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Engineered Living Nanoparticles for the Treatment of Inflammatory Diseases

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ENTITLED

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Engineered Living Nanoparticles for the Treatment of Inflammatory Diseases

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Abstract

Rheumatoid arthritis (RA) is a widespread, debilitating autoimmune disease characterized by painful inflammation of the joints. Current treatments for RA are either ineffective, expensive, or have undesirable effects, such as an adverse immune response. To mitigate these effects, we have designed an exosome-based treatment for inflammation. We chose to utilize exosomes for their longer half-life in the body, better penetrative capacity, and biocompatibility, thus improving upon previous RA treatments. To do this, we created a stable cell line to produce exosomes modified at the surface to express a tumor necrosis factor receptor (TNFR), which possesses the ability to act as a decoy and soak up soluble tumor necrosis factor alpha (TNFα), a notable cytokine responsible for inducing inflammation. Exosomes were then harvested from this cell line and characterized with various imaging techniques to confirm that our desired modifications had been made. Then we tested the efficacy of our experiment in two models: direct treatment and coculture. Both models showed decreased levels of inflammation with the addition of our modified, treatment exosomes. If proven to be clinically successful, this therapy has the potential to be the first ever exosome decoy treatment.

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1. Introduction

1.1 Background and Motivation

Over 1.3 million people suffer from rheumatoid arthritis (RA) in the United States. RA is a chronic autoimmune disease of unknown origin, the hallmark symptom of which is swollen, painful joints.[1] Eventually, this condition can result in the irreversible destruction of joints. Unfortunately, current treatments for RA are limited and have numerous drawbacks. The most effective treatments for RA are protein-based biologics that prevent inflammation-causing cytokines from binding to cells, however, use of these drugs can result in suppression of the immune system, leading to serious and sometimes fatal infections. For this reason, we aimed to develop and test an anti-inflammatory therapy for RA that utilizes biocompatible nanoparticles called exosomes.

1.2 Literature Review

1.2.1 Rheumatoid Arthritis

As a progressive autoimmune disease, RA results in chronic joint pain and stiffness that drastically reduces the quality of life in the people it affects. Typically, the disease manifests itself through severe discomfort in the hands and wrists, though it may also affect other regions of the body.[2] The underlying pathophysiology behind RA is the body's immune system attacking the joints, which leads to thickening of the synovium embedded in the joint capsule. In the disease mechanism, macrophages of the immune system secrete inflammatory cytokines: Interleukin-1 (IL-1), Interleukin-6 (IL-6) , and Tumor Necrosis Factor α (TNFα).[3] Together, these cytokines stimulate fibroblast-like synoviocytes to proliferate uncontrollably, in part by the phenotypic suppression of contact inhibition. These fibroblast-like synoviocytes attract other

[1] S. Cohen and P. Emery, "The American College of Rheumatology/European League Against Rheumatism Criteria for the Classification of Rheumatoid Arthritis: A Game Changer," *Arthritis & Rheumatism* 62 no. 9 (2010): 2592-2594, doi:10.1002/art.27583.

[2] "NIAMS Health Information on Rheumatoid Arthritis," National Institute of Arthritis and Musculoskeletal and Skin Disease, March, 21, 2018, Accessed June 10, 2018, https://www.niams.nih.gov/health-topics/rheumatoid-arthritis.

[3] Ankur Shah, *Harrison's Principle of Internal Medicine*, 18th ed., United States: McGraw Hill, 2738.

immune cells to the area, creating a chain of deleterious positive feedback that results in osteoclast and protease activation.[4] This contributes to the degradation of both protective cartilage and bone, as observed in RA pathology.

1.2.2 TNFα and the Inflammation Pathway

As indicated above, pro-inflammatory cytokines IL-1, IL-6, and TNFα represent major players in the RA transduction cascade. Of these cytokines, TNFα responds the most rapidly, only a few hours after stimulation, and is found in high concentrations in the synovial fluid of affected patients.[5] Further, research conducted on the interplay between these cytokines has illuminated the desirability of selective TNFα inhibition, which is sufficient in mitigating the downstream effects of IL-1 and IL-6, and thus reducing the inflammation response in RA.[6] At the cellular level, inflammation is mediated through the NFkB pathway. When TNFα binds to the TNF receptor on the exterior cell membrane, the receptor stimulates an enzyme that activates NFkB, a transcription factor consisting of p65 and p50. Translocation of NFkB to the nucleus allows it to bind upstream of quintessential inflammation response elements, promoting transcription and upregulation of inflammation inducing cascades.[7] Section 1.2.4 explains how we took advantage of this pathway to quantify inflammation in a cell culture model.

1.2.3 Exosomes and their Therapeutic Potential

Exosomes are naturally secreted nanovesicles roughly 30-100 nm in size originating from the intraluminal budding of multivesicular endosomes (MVEs).[8] Once thought to be a system of cellular waste elimination, exosomes have recently been characterized as a model for cell-cell communication, revitalizing interest in their drug delivery capacity.[9] Unlike liposomes and other synthetic nanoparticle vehicles, exosomes contain transmembrane and membrane-bound proteins

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[4] Shah, 2738.
[5] M. Feldmann et al, "Definition of TNFα as a Therapeutic Target for Rheumatoid Arthritis," *TNF-Inhibition in the Treatment of Rheumatoid Arthritis,* 2004, 1-22, doi: 10.3109/9780203624388.
[6] Feldmann.
[7] Z. Zhang et al, "AAV-Based Dual-Reporter Circuit for Monitoring Cell Signaling in Living Human Cells," *Journal of Biological Engineering* 11, no. 1 (2017), doi:10.1186/s13036-017-0060-9, 18.
[8] J. Kowal and M. Tkach, "Biogenesis and Secretion of Exosomes," *Current Opinion in Cell Biology 29* (2014): 116-125, Doi: 10.1016/j.ceb.2014.05.004.
[9] M. Rashed et al, "Exosomes: From Garbage Bins to Promising Therapeutic Targets," *International Journal of Molecular Science* 18, no. 3 (2017): 538, Doi: 10.3390ijms18030538.

that could promote the endocytosis and delivery of their internal content.[10] So far, exosomes have been suggested in a wide range of animal disease models, from cancer,[11] to parasitic infection,[12] and even as far as traumatic brain injury.[13]

1.2.3.1 Exosome Biogenesis

Exosome biogenesis is intimately associated with the endosomal system. Molecules on the plasma membrane are delivered to early endosomes in endocytic vesicles. Early endosomes mature into late endosomes where invagination of the membrane causes the formation of multivesicular bodies (MVBs) containing intraluminal vesicles.[14] Finally, fusion of MVBs with the plasma membrane causes the release of the intraluminal vesicles, now called exosomes.

