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EXOSOME INTERACTIONS IN ACUTE LYMPHOCYTIC LEUKEMIA

LAUREN SPADT
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This thesis was reviewed and approved by the following:

Cheng Dong
Professor of Biomedical Engineering
Thesis Supervisor

Jian Yang
Professor of Biomedical Engineering
Thesis Honors Advisor

ABSTRACT

Acute lymphocytic leukemia (ALL) is a pre-B cell malignancy characterized by rapid disease progression. To combat this disease's poor prognosis, innovative therapies are currently being developed. Cell-based therapy, like chimeric antigen receptor (CAR)-T cell immunotherapy, modifies patients' own T cell receptors to specifically attack cancer-specific antigens and spare non-cancerous cells. Rather than focusing solely on cell surface biomarkers, the current antigens targeted by most CAR-T cell immunotherapies, this project seeks to explore the insight given by exosomes. Exosomes, extracellular microvesicles with a size of 30-150 nm, are key players in extracellular communication, including tumor formation and metastasis [1]. Exosomes are abundantly distributed in the bodily fluids, with an average of around 10^{11} exosomes (as compared to 10^{7} leukocytes) per mL of blood [1,2]. Thus, it was hypothesized that exosome surface biomarkers' concentration gradients could lead CAR-T cells to cancerous cells. Past research has determined that CAR-T cells produce their own exosomes with significant therapeutic effects, but the interaction of cancer-derived exosomes with CAR-T cells and their associated exosomes is not yet well understood [3]. This research focused on comparing the surface biomarker expression of CD44, CD47, and CD19 on cancerous and non-cancerous cells and their associated exosomes. CD44 expression was lowered in cancerous vs. non-cancerous cells and their associated exosomes, so it is not recommended as a CAR-T cell target. CD47 was identified as a cell surface biomarker for ALL, indicating that it has the potential to be a CAR-T therapy target. Finally, the CD19 expression results validate the current CAR-T therapies that target CD19 antigens, and they also indicate that exosome surfaces have more differential expression of CD19 than cell surfaces [4].

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ACKNOWLEDGEMENTS

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Chapter 1

Introduction

Section 1.1 introduces the biological and physical frameworks that govern this project. Acute lymphocytic leukemia is the disease of interest, CAR-T therapy is the treatment of interest, and exosomes are the hypothesized means of improving existing treatments. The objectives of this thesis project are detailed in Section 1.2. Subsequently, Section 1.3 provides a summary of the organization of the remaining sections of this thesis.

1.1 Background

Acute lymphocytic leukemia (ALL) cannot be understood without knowledge of the various types of leukocytes. Lymphocytes differentiate into B cells, T cells, and natural killer (NK) cells, while myocytes differentiate into erythrocytes, neutrophils, macrophages, and more [5]. More details about ALL are discussed in Section 1.1.1. Although ALL is a disease within the immune system, it can be treated with immunotherapy like CAR-T therapy, which is described in more detail in Section 1.1.2. To improve upon existing immunotherapies, additional antigens must be identified. Since exosomes are key conduits of extracellular communication, including metastasis, they are the focus of this thesis and are introduced more thoroughly in Section 1.1.3.

1.1.1 Acute Lymphocytic Leukemia (ALL)

Acute lymphocytic leukemia (ALL) is a rapidly progressing malignancy originating in lymphocytes. Its acute progression is characterized by the presence of 20% or more blasts in

peripheral blood samples [6]. It is a priority to discover new treatments for ALL because it is the most common pediatric leukemia, and 80% of patients with ALL are children [6]. While there are existing therapies for ALL, including both induction and consolidation chemotherapy regimens and tyrosine kinase inhibitors (TKIs), these only yield long-term remission for 30-40% of adult patients [7]. For this reason, improved treatment options are necessary.

Current therapies are designed for either Philadelphia chromosome-positive (Ph+) ALL or Philadelphia chromosome-negative (Ph-) ALL [6]. The Philadelphia chromosome is the result of a common mutation involving a reciprocal translocation of an oncogene and a breakpoint cluster region, and it is found in 20-30% of ALL patients [8]. The distinction between these two forms of ALL is significant because Ph+ ALL is characterized by more aggressive disease progression and a higher likelihood of remission [8]. TKI-based combination therapies have found to be more effective in treating Ph+ leukemia than traditional chemotherapy, but they are not recommended for treatment of most Ph- leukemias [8].

