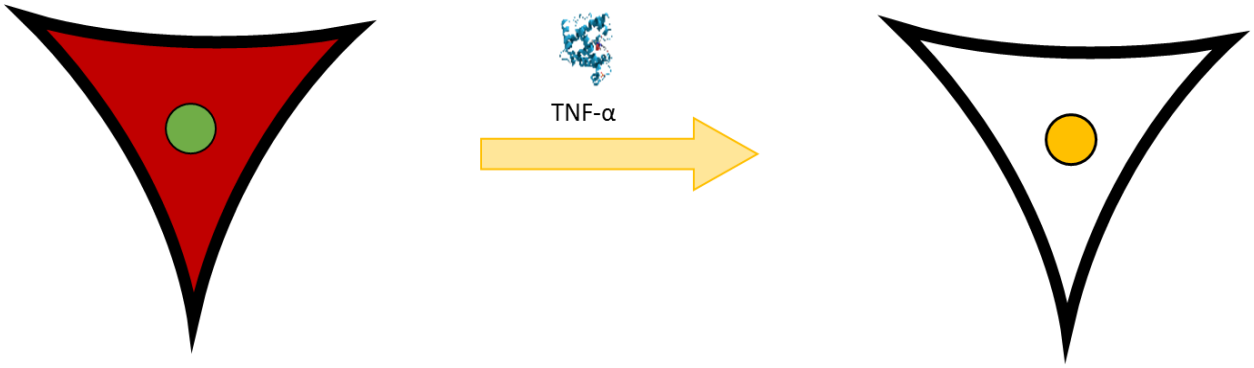

Figure 3-2: Visual change of 3T3 NF-κB reporter with exposure to TNFα. Red, green, and yellow represent RFP, GFP, and both RFP + GFP expression respectively.

3.2.2. HEK NF-κB GFP reporter

Using a dual reporter that we had previously helped develop (section 1.2.3.2), we were able to see one step after the migration of p65 into the nucleus. This reporter is measuring the expression of the gene downstream the NF-κB promoter region. As the cell transcribes what would usually be the genes coding for the inflammatory response to TNFα, it will now also transcribe the genes for both GFP and Firefly Luciferase. The presence of GFP was visually indicated by green fluorescence in the cytosol of the cell, as demonstrated in Figure 3-3.

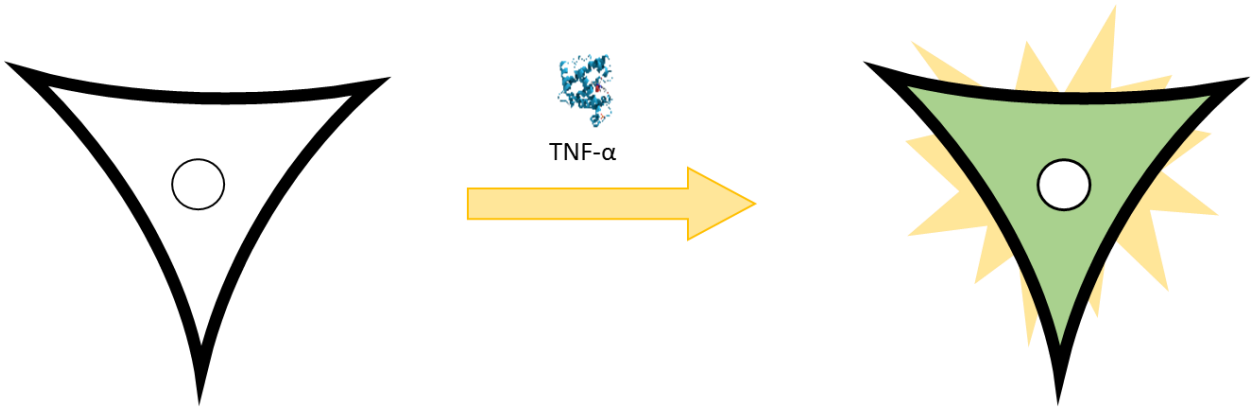

Figure 3-3: Visual change of HEK NF-κB dual reporter with exposure to TNFα. Green represents GFP expression while the yellow burst represents luminescence (discussed in section 3.2.3).

We were able to quantify the expression of GFP using flow cytometry, a technique that can measure the fluorescent intensity of each cell of a population individually. We designed an experiment where we used flow cytometry to measure the fluorescent intensity of our reporter cell line with and without TNFα addition. The results were compared to a control of unmodified HEK 293 cells.

3.2.3. HEK NF-κB Luciferase reporter

We then designed an experiment that utilized the second reporter protein expressed by our activate dual-reporter cell line: luciferase. Luciferase causes luminescence that cannot be detected by the naked eye. Thus, in order to measure the expression of luciferase, we lysed the cells and ran a luciferase assay that measures the luminescence of the firefly luciferase protein in a highly quantifiable format. Our experiment was designed as a dose response to TNFα so that we could identify the concentration of TNFα that would be most appropriate to test the efficacy of our therapy. The results were compared to a control of unmodified HEK 293 cells treated with the same TNFα doses.

3.3.Expected results

Due to past studies done using these reporters[17,18], we expected all three reporters to function. This entails migration of RFP to the nucleus in the 3T3 reporter, and both an increase in GFP and luminescence in the dual reporter in response to TNFα. We anticipated that the TNFα dose that would be best suited for the next phase to be somewhere between 0.1 and 10 ng/ml TNFα.

3.4.Backup plan

We chose to use three different reporting mechanisms to act as a back-up plan should one of them fail. In preparation for contamination, we made several frozen back-ups of each reporter cell line as well.

3.5.Materials and Methods: Verifying 3T3 NF-κB reporter

3.5.1. Materials

Table 3-1: Materials – Phase 2 – Verifying 3T3 NF-κB reporter

Material	Company/ Brand	Model #
Passaging Mammalian Cells Materials (Appendix A2-A)		
4 chamber glass bottom dishes	Cellvis	D35C4-20-0-N
DMEM + 10% FBS + PS media*	Created in lab	N/A
Recombinant Human TNFα Protein	R&D Systems	210-TA-020
Serum-Free Medium without L-Glutamine	BioWhittaker/ UltraCULTURE	12-725F

*Refer to Appendix A1: Making Media - DMEM + 10% FBS + PS

3.5.2. Special notices

[17] Tay, Savas. (2011). Single-cell NF-κB dynamics reveal digital activation and analog information processing in cells. *Nature*. *466*(7303), 267–271.
[18] Zhang, Z., Stickney, Z., Duong, N., Curley, K., & Lu, B. (2017). AAV-based dual-reporter circuit for monitoring cell signaling in living human cells. Journal of Biological Engineering, 11(1), 18.

- Proper laboratory attire and practice (further explained in the SCU EHS guidelines) is required.
- Safe and proper aseptic technique is required for this procedure.
- All reagent and cell handling is to be done in the fume hood.
- No lids or caps are to be left open unless presently in use.
- When not in use, live cells are stored in an incubator (5% CO_2, 37°C).
- Do *NOT* attempt to perform this procedure unless the above requirements are met.

3.5.3. Overview of procedure

Table 3-2: Overview of procedure – Phase 2 – Verifying 3T3 NF-κB reporter.