Another known fate of late endosomes is fusion with the lysosome organelle, a transfer considered to be unidirectional.[15] Soluble molecules within late endosomes, including intraluminal proteins tagged with ubiquitin, are transported to the lysosome for degradation.[16] Hence, molecules potentially secreted in exosomes could otherwise find themselves degraded. This alternate fate is important to note in our project to observe the extent of colocalization of our therapeutic exosomes within the lysosomal compartment. Unfortunately, not much is understood about cellular selection between the fates of late endosomes or MVBs since the population is thought to be distinctly heterogenous.[17]

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[10] S. Kamerkar, and V. LeBleu, "Exosomes Facilitate Therapeutic Targeting of Oncogenic KRAS in Pancreatic Cancer," *Nature,* 2017, *546:* 498-503, Doi: 10.1038/nature22341.
[11] D. Moris and E. Beal, "Role of Exosomes in Treatment of Hepatocellular Carcinoma," *Surgical Oncology* 26, no. 3 (2017): 219-228, doi: 10.1016/j.suronc.2017.04.005.
[12] F. Aline et al, "Toxoplasma Gondii Antigen-Pulsed-Dendritic Cell-Derived Exosomes Induce a Protective Immune Response Against T. Gondii Infection," *Infection and Immunity* 72, no. 7 (2004): 4127-37, doi: 10.1128/IAI.72.7.4127-4137.
[13] Y. Xiong et al, "Emerging Potential of Exosomes for Treatment of Traumatic Brain Injury," *Neural Regeneration Research* 12, no. 1: 19-22, doi: 10.4103/1673-5374.198966.
[14] N. Hessvik and A. Llorente, "Current Knowledge on Exosome Biogenesis and Release," *Cellular and Molecular Life Sciences* 75, no. 2: 193-208, doi:10.1007/s00018-017-2595-9.
[15] Hessvik.
[16] Hessvik.
[17] Hessvik.

1.2.3.2 Tetraspanin CD63

CD63 is the most abundant of tetraspanin proteins and it is considered a hallmark localizer of exosomes.[18] Tetraspanins consist of both intra and extra-vesicular domains, making them a great target for modifications.[19] Previous studies have shown that both RFP and GFP have been fused with tetraspanin CD63 and used to track the secretion and uptake of modified exosomes.[20]

1.2.4 Inflammation Reporter

In order to quantify inflammation in vitro, we utilized a reporter that signaled when the NF-kB pathway was initiated. Specifically, we used a HEK 293 NF-κB GFP/Luciferase reporter. The reporter uses a adeno-associated virus (AAV) helper system that contains transcription factor response elements (TREs) followed by a minimal CMV promoter and GFP-2A-Firefly-luciferase.[21] When this dual reporter cell line is in the presence of the inflammatory cytokine TNFα, Nf-KB is activated and binds to the TREs which leads to the expression of both GFP and luciferase, which can be quantified and related to cellular levels of inflammation.[22]

[18] Z. Stickney et al, "Development of Exosome Surface Display Technology in Living Human Cells," *Biochemical and Biophysical Research Communications* 472, no. 1 (2016): 53-59, doi: 10.1016/j.bbrc.2016.02.058, 53.
[19] Stickney, 53.
[20] Stickney, 54.
[21] Zhang, 2.
[22] Zhang, 5.

1.3 Drawbacks of Current Technologies

Given rheumatoid arthritis prevalence in the US population, there is a plethora of medications and therapies currently used for treatment. However, many have significant drawbacks impacting patient quality of life. In Table 1-1, we outline the mechanism of current treatments, list their drawbacks, and propose how an exosome-based therapy may circumvent these problems.

Table 1-1: Drawbacks of Current RA Treatments

Treatment	**Mechanism of action**	**Treatment side effects**	**Exosome-based therapy solutions**
Non-steroid anti-inflammatory drugs (NSAIDs)	Reduces vasodilation by inhibiting production of prostaglandins[23]	Adverse reactions in those with hepatic and renal sensitivities[24]	Exosomes will not block prostaglandins
Steroids	Mimics anti-inflammatory properties of naturally secreted hormones[10]	Can displace natural steroid production, creating dependency[10]	Localized exosome therapy will not displace natural exosomes
Disease modifying antirheumatic drugs (DMARDs)	Reduces proinflammatory cytokines by reducing folate levels[25]	Long-term liver toxicity[12]	Exosomes will not act on folate levels
Biologics	Inhibits inflammatory cytokines TNFα and IL-6[26]	Increased infection risk, drug becomes ineffective if doses are skipped[27]	Antibodies will not develop against treatment exosomes
Physical therapy	Maintains muscle strength and reduces inflammation[28]	Accessibility and cost	Not applicable
Joint replacement surgery	Removes joints too eroded to function[29]	Risks of surgery, long recovery time	Not applicable

[23] E. Ricciotti and G. Fitzgerald, "Prostaglandins and Inflammation," *Arteriosclerosis, Thrombosis, and Vascular Biology* 31, no. 5 (2011): 986–1000, doi: 10.1161/atvbaha.110.207449.
[24] L. Crofford, "Use of NSAIDs in Treating Patients with Arthritis," *Arthritis Research & Therapy* 15 (2013).
[25] M. Nurmohamed and B. Dijkmans, "Efficacy, Tolerability and Cost Effectiveness of Disease-Modifying Antirheumatic Drugs and Biologic Agents in Rheumatoid Arthritis," *Drugs* 65, no. 5 (2005): 661-694, doi: 10.2165/00003495-200565050-0006.
[26] J. Singh et al, "Adverse Effects of Biologics: A Network Meta-Analysis and Cochrane Overview," *Cochrane Database of Systematic Reviews* 2.
[27] L. Putte et al, "Adalimumab," *TNF-Inhibition in the Treatment of Rheumatoid Arthritis*, 2004, 71-88, doi: 10.3109/9780203624388-5.
[28] V. Kavuncu and D. Evcik, "Physiotherapy in Rheumatoid Arthritis," *Medscape General Medicine* 6, no. 2, doi: 10.3109/9780203624388-5.
[29] "Joint Replacement Surgery," National Institute of Arthritis and Musculoskeletal and Skin Diseases, October 4, 2017, Accessed June 11, 2018. https://www.niams.nih.gov/health-topics/joint-replacement-surgery.