1.1.2 CAR-T Therapy

Chimeric antigen receptor (CAR)-T cell therapy is a personalized immunotherapy where individual patients' own T cells are modified to specifically target and kill cancer cells [9]. This is done by designing CAR domains that bind specifically to cancer-associated antigens on the surfaces of cancer cells [9]. Then, these designed CAR molecules are conjugated to each patient's T cells (Figure 1-1A) [9]. Finally, the modified cells are reinfused into the patient's bloodstream, where the CAR molecules attach to cancer cell antigens and enable the associated T cells to kill the cancer cells (Figure 1-1B) [9]. This treatment is desirable because it reduces the non-specific cell killing caused by chemotherapy and reduces the chance of cell rejection by the host since the cells are derived from the individual being treated [9].

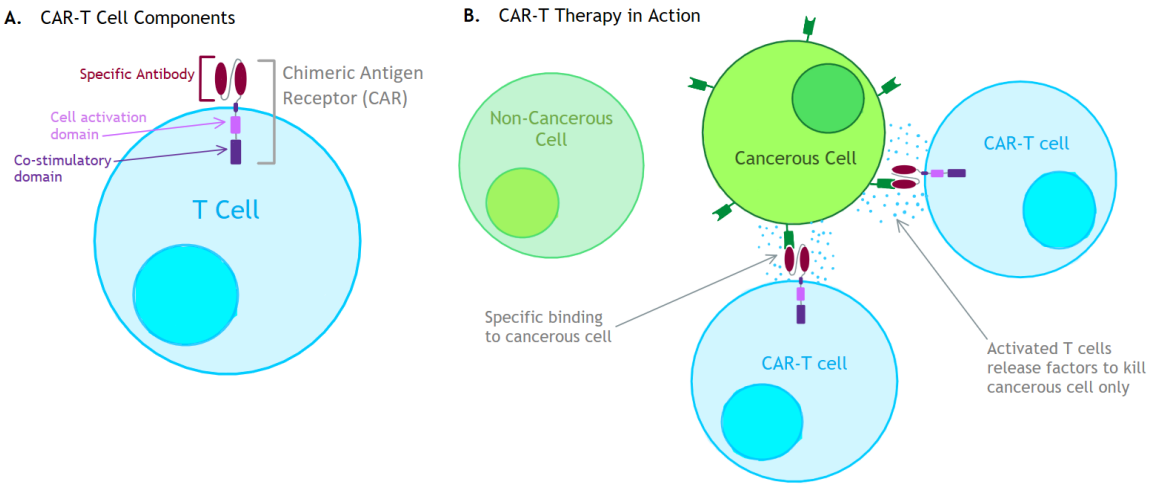

Figure **1-1**: CAR-T Cell Principles.

Multiple CAR-T therapies have already been developed for ALL, but all five FDA-approved treatments target the CD19 antigen [7]. These successes validate the potential of CAR-T therapy to kill cancer cells and even lead to remission, but more antigens need to be identified. This is because more longitudinal studies have found that patients who initially achieve remission from CAR-T therapy are at a high risk of relapsing soon after remission [10]. While there are many potential reasons for this high relapse rate, low CAR-T cell persistence *in vivo* and immune escape (where the relapse is CD19 negative) are two of the most discussed causes [10]. The discovery of more ALL-specific antigens could help mitigate immune escape effects and make CAR-T therapy more accessible to patients with different genetic backgrounds.

1.1.3 Exosomes

Exosomes are extracellular microvesicles of 30-150 nm in diameter [1]. While the contents of these vesicles were once considered to be only cellular waste, exosomes are now understood to play a major role in extracellular communication, including cancer metastasis [1].

1.1.3.1 Biological Origin & Properties

Extracellular vesicles can be delineated into two broad categories based on their method of formation: ectosomes and exosomes [11]. As shown in Figure 1-1, exosomes form from endosomes rather than directly from the plasma membrane [11]. Exosomes are abundant in lymph, blood, and urine. An average of around 10^{11} exosomes (as compared to 10^{7} leukocytes) is found in each mL of blood [1,2]. This far-reaching distribution is due in part to exosomes' small size of 30-150 nm [1].