	Step	Section
Day 1	Seed cells	1
Day 2	Treat cells with TNFα	2
	Image treated cells	3

3.5.4. Procedure

Day 1

1. Seed Cells
    1.1. Using a passaging mammalian cells protocol (Appendix A2-B), seed HEK 293 cells onto a 4 chamber glass bottom dish at a density of 1-5x10^5 cells/mL with DMEM + 10% FBS + PS media.
    1.2. Incubate at 37°C for 24 hours.

Day 2

2. Treat Cells with TNFα
    2.1. Add recombinant human TNFα protein to serum-free media to create a concentration of 10ng/mL TNFα.
    2.2. Aspirate the media in each of the chambers.
    2.3. Carefully add 1mL serum-free media to 2 chambers and 1mL of serum-free

media with 10ng/mL TNFα to the other 2 chambers.

2.4. Incubate at 37°C for 1 hour.

3. Image treated cells

3.1. Using a confocal microscope take phase, GFP, and RFP images of the cells at 20x for each chamber.

3.6.Materials and Methods: Verifying HEK NF-κB GFP reporter

3.6.1. Materials

Table 3-3: Materials – Phase 2 – Verifying HEK NF-κB GFP reporter

Material	Company/ Brand	Model #
Passaging Mammalian Cells Materials (Appendix A2-A)		
Multiwell culture plates, 12 well	Sigma-Aldrich/ Greiner CELLSTAR	665102
DMEM + 10% FBS + PS media*	Created in lab	N/A
Recombinant Human TNFα Protein	R&D Systems	210-TA-020
Serum-Free Medium without L-Glutamine	BioWhittaker/ UltraCULTURE	12-725F
Phosphate-Buffered Saline pH 7.4 (PBS)	Thermo Fisher Scientific	AM9625
Trypsin 0.25% with phenol red	Thermo Fisher Scientific	15050065
15 mL conical-bottom centrifuge tubes	VWR	89039-666
Disposable glass tubes 12 mm, 6 mL	Kimble™ / KIMAX™	735001275
Cytometer Setup & Tracking Beads FL 1-3	BD Biosciences/ BD CS&T	661414
Sheath, Detergent, FACSClean Solution Bottle, Filters	BD Biosciences	660322
Flow Cytometer	BD Biosciences/ BD Accuri C6 Plus	661311

*Refer to Appendix A1: Making Media - DMEM + 10% FBS + PS

3.6.2. Special notices

- Proper laboratory attire and practice (further explained in the SCU EHS guidelines) is required.
- Safe and proper aseptic technique is required for this procedure.
- All reagent and cell handling is to be done in the fume hood.
- No lids or caps are to be left open unless presently in use.
- When not in use, live cells are stored in an incubator (5% CO_2, 37°C).
- Do *NOT* attempt to perform this procedure unless the above requirements are met.

3.6.3. Overview of procedure

Table 3-4: Overview of procedure – Phase 2 – Verifying HEK NF-κB GFP reporter

	Step	**Section**
Day 1	Seed cells	1
Day 2	Treat cells with TNFα	2
Day 3	Image transfected cells	3
	Flow cytometry analysis	4

3.6.4. Procedure

Day 1

1. Seed Cells

1.1. Using a passaging mammalian cells protocol (Appendix A2-B), seed cells into 7 wells of a 12-well plate at a density of 1-5x10^5 cells/mL with DMEM + 10% FBS + PS media.

1.1.1. Plate 1 well with HEK 293 cells and the other 6 wells with the HEK 293 NF-κB GFP/Luciferase Dual Reporter System cells.

1.2. Incubate at 37°C for 24 hours.

Day 2

2. Treat Cells with TNFα
    2.1. Add recombinant human TNFα protein to serum-free media to create a stock concentration of 10ng/mL TNFα.
    2.2. Aspirate the media in each well.
    2.3. Carefully add 1mL of new media to the 7 wells with cells.
        2.3.1. Use 10ng/mL TNFα serum-free media for 3 of the wells with the reporter cells.
        2.3.2. Use normal serum-free media for the other 3 wells with the reporter cells and the 1 well with HEK 293.
    2.4. Incubate at 37°C for 24 hours.

Day 3

3. Image treated cells (for each different condition)
    3.1. Using a fluorescent microscope take phase and GFP images of the cells at 4x.
4. Flow cytometry analysis
    4.1. Carefully aspirate the media off of the dish.
    4.2. Carefully add 0.5mL of trypsin to each well with cells.
    4.3. Incubate at 37°C for 2 minutes.
    4.4. Deactivate trypsin by adding 1.5mL of DMEM + 10% FBS + PS media to one well.
    4.5. Collect all media in that well into a 15mL centrifuge tube.
    4.6. Repeat for remaining wells with cells.
    4.7. Centrifuge at 1500 RPM for 5 minutes.
    4.8. Carefully aspirate off the supernatant.
    4.9. Resuspend the pellet in 500-1000µL of PBS.
    4.10. Filter each sample through a 40 micron filter into a 6mL glass tube.
    4.11. Turn on the computer and flow cytometer.
    4.12. Place empty glass tube on SIP and run backwash.
    4.13. Run DI water through the flow cytometer for 2 minutes.
    4.14. Run a solution of DI water and FL1-3 tracking beads through the flow cytometer for 2 minutes.

4.15. Run each sample through the flow cytometer until 30,000-50,000 events have been recorded.

4.15.1. Between each sample, run DI water through the flow cytometer for 2 minutes.

4.16. Run cleaning solution through the flow cytometer for 2 minutes.

4.17. Run DI water through the flow cytometer for 2 minutes.

4.18. Leave the glass tube with DI water on the SIP.

3.7.Materials and Methods: Verifying HEK NF-κB Luciferase reporter

3.7.1. Materials

Table 3-5: Materials – Phase 2 – Verifying HEK NF-κB Luc reporter

Material	Company/ Brand	Model #
Passaging Mammalian Cells Materials (Appendix A2-A)		
DMEM + 10% FBS + PS media*	Created in lab	N/A
96 well plate, clear bottom	Sigma-Aldrich/ Corning	3340
Serum-Free Medium without L-Glutamine	BioWhittaker/ UltraCULTURE	12-725F
Recombinant Human TNFα Protein	R&D Systems	210-TA-020
Plate reader	BMG Lab Tech/ LUMIstar Omega	S/N 415-1717
Luciferase assay system w/ luciferase assay buffer (LAB), luciferase assay substrate (LAS), and passive lysis buffer (PLB)	Promega	E1501

*Refer to Appendix A1: Making Media - DMEM + 10% FBS + PS

3.7.2. Special notices

- Proper laboratory attire and practice (further explained in the SCU EHS guidelines) is required.
- Safe and proper aseptic technique is required for this procedure.
- All reagent and cell handling is to be done in the fume hood.

- No lids or caps are to be left open unless presently in use.
- When not in use, live cells are stored in an incubator (5% CO_2, 37°C).
- Do *NOT* attempt to perform this procedure unless the above requirements are met.

3.7.3. Overview of procedure

Table 3-6: Overview of procedure –Phase 2 – Verifying HEK NF-κB Luc reporter

	Step	Section
Day 1	Seed cells	1
Day 2	Treat cells with TNFα	2
Day 3	Image transfected cells	3
	Luciferase assay	4

3.7.4. Procedure

Day 1

1. Seed Cells

1.1. Using a passaging mammalian cells protocol (Appendix A2-B), seed HEK 293 cells into 18 wells of a 96-well plate at a density of 1-5x10^5 cells/mL with DMEM + 10% FBS + PS media.