1.4 Project Goals and Constraints

Our project goal is to take the specificity of biologics one step further using naturally secreted nanoparticles. However, it is important to note that our project is only one step in a long line of research necessary to safely implement a novel RA therapeutic. For instance, we cannot measure general clinical outcomes such as liver toxicity due to institutional limitations on animal testing, nor do we have the means to perform purity analysis. Our project, furthermore, is far from all-encompassing; we do not aim to replace physical therapy or joint replacement surgery with our exosomal therapy, as they are required in extreme circumstances. Instead, we evaluated the efficacy of our exosomes in a cell culture model, extrapolating existing literature on exosomal treatment to make clinical predictions when appropriate. For this reason, we propose a proof of concept for reducing inflammation *in vitro.*

Our project is nominally divided into three phases or goals. The first goal of our project was to create a stable cell line that produced engineered exosomes. The second goal involved confirmation of the desired modifications via fluorescence imaging. Following successful production of our therapeutic exosomes, we tested their efficacy in a quantitative assay in the third and final goal.

1.4.2 Phase 1: Production

In phase 1, we focused on creating a stable cell line that produced engineered exosomes capable of preventing inflammation. Next, we harvested these exosomes and store them for phases 2 and 3 of our project.

1.4.3 Phase 2: Characterization

In phase 2, we aimed to confirm that we had made the desired modifications to our stable cell lines. We followed the biogenesis of exosomes using various markers to ensure that our TNFR had been added to the surface of the exosomes.

1.4.4 Phase 3: Testing

In phase 3, we utilized our reporter cell line to determine the effect of our engineered exosomes on the inflammatory response of human cells in vitro.

1.5 Back-Up Plan

Despite careful aseptic technique, research with mammalian cell lines is notably susceptible to unforeseen contamination in the form of bacteria or fungi. In order to prevent contamination that could jeopardize the timeline of our project, we continually made frozen copies of our stable cell lines to store at -80C. Although this procedure was critically important to staying ahead of deadlines, it is hardly unique to our project. Project specific back-up plans include the option to engineer TNFR onto a different scaffold, such as VSVG or RD114, if our CD63-TNFR-GFP exosomes are not successful in significantly reducing inflammation. Secondly, we could refocus our attention on other inflammatory cytokines implicated in the RA transduction cascade like IL-1 and IL-6.

1.6 Significance

If our proposed therapy is successful in preventing inflammation in-vitro, it could be one of the first exosome-based therapies to be tested in animals and clinical trials. Due to the natural stability of exosomes in the body and the fact that they do not initiate an adverse immune response, our proposed therapy has the potential to be as effective as DMARDs or biologics without the significant drawbacks of those classes of drugs. The technology utilized by our proposed exosome therapy could also be applied to a number of other therapeutic uses, such as gene therapies, immunotherapies, and targeted drug delivery.

1.7 Team Management

As a team, we have shared equal responsibility maintaining our cell lines, designing and performing experiments, and analyzing data. Dr. Lu provides guidance through regular correspondence and weekly meetings.

1.8 Budget

See Table 1-2 below for our project budget.

Table 1-2: Project Budget

Flasks and Plates	$250
Fetal Bovine Serum	$250
Culture Media	$500
TNFα	$210
Serum Free Media	$120
Luciferase Assay	$250
Endosomal Stains	$500
Transfection Reagent	$1000
Total Cost	**$3080**

1.9 Timeline

Our project timeline is outlined below in Table 1-3.

Table 1-3: Project Timeline

	Fall Quarter	Winter Quarter	Spring Quarter
Establish Cell Lines	X		
Maintain Cell Lines	X	X	X
Characterize and Image Exosomes	X	X	
Track exosomes through endosome pathway		X	
Evaluate Dose Response		X	X
Write Thesis		X	X
Present Results			X

2. Creating Stable Cell Lines to Produce Therapeutic Exosomes

2.1 Design Description

In order to produce and collect modified exosomes efficiently, we decided to create two stable cell lines. Our treatment stable cell line utilizes CD63 tetraspanin to anchor a TNFR along with GFP to the surface of exosomes. Based on prior experimentation, we anticipated that this TNFR on the surface of the exosomes would soak up excess TNFα and thereby prevent the initiation of inflammation in cells. We also created a control stable cell line for experimentation purposes, replacing the TNFR with RFP. Both cell lines utilized HEK 293 cells transfected with our desired DNA constructs and a puromycin resistance gene in order to select for our transfected cells.

2.2 Key Constraints

To prevent cells without our desired construct from growing, we grew our stable cell lines in media containing the antibiotic puromycin for a period of 10 weeks. Antibiotics can be harsh on cells, so finding the correct dose that would kill cells without our desired modifications but would also allow the resistant cells to thrive can be difficult. One must consistently monitor the cells to ensure that only the cells without the desired modifications (the cells that do no fluoresce green) are killed off.

2.3 Expected Results

We expected to see GFP expressed in all of our HEK 293 CD63-TNFR-GFP cells and both GFP and RFP expressed in all of our HEK 293 CD63-RFP-GFP cells after the 10 week treatment with puromycin. This period of treatment should have been sufficient for killing off any untransfected cells. Final results are shown in section 6.1.

2.4 Materials and Methods

The following table (Table 2-1) contains the necessary materials to create our stable cell lines.

Table 2-1: Materials for Creating Stable Cell Lines

Material	Company/Brand	Model #
Passaging Mammalian Cells Materials (See Appendix)		
HEK 293 cells		N/A
HEK 293 CD63-RFP-GFP Stable Cell Line		
DMEM + 10% FBS + PS media		
Puromycin	ThermoFisher Scientific	A1113802
FuGene HD Transfection Reagent	Promega	E231A
Microcentrifuge tubes	Sigma-Aldrich/ Eppendorf Safe-Lock	T9661
Opti-MEM Reduced Serum Media	ThermoFisher Scientific	31985062
DNA Plasmids (CD63-TNFR-GFP and CD63-RFP-GFP)	Genscript	

Methods

Day 1

1. Use protocol for passaging mammalian cells onto a 60mm x 15mm dish and incubate at 37°C for 24 hours.

Day 2

2. After 24 hours, combine reduced-serum media (Opti-MEM) and FuGene HD Transfection Reagent at a ratio of 2µg of DNA per 4µl of transfection reagent.

3. Incubate at room temperature for 5 minutes.

4. Carefully add 1.5µg/mL of DNA plasmid into the reduced-serum media (Opti-MEM) and transfection reagent mixture.

5. Incubate the mixture at room temperature for 20-30 minutes.

6. Carefully add the mixture to the cell culture dish.

7. Incubate the dish at 37°C for 24 hours.

Days 4-70

8. Add 5µg/ml of puromycin to 50 mL of DMEM + 10% FBS + PS media and repeat as needed.

9. Use protocol for passaging mammalian cells and plate the cells in the media created in step 8.

3. Image Characterization of Therapeutic Exosomes

3.1 Design Description

After creating our treatment stable cell line (HEK 293 CD63-TNFR-GFP), we wanted to ensure that the exosomes it produced had been successfully modified. To do this, we decided to track the exosomes at each stage of their biogenesis. As discussed in the introduction, exosomes originate from the intraluminal budding of multivesicular endosomes. Therefore, we decided to utilize RFP stains for both early and late stage endosomes to visualize if our modified exosomes followed this path of biogenesis. We also utilized a lysosome tracker, as late stage endosomes can be digested by lysosomes. Finally, we performed a co-transfection of our stable cell line with an exosome localizer and RFP to ensure that our TNFR-GFP construct had successfully localized onto the surface of exosomes. All of the stains and the co-transfection were performed in a 4-chamber glass bottom plate, as shown in Figure 3.1.1.

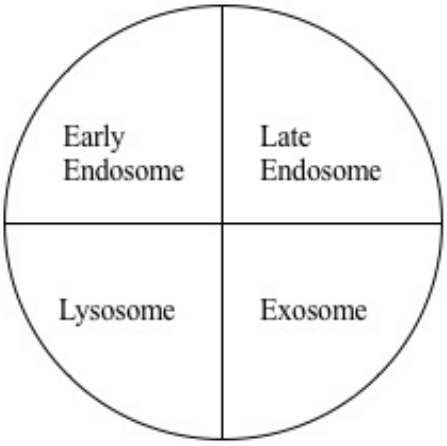

Figure 3.1.1: Schematic of characterizing stains in a four-chamber plate.

3.2 Key Constraints

The key constraints for this portion of the project centered on the transfection and staining efficiency of our reagents. Some factors affecting this are cell confluency, reagent age, and seeding uniformity. Cell confluency and seeding uniformity can also affect image quality.

3.3 Expected Results

We expected to see the GFP on our modified exosomes colocalize with the both the early and late endosome stains, as well as the RFP exosome localizer. When overlaying images taken with RFP and GFP filters, we expected to see the two colors overlap and appear yellow. Final results are shown in section 6.2.

3.4 Materials and Methods

The following tables (Table 3-1) includes the necessary materials to perform the characterization experiments outlined in section 3.1.

Table 3-1: Materials for Characterization

Material	Company/Brand	Model #
Passaging Mammalian Cells Materials (See Appendix)		
CD63-TNFR-GFP stable cell line	Created in lab	N/A
Early Endosomes-RFP, BacMam 2.0	ThermoFisher/CellLight	C10587
Late Endosomes-RFP, BacMam 2.0	ThermoFisher/CellLight	C10589
LysoTracker Red DND-99	ThermoFisher/Invitrogen	L7528
Opti-MEM Reduced Serum Media	ThermoFisher Scientific	31985062
4-chamber 35 mm glass-bottom plate	In Vitro Scientific	D35C4-20-1.5-N
1 g/L polyethylenimine (PEI) transfection reagent	Created in lab	N/A
CMV-XP-RFP-EF1α expression vector	System Biosciences/XPack	XPAK531PA-1
35 mm glass-bottom plate	Matsunami Glass	D35-14-1.5-U

Methods

Day 1

1. Seed cells for endosome imaging

    1.1. Use protocol for passaging mammalian cells to seed HEK 293 CD63-TNFR-GFP cells into all chambers of the 4-chamber 35 mm glass bottom plate at a density of $2x10^5$ cells/ml with DMEM + FBS + PS media.

        1.1.1. Each chamber of the glass-bottom plate contains 500 µL; add cells one chamber at a time to ensure equal cell density.

    1.2. Designate the 4 chambers as early endosome, late endosome, lysosome, and no transfection.

    1.3. Incubate at 37°C for 24 hours.

2. Seed cells for exosome imaging

    2.1. Use protocol for passaging mammalian cells to seed HEK 293 CD63-TNFR-GFP cells into five 35 mm glass bottom plates at a density of $2x10^5$ cells/ml with DMEM + FBS + PS media.