1.2. In the same 96-well plate, repeat 1.1 for HEK 293 NF-κB GFP/Luciferase Dual Reporter cells.

1.3. Incubate at 37°C for 24 hours.

Day 2

2. Treat Cells with TNFα

2.1. Add recombinant human TNFα protein to serum-free media to obtain concentrations of 10, 5, 1, 0.5, 0.1, and 0 ng/mL TNFα.

2.2. Aspirate the media in each well.

2.3. Carefully add 100µL of serum-free media to each well with the following concentrations of TNFα: 10, 5, 1, 0.5, 0.1, and 0 ng/mL.

2.3.1. Each concentration is run in triplicate for both cell lines.

2.4. Incubate at 37°C for 24 hours.

Day 3

3. Image treated cells (for each different condition)

3.1. Using a fluorescent microscope take phase and GFP images of the cells at 4x.

4. Luciferase assay

4.1. Prepare Luciferase Assay Reagent (LAR) by combining 10mL of LAB with an entire vial of LAS. Pipette up and down to mix.

4.2. Dilute the PLB by combining stock with 4x volume of DI water.

4.3. Add 20μL of diluted lysis buffer to each well.

4.4. Mix with gentle rotations for 10 minutes.

4.5. Turn on computer and plate reader.

4.6. Set up plate reader to measure luminescence with a read time of 10 seconds.

4.7. Quickly add 100μL of LAR to each well.

4.8. Immediately run the plate in the plate reader.

4.8.1. Ensure that the order in which you add the LAR is the same order in which the plate reader reads the samples.

3.8.Results

3.8.1. 3T3 NF-κB reporter

Figure 3-4 shows the results of our first attempt to measure inflammatory response in a cell culture model using the RFP-based nuclear localization reporting system.

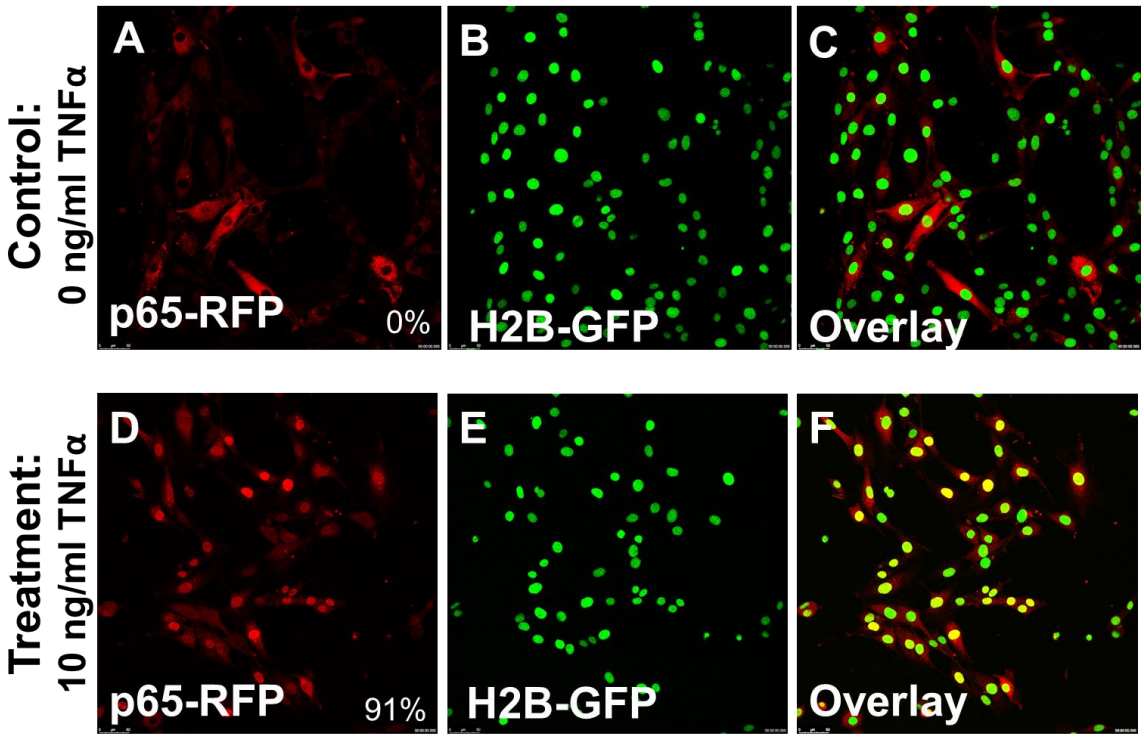

Figure 3-4: Confocal images of 3T3 p65-RFP H2B-GFP reporter. Photos were taken 1 hour after treatment with TNFα. The percentage of cells that present nuclear RFP is displayed on the RFP images.

The images show that this reporter system was successful in indicating the effects of TNFα on the cell. The cells to which no TNFα was added showed no nuclear localization of p65-RFP, as evident from the lack of yellow nuclei in Figure 3-4. Conversely, many of the cells to which TNFα was added show a migration of RFP to the nucleus and this is verified by their yellow nuclei in the overlay (Figure 3-4). Counting the number of yellow nuclei in the overlay indicated the reporter's efficiency was approximately 91%.

3.8.2. HEK NF-κB GFP reporter

Figure 3-5 shows the results of our second method of measuring inflammatory response in a cell culture model by measuring GFP intensity of our GFP-Luc dual reporter system.