    2.2. Designate 4 plates to be transfected with CMV-XP-RFP-EF1α DNA and one plate to remain untransfected for control.

    2.3. Incubate at 37°C for 24 hours.

Day 2

3. Transfect cells with late and early endosome markers

    3.1. Estimate the number of cells in each chamber via light microscopy.

    3.2. Add 2 µL of early or late endosome mix per 45,000 cells to respective chambers.

    3.3. Incubate at 37°C for 16-24 hours.

4. Transfect cells with exosome marker (for each transfected dish)

    4.1. Aliquot 100 µL of opti-MEM media.

    4.2. Since each dish requires about 2µg of CMV-XP-RFP-EF1α DNA and 5 µL of 1 g/L PEI is required to encapsulate 1 µg of DNA, add 10 µL of PEI to opti-MEM.

    4.3. Allow PEI and opti-MEM mixture to sit at room temperature for 5 minutes.

    4.4. Add 2µg of CMV-XP-RFP-EF1α DNA to mixture and allow to sit at room temperature for 20 minutes.

    4.5. Add 100 µL of mixture to each transfected dish.

4.6. Incubate dishes at 37°C for 24 hours.

Day 3

5. Stain lysosomes and image all cells

5.1. Dilute stock LysoTracker Red DND-99 to a concentration of 50-75nM in cell culture media.

5.2 Add 1μL of lysosome stain to designated chamber.

5.3 Incubate at 37°C for 45 minutes.

6. Capture phase contrast, RFP, and GFP images of each transfection at 40x

4. Testing Efficacy of Therapeutic Exosomes: Direct Treatment

4.1 Design Description

After creating our stable cell line, we wanted to test the efficacy of our CD63-TNFR-GFP exosomes. To do this, we added TNFα to half of the wells of our HEK 293 Nfkb dual reporter cells to simulate inflammation as well as varying concentrations (0.5mg/ml, 0.1mg/ml, and 0 mg/ml) of our CD63-TNFR-GFP treatment exosomes or our CD63-RFP-GFP control exosomes. Each experimental condition was run in triplicate. We then utilized a luciferase assay to determine whether our treatment exosomes had successfully inhibited TNFα initiated inflammation in vitro.

4.2 Key Constraints

The key constraints for this portion of the project centered on the response sensitivity and volume of the HEK 293 Nfkb dual reporter cells. These constraints were controlled by reporter system expression and the number of seeded cells, respectively. A second constraint was the degradation of the luciferase assay reagent (LAR). LAR is time and light sensitive, therefore our results also depended on this substrate's quality.

4.3 Expected Results

We expected to see a statistically significant reduction in luciferase fluorescence--corresponding to a reduction in inflammation--only when we add CD63-TNFR-GFP exosomes. We also expected this response to be dependent on the exosome concentration. Final results are shown in section 6.3.

4.4 Materials and Methods

The following table (Table 4-1) includes the materials required to perform the direct treatment efficacy assay.

Table 4-1: Materials for Efficacy of Design Experiment: Direct Treatment

Material	Company/Brand	Model #
Passaging Mammalian Cells Materials (See Appendix)		
DMEM + 10% FBS + PS media	Created in lab	N/A
Serum-Free Medium without L-Glutamine	BioWhittaker/ UltraCULTURE	12-725F
96 well plate, clear bottom	Greiner Bio-one/Cellstar	655180
Recombinant Human TNFα Protein	R&D Systems	210-TA-020
Materials for Luciferase Assay (See Appendix A4)		

Methods

Day 1

1. Seed cells

    1.1 Use protocol for passaging mammalian cells to seed HEK 293 NfKB reporter cells into 36 wells of the 96-well plate at a density of $1x10^5$ cells/ml with DMEM + FBS + PS media.

    1.2 Incubate the plate at 37°C for 24 hours.

Day 2

2. Treat cells with recombinant human TNFα and exosomes.

    2.1 Add recombinant human TNFα protein to serum-free media to create a stock concentration of 100 ng/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. Create stock concentrations of CD63-RFP-GFP exosomes of 0, 0.1, and 0.5mg/mL in serum-free media.

2.4. Add stock TNFα protein solution to appropriate exosome solutions to create a working concentration of 1 ng/mL TNFα.

2.5 Tilt the plate and carefully aspirate the media off each well.

2.6. Carefully add 100μL of the various serum-free media conditions to each well.

2.7. Incubate at 37°C for 24 hours.

Day 3

3. Perform luciferase assay (See Appendix A4).

5. Testing Efficacy of Therapeutic Exosomes: Coculture

5.1 Design Description

In this experimental model, we wanted to determine the effect our CD63-TNFR-GFP exosomes would have in reducing inflammation in coculture with the HEK 293 Nfkb dual reporter cells. The cells were seeded in a 3:1 ratio of exosome producing cells to reporter cells. This allowed for a sufficient number of modified exosomes to be produced and released from the stable cell lines. HEK 293 CD63-RFP-GFP cells were used as control and plain HEK cells were used as background. Six wells of each coculture condition were plated and TNFα was added to half of the wells. A luciferase assay was then performed to determine the extent to which our exosomes inhibited TNFα.

5.2 Key Constraints

The biggest constraint with this experimental model was that we did not know how many exosomes were produced by the cells in coculture with the reporter cell line. Therefore, we were unable to determine an effective dosage of CD63-TNFR-GFP exosomes using this model.

5.3 Expected Results

We expected that the exosomes secreted by our stable cell line in coculture with the reporter cell line would effectively decrease levels of TNFα. However, since the overall concentration of treatment exosomes was likely lower in this model than in the direct treatment model, the effect was not as pronounced. Final results are shown in section 6.3.

5.4 Materials and Methods

Table 5-1 includes the materials necessary to perform the coculture efficacy assay.

Table 5-1 Materials for Efficacy of Design Experiment: Coculture

Material	Company/Brand	Model #
Passaging Mammalian Cells Materials (See Appendix)		
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
96 well plate, clear bottom	Greiner Bio-one/Cellstar	655180
Materials for Luciferase Assay (See Appendix A4)		N/A

Methods

Day 1

1. Seed cells

    1.1 Create stock concentrations of exosome producing cells (HEK 293, HEK 293 CD63-RFP-GFP, HEK 293 CD63-TNFR-GFP) and reporter cell line HEK 293-NfKB Dual Reporter in suspension at $1x10^5$ cells/ml.