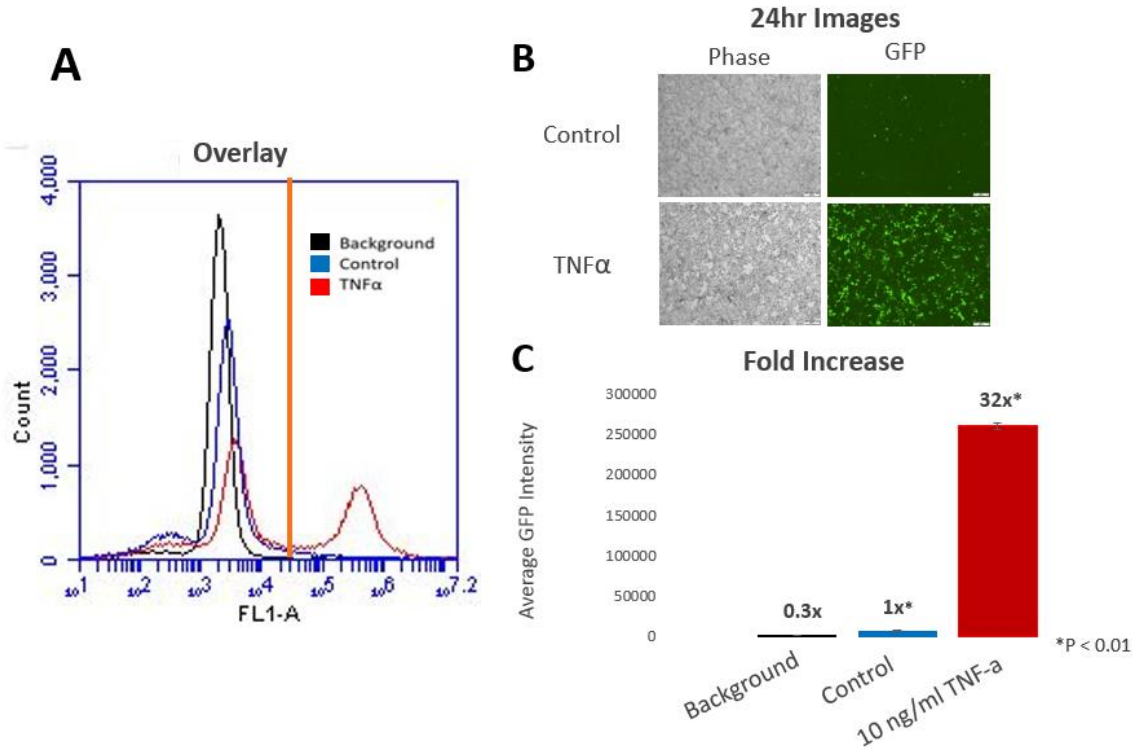

Figure 3-5: Flow cytometry data for HEK NF-κB GFP reporter. HEK293 cells were transfected with the reporter and treated with 0 or 10 ng/ml TNFα. 24 hours after treatment, images were taken (left) and the fluorescence was measured using flow cytometry (all graphs).

Evinced in Figure 3-5-A there are two distinct populations of cells in the treatment group: those expressing GFP (the peak to the right of the vertical line) and those not expressing GFP (the peak to the left of the vertical line). The left peak was consistent with the control and background conditions, while the right peak showed a significant increase in measurable GFP intensity. Visual representations of this increase in GFP expression are evident in the fluorescent microscope images in Figure 3-5-B. Overall fold differences in the average GFP intensity of each population are displayed in Figure 3-5-C. We saw a statistically significant 32-fold increase in average GFP intensity between the reporter cells treated with TNFα compared to those untreated.

3.8.3. HEK NF-κB Luciferase reporter

Figure 3-6 shows the results of our second method of measuring inflammatory response in a cell culture model by measuring luciferase expression from a dose response of TNFα using our GFP-Luc dual reporter system.

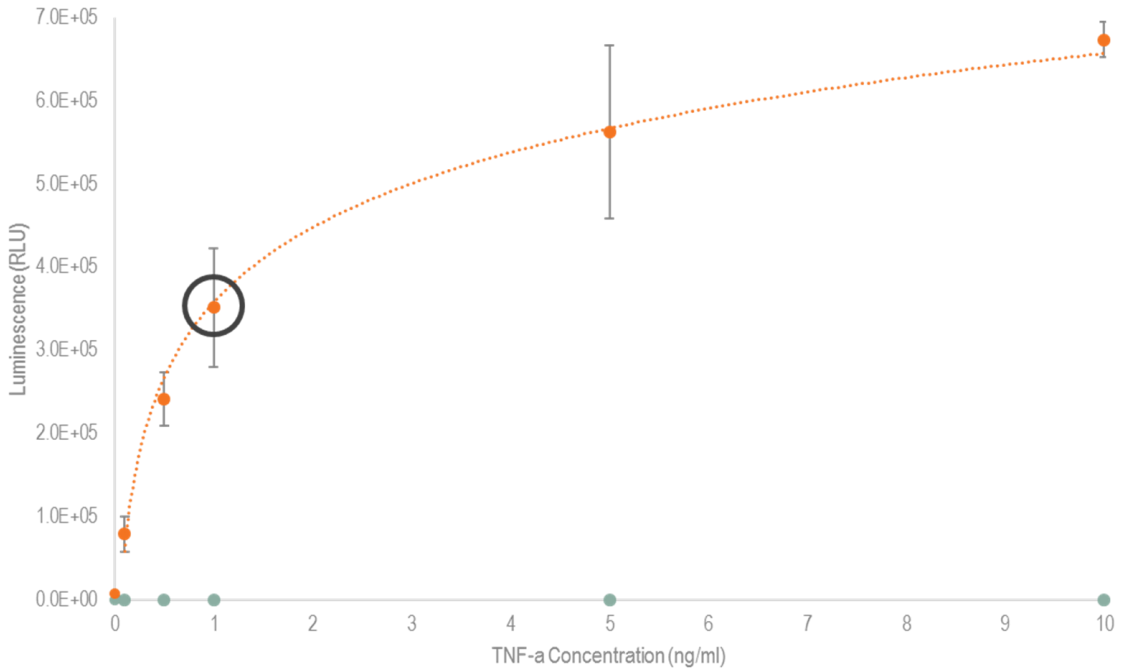

Figure 3-6: Luciferase assay for HEK NF-κB Luc reporter. The control, unmodified HEK 293 cells (green), provided the luminescence baseline. Each condition was run in triplicate, and error bars represent ± one standard deviation.

There was a logarithmic trend between the concentration of TNFα and luminescence in our dual reporter cells as demonstrated in Figure 3-6. The TNFα dose response to the control group showed no significant upward trend in luciferase expression, confirming that TNFα on its own does not increase luminescence readings in a luminometer. We selected 1 ng/ml TNFα as the dose for which we will test our treatment because it is well above baseline, but a decrease in TNFα concentration would be clearly measurable by a decrease in luminescence. Also, this concentration of TNFα is still ten times higher than that found in Rheumatoid synovium[19], making it a good choice for a proof of concept.

[19] Manicourt, D., Triki, R., Fukuda, K., & Devogelaer, J. (n.d.). Levels of circulating tumor necrosis factor a and interleukin-6 in patients with rheumatoid arthritis Relationship to Serum Levels of Hyaluronan and Antigenic Keratan Sulfate, 490–499.

3.9.Discussion

The results from the above three inflammatory reporter verification experiments have shown us that we can measure inflammation in a variety of ways, thus showing us the inflammation pathway at two distinct stages. The experiment that imaged nuclear localization was not repeatable during our timeframe since the images were taken during a confocal microscope demo session and the microscope was not delivered to the lab until after winter quarter. The flow cytometry experiment that measured GFP intensity was limited in the fact that our TNFR-exosome complexes also contained GFP markers, thus the basal level GFP intensity would be high and any changes would be more difficult to measure. For these reasons, we chose to use the luciferase assay to test our therapy in the following phase of our project.

4. Testing Efficacy of Design

Figure 4-1: Timeline of project phases. This chapter focuses on Phase 3.

The goal of this stage was to determine whether our modified exosomes are capable of preventing inflammation. Using the inflammation reporter verified in Phase 2, we measured the effect of the modified exosomes stored from Phase 1 on the inflammatory response of live mammalian cells.

4.1. Key constraints

The key constraints for this phase of our project was primarily the quantity of exosomes. Although our store of exosomes was too low to run treat cells in 12-well plate for flow cytometry, we had enough to treat cells in a 96-well plate for a luciferase assay.

4.2. Design description

We designed this experiment using the luciferase assay method of monitoring inflammation for the reasons expressed in section 3.8 above. The luciferase assay measures the intensity of firefly luciferase, which increases with the initiation of the inflammation pathway after exposure to TNFα, as explained in section 3.7.2. Successful inhibition of inflammation would be indicated by a decrease in luciferase expression with the addition of treatment.