    1.2 Plate 100 ul per well comprised of 75 ul of exosome producing suspension and 25 ul of reporter cell suspension across the following groups: HEK 293 vs Reporter, RFP-GFP vs Reporter, TNFR-GFP vs Reporter.

    1.3 Incubate 48 hours

Day 3

2. Treat half the groups with Recombinant Human TNFα Protein at 1 ng/ml. Incubate 24 hours at 37°C.

Day 4

3. Perform Luciferase Assay (See Appendix A4).

6. Results

6.1 Creation of Stable Cell Lines

We succeeded in creating a stable cell line that produces our modified, decoy exosomes, as well as a second stable cell line for experimental control. Figures 6.1.1 and 6.1.2 show the expression of our desired DNA constructs in HEK 293 cells. The localization of fluorescence to small dots outside the cells' nuclei shows that our constructs have been integrated into the membranes of exosomes.

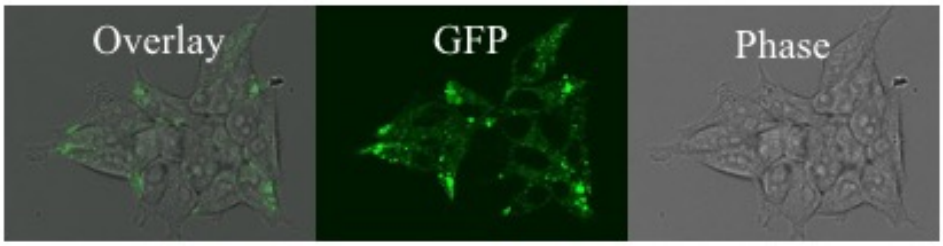

Figure 6.1.1: HEK 293 CD63-TNFR-GFP stable cell line. Modified exosomes represented by GFP. Images taken at 40x.

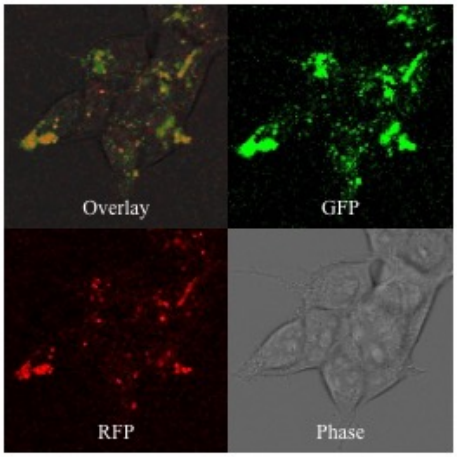

Figure 6.1.2: HEK 293 CD63-RFP-GFP stable cell line for experimental control. Images taken at 40x.

6.2 Characterization

Imaging confirmed that our desired modifications had been made to the surface of exosomes produced by the HEK 293 CD63-TNFR-GFP stable cell line. Images show colocalization of exosomes with both early and late stage endosomes (Figure 6.2.1 A and B), which demonstrates that our modifications are following the path of exosome biogenesis. As discussed in 1.2.3.1, exosomes may follow an alternate path and reside within lysosomes. Imaging shows that our modified exosomes also colocalize with lysosomes (Figure 6.2.1 C). An exosome-specific marker (XPACK-RFP) also colocalizes with our CD63-TNFR-GFP exosomes (Figure 6.2.2). Colocalization of each of the RFP markers with GFP is represented as yellow in the overlaid images.

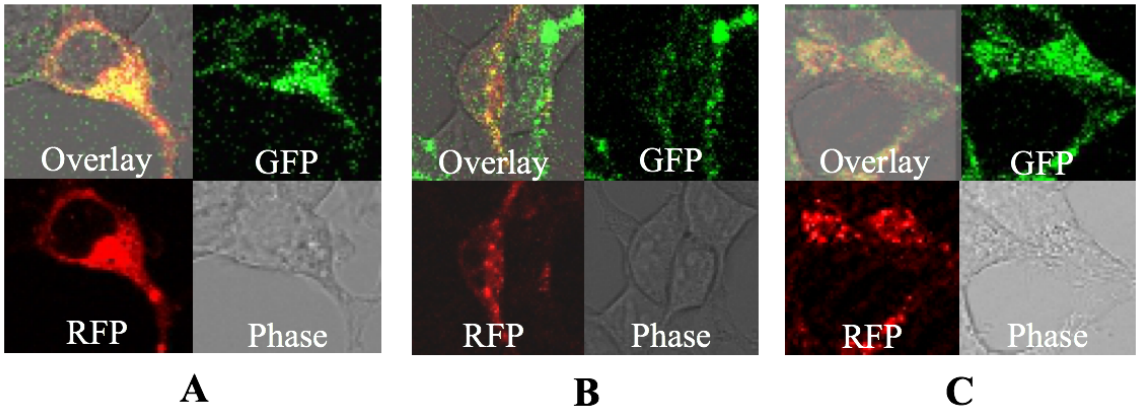

Figure 6.2.1: HEK 293 CD63-TNFR-GFP cells with A: Early Endosome RFP Marker, B: Late Endosome RFP Marker, and C: Lysosome RFP Marker. All images taken at 40x.

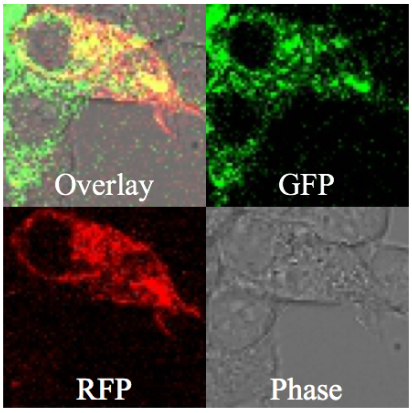

Figure 6.2.2: HEK 293 CD63-TNFR-GFP cells with XPACK-RFP, an exosome localizer. Images taken at 40x.

6.3 Efficacy

The presence of our treatment exosomes was successful in reducing levels of TNFα in both the coculture and direct treatment models. Inflammation was quantified in both models using the HEK 293 Nfkb dual reporter cell line. A decrease in levels of TNFα corresponds to a decrease in luminescence detected during our assay.