To robustly assess the effects of our therapy, we designed an experiment with several controls and varying concentrations of exosomes. We also ran each group in triplicate for statistical significance. We added two different types of exosomes to our dual-reporter cell line; CD63-RFP-GFP and CD63-TNFR-GFP exosomes that were collected during Phase 1.

With RFP in place of the TNFR protein in our therapeutic exosomes, these exosomes could act as a control group to show what effect exosomes themselves have on the luciferase assay without the inclusion of the therapeutic TNFR protein. The CD63 TNFR-GFP exosomes were our therapeutic design. Each of these were added in three concentrations: 0.5 mg/ml, 0.1 mg/ml, and 0 mg/ml (negative control). These concentrations were selected based on previous experiments we did in our lab for different projects in the past. Each experimental group was run in media with no TNFα and 1 ng/ml TNFα.

4.3.Expected results

We expected that the TNFα treated wells in which we added our TNFR exosomes would have a decreased luciferase luminescence compared to those without exosomes by at least 50%. We predicted that our therapeutic exosomes would bind to TNFα, effectively reducing its concentration and thus reducing inflammatory response. We did not expect to see a significant decrease in luminescence with the addition of the control exosomes because they lacked TNFR to specifically bind to and soak up the TNFα added.

4.4.Backup plan

Our back up plan was to use one of the other reporters tested in the previous phase of the experiment. Though not ideal, they would be sufficient in providing a proof of concept for further experimentation.

4.5.Materials and Methods

4.5.1. Materials

Table 4-1: Materials – Phase 3

Material	**Company/ Brand**	**Model #**
Passaging Mammalian Cells Materials (Appendix A2-A)		
DMEM + 10% FBS + PS media*	Created in lab	N/A
96 well plate, clear bottom	Sigma-Aldrich/ Corning	3340
Serum-Free Medium without L-Glutamine	BioWhittaker/ UltraCULTURE	12-725F
Recombinant Human TNFα Protein	R&D Systems	210-TA-020
CD63-TNFR-GFP exosomes	Created in lab in Phase 1	N/A
CD63-RFP-GFP exosomes	Created in lab in Phase 1	N/A
Plate reader	BMG Lab Tech/ LUMIstar Omega	S/N 415-1717
Luciferase assay system w/ luciferase assay buffer (LAB), luciferase assay substrate (LAS), and passive lysis buffer (PLB)	Promega	E1501

*Refer to Appendix A1: Making Media - DMEM + 10% FBS + PS

4.5.2. Special notices

- Proper laboratory attire and practice (further explained in the SCU EHS guidelines) is required.
- Safe and proper aseptic technique is required for this procedure.
- All reagent and cell handling is to be done in the fume hood.
- No lids or caps are to be left open unless presently in use.
- When not in use, live cells are stored in an incubator (5% CO_2, 37°C).
- Do *NOT* attempt to perform this procedure unless the above requirements are met.

4.5.3. Overview of procedure

Table 4-2: Overview of procedure – Phase 3

	Step	Section
Day 1	Seed cells	1
Day 2	Treat cells with TNFα + exosomes	2
Day 3	Image transfected cells	3
	Luciferase assay	4

4.5.4. Procedure

Day 1

1. Seed Cells

1.1. Using a passaging mammalian cells protocol (Appendix A2-B), seed cells into 27 wells of a 96-well plate at a density of 1-5x10^5 cells/mL with DMEM + 10% FBS + PS media according to the figure below.

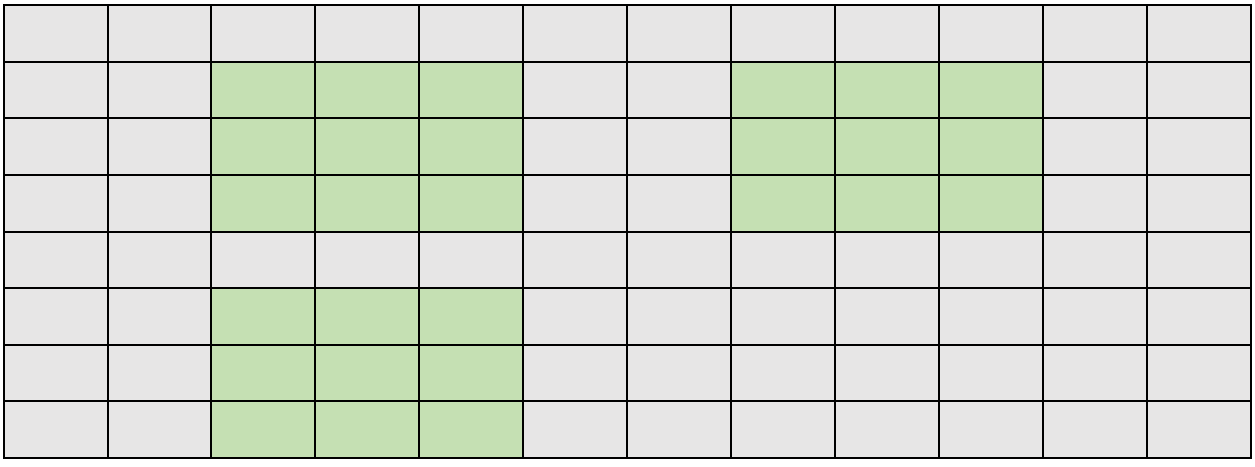

Figure 4-2: Diagram for plating cells in a 96-well plate. Seed green wells with HEK GFP/Luciferase Dual reporter cells.

1.2. Incubate at 37°C for 24 hours.

Day 2

2. Treat Cells with TNFα + exosomes
    2.1. Add recombinant human TNFα protein to serum-free media to create a stock concentration of 1ng/mL TNFα.
    2.2. Create stock concentrations of CD63-TNFR-GFP exosomes of 0, 0.1, and 0.5mg/mL in serum-free media.
    2.3. Repeat 6.2 in 1ng/mL TNFα serum-free media.
    2.4. Perform steps 6.2-3 for CD63-RFP-GFP exosomes.
    2.5. Aspirate the media in each well.
    2.6. Carefully add 100µL of the various serum-free media conditions to each well according to the following diagram.

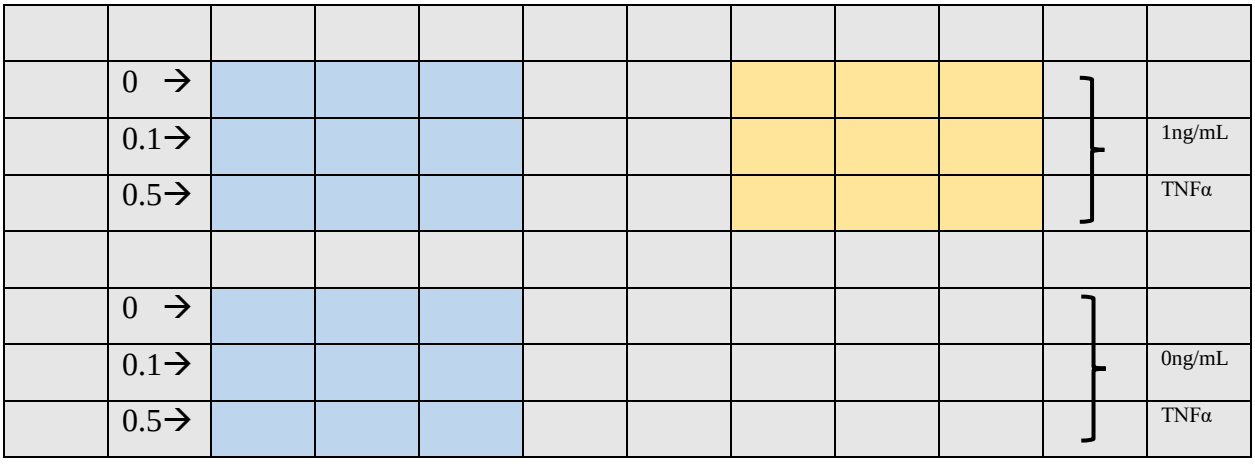

Figure 4-3: Diagram for treating cells in a 96-well plate. The concentrations of exosomes are noted on the left and apply to the entire row. Add the corresponding concentrations of CD63-TNFR-GFP exosomes to blue wells and CD63-RFP-GFP exosomes to yellow wells. The top 2 blocks should have 1ng/mL TNFα while the bottom block has 0ng/mL TNFα.