As shown in Figure 6.3.1, our exosomes produced in coculture with the reporter cell line were able to significantly reduce (p=0.036) the levels of TNFα compared to the experimental condition with natural, HEK exosomes only.

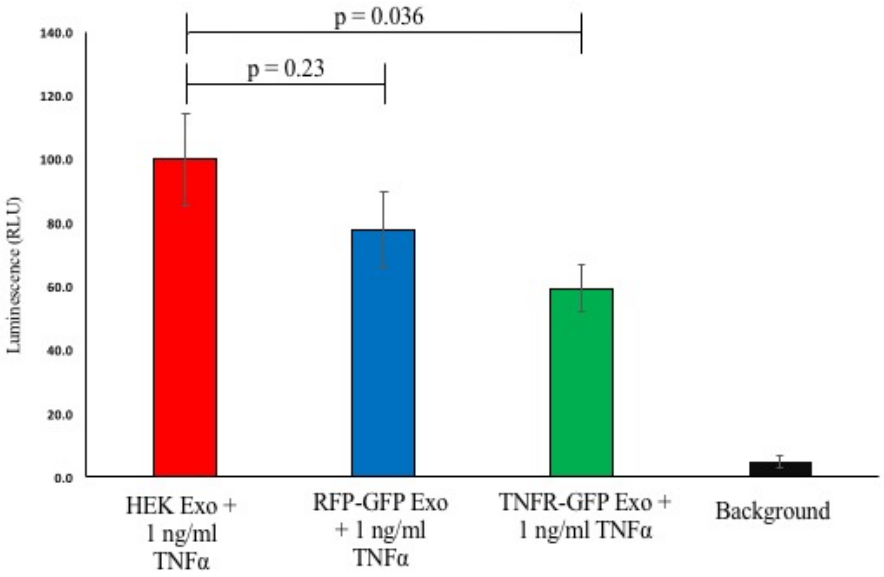

Figure 6.3.1: Efficacy of exosome producing stable cell lines in coculture with HEK 293 Nfkb dual reporter cells. N=9 from three separate trials. Error bars represent standard error of the mean.

The direct treatment model also showed a significant decrease in TNFα levels with the addition of 0.5mg/ml of CD63-TNFR-GFP exosomes (Figure 6.3.2).

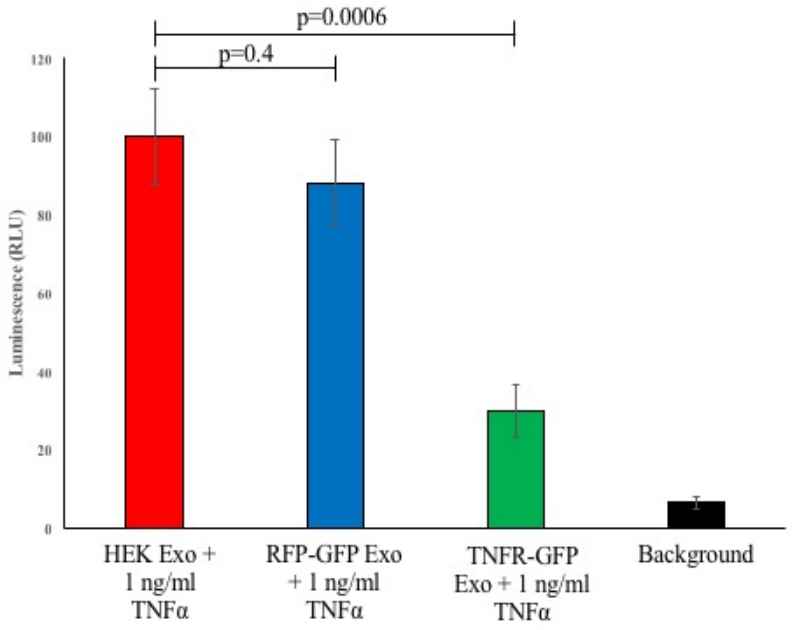

Figure 6.3.2: Efficacy of 0.5mg/ml exosomes added directly to HEK 293 Nfkb dual reporter cells. N=9 from three separate trials. Error bars represent standard error of the mean.

7. Discussion and Conclusion

Our results show that the concept of an effective, exosomal decoy treatment for inflammation is possible. In the production phase of our project, we find that our stable cell lines expresses GFP and RFP, as expected. In the verification phase, we confirm that the particles our cell lines produce are exosomes due to colocalization features with other cell compartments. Most importantly, we prove that both models of efficacy experiments show a significant decrease in inflammation due to the presence of the exosomes we created. This fact, combined with the penetrative capacity known of exosomes and their longer biological half-life, indicates a solid footing into further exosome research for RA. If our treatment is proven to work in animal and human models, it has the potential to be one of the first exosomal treatments. Future work, such as purity testing, high-throughput production design, and observation of cytotoxic and organ system effects would be the best way to continue this project, but would require outside help and better equipment.

We hope that our project will trigger more research into exosomal decoy therapies for other human diseases. Besides RA, exosomes have a promising ability to deliver enzymes to the lumen of the lysosomes for patients with a wide-variety of lysosomal storage diseases such as Gaucher's or Tay Sachs.

8. Engineering Standards and Realistic Constraints

8.1 Social impact

By utilizing exosomes for our treatment, we believe that the therapeutic effects of our anchored TNF receptor will be superior to treatments utilizing soluble TNFR. This would primarily be due to exosomes' long half-life in the body, as well as their ability to deeply penetrate inflamed tissues. This targeted therapeutic will also be less wasteful than current soluble TNFR treatments; soluble TNFR will degrade much faster than TNFR anchored to an exosomal membrane. Hopefully, this will result in the patient requiring fewer injections, which will improve their quality of life by decreasing the number of times they have to visit a doctor.

8.2 Health and Safety

Any medical therapy must undergo rigorous testing before being administered to humans. Our project is focused on manufacturing, characterizing, and testing therapeutic exosomes in vitro, and can therefore be classified as pre-clinical. The first step to ensuring the safety of our therapeutic would be to perform a toxicity assay in a mammalian cell culture model. Unfortunately, our lab does not have the resources to move forward with the next steps in safety testing. However, if our therapeutic were to proceed to clinical trials, it would be tested in animals before beginning four phases of human trials.