    2.7. Incubate at 37°C for 24 hours.

Day 3

3. Image treated cells (for each different condition)
    3.1. Using a fluorescent microscope take phase and GFP images of the cells at 4x.
4. Luciferase assay
    4.1. Lyse
    4.2. Plate reader

4.3. Prepare Luciferase Assay Reagent (LAR) by combining 10mL of LAB with an entire vial of LAS. Pipette up and down to mix.

4.4. Dilute the PLB by combining stock with 4x volume of DI water.

4.5. Add 20µL of diluted lysis buffer to each well.

4.6. Mix with gentle rotations for 10 minutes.

4.7. Turn on computer and plate reader.

4.8. Set up plate reader to measure luminescence with a read time of 10 seconds.

4.9. Quickly add 100µL of LAR to each well.

4.10. Immediately run the plate in the plate reader.

4.10.1. Ensure that the order in which you add the LAR is the same order in which the plate reader reads the samples.

4.6.Results

Figure 4-4 shows the results from the efficacy test of our TNFR exosome treatment experiment.

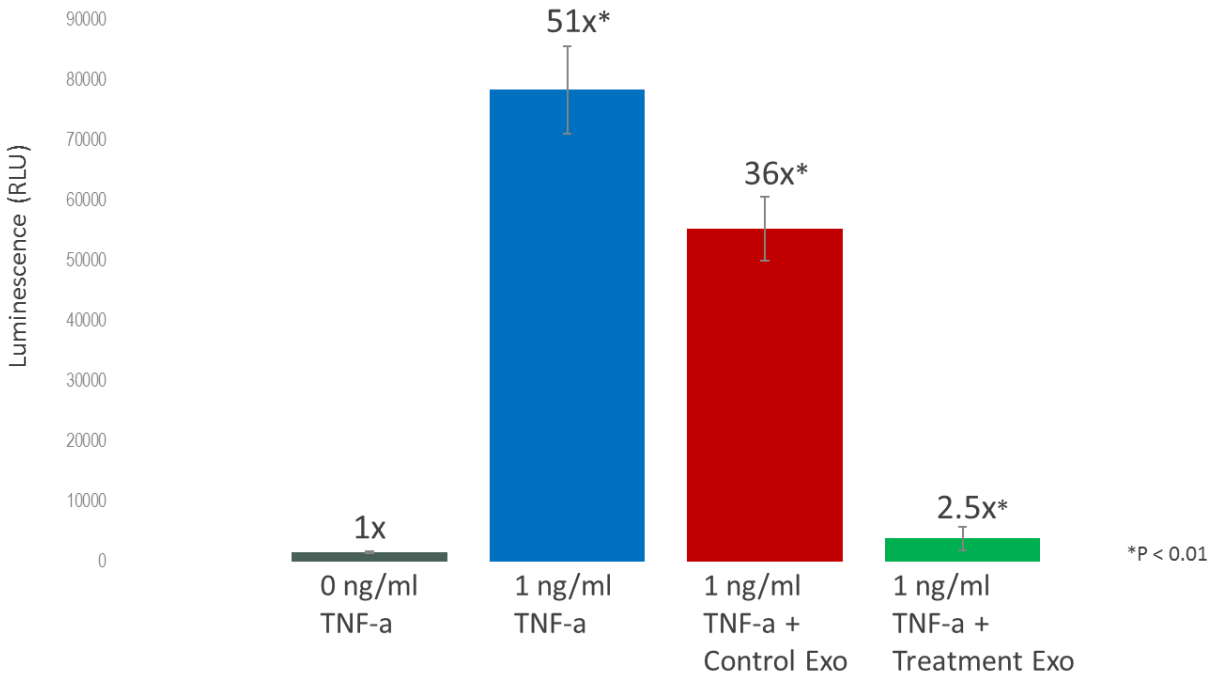

Figure 4-4: Efficacy of exosome membrane bound TNFR in preventing inflammation. Each group was run in triplicate, and error bars represent ± one standard deviation.

The treatment with TNFα caused an increase in luminescence, consistent with the previous phase of our project, confirming that the luciferase assay was run correctly. The addition of the control exosomes to TNFα treated cells yielded a decrease in luminescence by about 30%. The addition of our treatment exosomes reduced the luciferase expression by 95%, almost to the baseline expression with no TNFα addition. Run in triplicate, the differences between all three conditions were deemed statistically significant with a P value less than 0.01 from an analysis of variance (ANOVA). This indicated that the both exosome groups decreased the effect of TNFα on the inflammatory response in our dual-reporter cells. The much larger decrease in luminescence in the group with our TNFR exosomes indicate that they successfully inactivated more TNFα than the control exosomes.

4.7.Discussion

The results indicate that our TNFR-exosomes were more efficacious in reducing the inflammatory response in our HEK 293 cells than we had expected. While we hoped for at least a 50% decrease, our data demonstrates a 95% decrease. We did not anticipate the reduction in inflammatory response from the control exosomes. However, we believe this decrease is most likely because the exosomes may non-specifically absorb TNFα, reducing the inflammation to a lesser extent than the specific binding by our TNFR-exosomes. Overall, the results clearly show that our product has incredible therapeutic potential and can effectively reduce the initiation of the inflammation pathway in this mammalian cell culture model.

5. Conclusion

5.1. Summary

The primary symptom of rheumatoid arthritis is the swelling of the synovium which causes pain and can lead to further joint deformity. A key player in the initiation of the inflammation pathway in RA is TNFα. There are a few current treatments that target TNFα; however, these options come with limitations including a significant immune response and/or short-term relief. Exosomes, however, are naturally secreted nanovesicles that could be used to avoid these limitations. We have confirmed that exosome membrane bound TNF-receptors have the potential to be an effective treatment for rheumatoid arthritis with each phase of our project.

- Phase 1: Design Production— we demonstrated that anchoring TNFR onto tetraspanin CD63 allowed for production of exosomes with TNFRs.
- Phase 2: Verification of Inflammation Reporters— we identified 3 different methods of measuring inflammation in live mammalian cells.
- Phase 3: Test Efficacy of Design— using one of the reporters tested in Phase 2, we confirmed that the CD63-TNFR-GFP exosomes have the ability to prevent inflammation in live mammalian cells.

The success of our research could lead to the development of a novel treatment of rheumatoid arthritis as well as serve as a foundation for further research into engineering exosomes for therapeutic purposes (e.g., targeted drug delivery).

5.2. Future work

With the success of our project thus far, we hope to further this research with the following endeavors tackled by us or future groups:

5.2.1. Creation of a stable cell line

A stable cell line is a group of mammalian cells that permanently have a specific genetic modification. We are creating a line of HEK 293 cells that have been permanently modified to produce the CD63-TNFR-GFP exosomes. This would make manufacturing and collecting the modified exosomes much quicker and easier for future experimentation.

5.2.2. Toxicity assay

For a treatment that will be used in humans, it is extremely important to confirm that the treatment will not harm the patient. Thus, we would like to run a toxicity assay with our CD63-TNFR-GFP exosomes. This involves measuring the viability of mammalian cells to varying concentrations of our modified exosomes.

5.2.3. Testing design's ability in reversing inflammation

We have demonstrated that our modified exosomes can prevent inflammation. However, it is necessary to confirm whether or not they can reverse inflammation in cells that have already initiated the inflammation pathway. This could be tested by adding the CD63-TNFR-GFP exosome at after TNFα (as opposed to simultaneously). The luciferase could be measured at various time points: before TNFα treatment, after TNFα treatment, and several times after addition of CD63-TNFR-GFP exosomes.

5.2.4. Verifying efficacy of design in other cell lines

We have demonstrated that our modified exosomes have the ability to prevent inflammation in human embryonic kidney cells. HEK 293 cells were used for this project because they are easy to work with and commonly used in a research environment. However, the primary cells of concern in rheumatoid arthritis are cells surrounding the joints (not kidney cells). Thus, future groups should repeat our experimentation in cell lines relating to joints (e.g., chondrocytes).

5.2.5. Comparison to soluble TNFR

Soluble TNFR is currently the primary treatment for RA. Thus, future groups should run an experiment comparing sTNFR to our CD63-TNFR-GFP exosomes in lowering levels of inflammation. This would indicate whether or not our design would be competitive in the pharmaceutical industry.

5.2.6. Testing other scaffolds

We chose tetraspanin CD63 as our scaffold for anchoring TNFRs onto the exosome surface because of its prevalence on the exosome surface. However, there are many other scaffolds that can be used with different benefits. For example, a VSV-G scaffold could be used that might enhance exosome production. Thus, future groups should test other possible designs to determine which scaffold would be the best to continue with development of a clinical treatment.

6. Engineering Standards and Realistic Constraints

6.1. Social impact

As engineers our goal was to use our knowledge and expertise to develop a product for the betterment of society. We hope our exosomal TNFR therapy will provide three types of societal benefit.

6.1.1. Increased efficacy

By anchoring the TNFR to exosome membranes, we firmly believe that the therapeutic effects will be greater than those with current treatments such as soluble TNFR receptors. This would be due to their ability to deeply penetrate the tissue and spread their effects throughout the joint. Should this be the case, then our therapy would benefit those receiving our therapy with a more significant reduction in swelling and therefore less damage and suffering. Should appropriate safety testing be successful (see section 6.2 for more details) and the product cause no harm to the patients, it is clear that this would be a net positive impact on the well-being of society. The only members of society who would face negative consequences of a safe and more efficacious treatment for RA would be those selling competing therapies; however their loss is heavily outweighed by the gain of the patients.

6.1.2. Optimized efficiency

Another benefit of anchoring TNFR to an exosome is the natural stability the exosome provides to the protein. A more stable protein does not degrade as easily and lasts longer in the body. Combine this with the exosomes ability to penetrate the tissue and the result is a more efficacious treatment that is less wasteful and longer lasting than current treatments. The bottom line is that a patient will need far fewer injections and far fewer wasted proteins than typical of a soluble TNFR therapy. This will make it easier for the patient by helping them save money (see section 6.3 for more details) and time, by mandating fewer doctor visits. Not only does this provide a social benefit by making it easier for those who are

already undergoing treatment with current therapies, but it will also increase the number of people who can afford effective treatment.

6.1.3. Encourage adaptations

One of the most impactful social benefits of our project is the potential for future adaptations of our work. We hope to publish our work in an impactful journal such that our project can act as a proof of concept for engineering therapeutic proteins onto the surface of exosomes. The same benefits that exosomes provided for RA treatments mentioned above could be applied to many other protein-based therapeutics for diseases such as cancers, alzheimer's, and many more. Should our research provide inspiration for further research in labs around the world, our project could have indirect, yet far-reaching social benefit.

6.2. Health and safety concerns

As with all new medical therapies, there are innumerable health and safety concerns. The lack of complete understanding of the human body and how it will react to novel therapeutics mandates a rigorous process of safety testing regulated by the FDA. In the current stage of its development, our project represents a preclinical efficacy study. Our experimentation indicates promising efficacy that will need to be replicated in various cell types and with various reporters. This data will then have to be compared to similar tests run using the current leading therapy (soluble TNFR). Once further testing has confirmed the improved efficacy of our novel therapy, then safety testing can begin.

The first stage of safety testing will most likely involve a toxicity assay in a mammalian cell culture model with various human cell types. Then FDA clinical trials begin with Phase 0, animal models, for both safety and efficacy testing. This is followed by four phases of clinical trials on human subjects. Because such trials are far beyond the scope of our project, they would need to be taken up by another lab with more resources. Nevertheless, the regulations in place by the FDA will help ensure that if a clinical therapy evolves from our project it is safe and will not cause more harm to those who will receive it.

6.3.Economic

We feel optimistic about the financial prospects of our project. However, as with most advances in the medical field, there will be high research and development costs and a sizeable overhead. Although we anticipate that the necessity for less frequent injections (as mentioned in section 6.1.2, above) will reduce the cost to the consumer in the long run, it will not reduce the cost to develop or manufacture the product. The costs involved with meeting the FDA requirements (mentioned in section 6.2, above) are often quite large. Nevertheless, this is an economic obstacle faced by all up-and-coming therapies.

It might be assumed that because of the reduced frequency of injections that the costly overhead would yield a lower net profit, but we anticipate that the reduced cost to consumer will widen the market to encompass those who are not able to afford current treatment methods. This would make up for any presumed losses by increasing the number of injections sold while having a larger overall benefit to society. Thus we believe that the benefits of our novel therapy will make it desirable enough to be pursued by a successful pharmaceutical company once the proof of concept is firmly established. Our optimism has led us to pursue a patent on our therapy that we believe could be fruitful in the coming years.

6.4.Manufacturability

Manufacturing of our therapy remains to be the most significant roadblock. At this current stage, the exosome manufacturing method that we used for our product (see section 2.1 for details) is not optimized. It is inefficient and uses large quantities of resources to obtain small amounts of exosomes. This method is sufficient for proof-of-concept research such as ours, yet optimization will be necessary for mass production in the later stages of the therapy's development. Fortunately, our literature studies have indicated that it is safe to assume that with the publication of our research and with the work of others who are beginning to use exosomes for therapeutic purposes, a more efficient exosome harvesting method will soon be pioneered.