8.3 Manufacturability

A major issue encountered in our design project was the efficient and reliable production of engineered exosomes at a rate that would warrant its use as a therapeutic. Previous to our work on the project, the procurement of therapeutic exosomes required transient transfection of our construct immediately prior to each harvest being performed. Sustained gene expression guaranteed by stable cell lines allowed us to more quickly and reliably harvest therapeutic exosomes and cut down the cost incurred through prodigal use of transfection reagents. Further improvements to exosome harvest and purification could allow more efficient manufacturability in the future. Cellular exosome yield could be drastically heightened with the introduction of better, more suitable conditions using a controlled bioreactor. Modulation of pH,

temperature, or agitation may change the ability of our cells to produce exosomes. Additionally, the efficacy experiments indicated that a high concentration of exosomes induced a modest reduction in the inflammation pathway response. However, we have reason to believe that the concentration indicated by the nanodrop lite may erroneously register the additive effect of artifacts and thus display false readings. A better protein expression system to gauge the purity and true concentration of our purified exosomes is needed to achieve desired manufacturability.

8.4 Economic

As we noted in our introduction, novel biologics that work via inhibition of inflammatory cytokines IL-6 and TNFα have the potential to induce antagonistic antibody production or allow the reactivation of latent infections such as tuberculosis. These could lead to far more expensive medical costs. Furthermore, effective use of these therapies requires regular and expensive treatment. Our exosomes have the potential to reduce cost by limiting the number of times a patient must return for treatment. Exosomes boast a longer half-life in the body, and do not threaten to evoke deleterious immune responses that pile up the medical bills.

8.5 Ethical implications

We chose to work on this project because we believed that this novel treatment had the potential to impact the millions of people suffering from a disease as debilitating as rheumatoid arthritis. A chronic illness severely limits patients' autonomy and can prevent them from living a satisfying life. We hope that a therapy that aids symptom management for RA will promote patients' autonomy. From a utilitarian point of view, developing a new treatment that will be less expensive for patients, ease their pain, and require them to visit the doctor less often is doing moral good.

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Appendix

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

Table A1-1: Materials for Making Media

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

1. Add 50 mL of FBS into a 500ml bottle of DMEM with L-Glutamine.
2. Add 5 mL of PS to the combined FBS and DMEM.
3. Mix thoroughly.
4. Store at 4°C until needed.

A2: Passaging Cells

Table A2-1: Materials for Passaging Cells

Material	Company/Brand	Model #
DMEM + 10% FBS + PS media	N/A	N/A
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

1. Aspirate depleted media
2. Wash with 3 mL of PBS. Pipet gently into side of plate.
3. Aspirate PBS.
4. Add 1.5 mL of trypsin to plate.
5. Incubate at 37°C for 2 minutes.
6. Deactivate trypsin with 4.5 mL of DMEM + 10% FBS + PS media.

7. Collect media in 15 mL centrifuge tube, spin at 1500 RPM for 5 minutes.
8. Aspirate off supernatant.
9. Resuspend the pellet in DMEM + 10% FBS + PS media.
10. Plate at desired density.

A3: Harvesting Exosomes

Table A3-1: Materials for Exosome Harvest

Material	Company/Brand	Model #
Passaging Mammalian Cells Materials (See Appendix A1)		
Dish 145mmx20mm	Sigma-Aldrich/ Greiner	639160
Serum-Free Medium without L-Glutamine	BioWhittaker/ UltraCULTURE	12-725F
50 mL conical-bottom centrifuge tubes	VWR	89039-658
Syringe, 30 mL	BD Biosciences/ BD Luer-Lok	
Sterile 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

Day 1

1. Seed Cells

    1.1. Using a passaging mammalian cells protocol (Appendix A2-B), seed stable cell lines onto 145mmx20mm dishes at 40-60% confluency (2-3x10^5 cells/mL) with DMEM + 10% FBS + PS media.

    1.2. Incubate at 37°C for 24 hours or until 70-80% confluent.

Day 2:

2. Change media

2.1. Aspirate off DMEM + 10% FBS + PS media and replace with equal volume of serum-free media without L-glutamine.

2.2. Incubate at 37°C for 48 hours.

Day 4:

4. Exosome Harvest Part I

4.1. Collect the serum-free medium into a 50 mL centrifuge tube.

4.2. Centrifuge the 50 mL centrifuge tube at 1500xg for 10 minutes.

4.3. Filter the supernatant through a 0.2-micron filter with a sterile 30 mL syringe into a new 50 mL centrifuge tube.

4.4. Add 1⁄4 of the supernatant volume of exosome precipitation solution.

4.5. Incubate at 4°C for 24 hours.

Day 5:

5. Exosome Harvest Part II

5.1. Centrifuge the 50 mL centrifuge tube from Day 4 at 3000xg for 45-90 minutes.

5.2. Carefully aspirate off the supernatant, taking care to not disturb the pellet on the side of conical tube.

5.3. Resuspend the pellet in 50 µl-100 µl of PBS.

5.4. Store in a cryogenic vial at -80°C until needed.

A4: Luciferase Assay

Table A4-1: Materials for Luciferase Assay

Material	Company/Brand	Model #
Plate Reader	BMG Lab Tech/LUMIstar Omega	S/N 415-1717
Luciferase Assay Buffer (LAB)	Promega	E1501
Luciferase Assay Substrate (LAS)	Promega	E1501
Passive Lysis Buffer (PLB)	Promega	E1501

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

2. Dilute the Passive Lysis Buffer (PLB) by combining stock with 4x volume of DI water.
3. Add 20μL of diluted lysis buffer to each well.
4. Mix with gentle rotations for 10 minutes.
5. Turn on computer and plate reader.
6. Set up plate reader to measure luminescence with a read time of 10 seconds and set the attenuation to none.
7. Quickly add 100μL of LAR to each well, ensuring that the order in which you add the LAR is the order in which the plate reader will read the wells.
8. Immediately run the plate in the plate reader.