6.5.Ethical Implications

6.5.1. Ethical justification

At the beginning of the year, we met to discuss our interests and pick project. During this discussion, it was agreed that we wanted to focus on a project that accomplished two goals: (1) progressed scientific understanding and (2) laid the groundwork for the development of a novel medical therapy. Our main driver for these goals was our desire to help those suffering from diseases or disorders. We decided to pursue exosomes due to their promising therapeutic nature and because they have yet to be incorporated into a successful therapy. Our efforts would therefore contribute to the advancement of leading edge biomedical engineering. Rheumatoid arthritis in particular was our focus because it is a widespread and debilitating disease for which a therapeutic advancement would directly help many patients by reducing their pain, suffering, and the physical damage associated with inflammation.

We analyzed the moral aspects of this project from a compounded ethical framework, assessing the project's ethicality from a utilitarian, rights, justice, virtue, and common good standpoint. Using the utilitarian approach, we hoped our success would yield benefit to the millions who suffer from RA or any other disease that our exosomal therapeutic techniques may inspire a therapy for. Considering a rights approach we hoped our success would increase patients' access to their right good health. From a justice viewpoint, we hoped that in making a longer lasting therapeutic injection, we would allow for a more affordable treatment that would result in a more just distribution of treatment for those who currently cannot afford it. From a virtue ethics approach, we felt that by using our expertise in biological sciences we were being our best selves by committing our work to alleviating the suffering of others. Lastly, we hoped to benefit the common good with our therapy by contributing to the progress of the medical field and providing a therapy for RA.

6.5.2. Virtues of a good engineer

Through this project we have learned to incorporate the following ethical habits, as discussed by Charles Harris in his article "The Good Engineer: Giving Virtue Its Due in

Engineering Ethics."[20]

6.5.2.1. Techno-social sensitivity

We considered the interplay between technology and society; that technological changes have an effect on society and societal changes have an effect on technology. It was obvious how society has advanced technology—as is frequently said, "necessity is the mother of invention," – and we saw how there was a need for cheaper and more effective RA treatments in our society and sought to develop a technology to accommodate that. We then hoped that with our technology we would yield societal benefit by helping those suffering with RA and by spurring research to help others suffering from other diseases and conditions.

6.5.2.2. Commitment to the public good

As mentioned in section 6.5.1 above, the motivation behind our project was primarily to help those suffering and to advance medical therapeutic techniques. We firmly believe that our research will allow this to happen, and in turn have a positive effect on the public.

6.5.2.3. Teamwork

In working together, we have learned the values that allow for strong teamwork. Primarily is stressing effective communication. This not only encompasses clear note taking and email conversations, but also communicating thoughts regarding the project. As a group of two, communication was relatively straightforward and helped with the success of our project. Another requirement is delegation and trust, which we achieved by distributing the workload fairly and keeping each other apprised of issues and situations.

6.5.3. Safety and risk assessment

There are two aspects to safety and risk assessment when ethically considering a project. First is the safety to those working on the project, we addressed this by completing the lab safety training and rigidly abiding by the guidelines set out by the Environmental Health

[20] Harris, Charles E. "The Good Engineer: Giving Virtue its Due in Engineering Ethics." Science and Engineering Ethics 14, no. 2 (2008): 153-64.

and Safety department at Santa Clara University. Secondly, we had to consider the safety of our product on those who would be using it. Unfortunately, due to our limited resources and time, we were only able to establish a proof-of-concept and cell culture model in our project. However, as discussed in section 6.2, our project's future holds a rigorous course of safety testing as it would seek to obtain FDA approval.

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Appendix A: Additional Materials and Methods

Special Notices

- Proper laboratory attire and practice (further explained in the SCU EHS guidelines) is required.
- Safe and proper aseptic technique is required for this procedure.
- All reagent and cell handling is to be done in the fume hood.
- No lids or caps are to be left open unless presently in use.
- When not in use, live cells are stored in an incubator (5% CO_2, 37°C).
- Do *NOT* attempt to perform any procedures unless the above requirements are met.

Appendix A1: Making Media – DMEM + 10% FBS + PS

A1-A. Materials

Table A-1: Materials for making DMEM + 10% FBS + PS.

Material	Company/ Brand	Model #
Dulbecco's Modified Eagle Medium with L-Glutamine (DMEM)	Thermo Fisher Scientific/ Gibco	11965092
Fetal bovine serum (FBS)	Thermo Fisher Scientific/ Gibco	10438034
Penicillin-Streptomycin (PS)	Thermo Fisher Scientific/ Gibco	15140122

A1-B. Methods

1. Add 50mL of fetal bovine serum into 1 500mL bottle of Dulbecco's Modified Eagle Medium with L-Glutamine.
2. Add 5mL of Penicillin-Streptomycin to the combined FBS and DMEM.
3. Mix thoroughly.
4. Store at 4°C until needed.

Appendix A2: Passaging Mammalian Cells

A2-A. Materials

Table A-2: Materials for passaging mammalian cells.

Material	Company/ Brand	Model #
DMEM + 10% FBS + PS media*	Thermo Fisher Scientific/ Gibco	11965092
Phosphate-Buffered Saline pH 7.4 (PBS)	Thermo Fisher Scientific	AM9625
Trypsin 0.25% with phenol red	Thermo Fisher Scientific	15050065
15 mL conical-bottom centrifuge tubes	VWR	89039-666

* Refer to Appendix A1: Making Media – DMEM + 10% FBS + PS

A2-B: Methods

1. Retrieve 60mmx15mm dish with cells from incubator.
2. Carefully aspirate media off of dish.
3. Carefully wash cells by adding 3mL of PBS.
4. Carefully aspirate off PBS of dish.
5. Add 1.5mL trypsin into dish.
6. Incubate at 37°C for 2 minutes.
7. Deactivate trypsin by adding 4.5mL of DMEM + 10% FBS + PS media to the dish.

8. Collect all media in the dish into a 15mL centrifuge tube.
9. Centrifuge at 1500 RPM for 5 minutes.
10. Aspirate off the supernatant.
11. Resuspend the pellet in DMEM + 10% FBS + PS.
12. Plate as desired.

Appendix A3: Transfection

A3-A. Materials

Table A-3: Materials for transfection.

Material	**Company/ Brand**	**Model #**
Passaging Mammalian Cells Materials (Appendix A2-A)		
Transfection Reagent	Thermo Fisher Scientific/ Lipofectamine 2000	11668019
Microcentrifuge tubes	Sigma-Aldrich/ Eppendorf Safe-Lock	T9661
Reduced Serum Medium	Thermo Fisher Scientific/ Opti-MEM	31985070
DNA plasmid of choice		

A3-B. Methods

1. Passage according to Appendix A2: Passaging Mammalian Cells onto the desired dish/ well plate.
    a. Volumes for the following steps depend on the dish or well plate used and are specified in the Table A-4.

Table A-4: Specified volumes for transfections.

	Reduced-Serum Media	**Transfection Reagent**	**DNA plasmid**
12 well plate	100 ul / well	5 µl / well	1-3 µg / well
145mmx20mm dish	1 mL	60 µl	30-40 µg

2. Incubate at 37°C for 24 hours.
3. After 24 hours, combine reduced-serum media and transfection reagent in a microcentrifuge tube.
4. Incubate at room temperature for 5 minutes.
5. Carefully add DNA plasmid into the reduced-serum media and transfection reagent mixture.
6. Incubate at room temperature for 20 minutes.
7. Carefully add the mixture to the dish/ well.
8. Incubate the dish/ well plate at 37° for 24 hours.