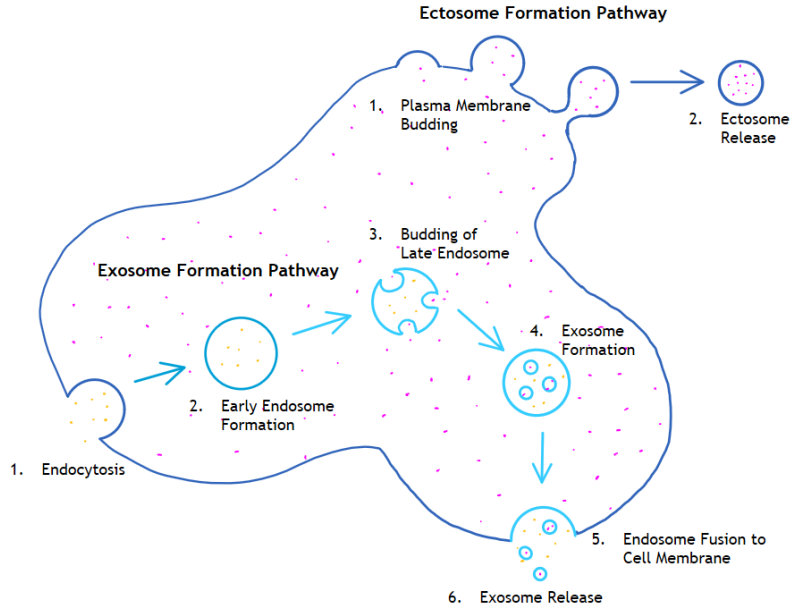

Figure **1-2**: Exosome Formation in Contrast to Ectosome Formation.

Due to their origin within endosomes, exosome membranes express higher levels of endosome-associated tetraspanins such as CD9, CD63, and CD81 [12]. Thus, CD9, CD63, and CD81 are considered pseudo markers for exosomes. The presence of these tetraspanins was monitored throughout experimentation since their expression indicates that exosomes (rather than any other type of extracellular vesicle) were isolated and analyzed.

1.1.3.2 Isolation Principles

Exosomes are unique in their size and origin, so they can be isolated through a variety of different methods. Differential ultracentrifugation (DUC) is the most common means of exosome isolation, since it can yield large volumes of exosomes with few reagents [13]. However, this method is instrument-dependent and contamination prone [13]. Other mainstream exosome isolation methods include chromatography, ultrafiltration, and precipitation-based methods [13]. However, chromatographic methods require many purification steps for low volume yields, ultrafiltration yields the same exosome-sized contaminations as DUC, and precipitation-based methods can lead to membrane fusion [13].

Thus, immunoaffinity capture methods are gaining popularity because they can yield relatively pure exosomes in few steps [13]. These methods utilize knowledge of exosome pseudo markers. Antibodies that bind to these pseudo markers are attached to either stationary surfaces or larger beads, and exosomes are specifically pulled out of solution as their antigens bind to these antibodies [13,14]. In this project, beads were utilized to isolate exosomes due to their specificity and their ability to be visualized via flow cytometry.

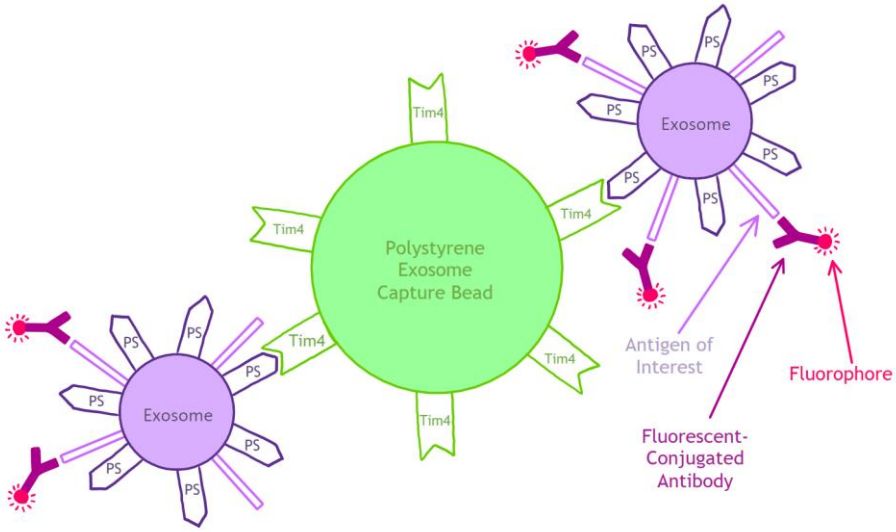

Figure **1-3**: Interaction Between Exosomes and Tim4-Conjugated Beads.

The beads utilized for this project targeted phosphatidyl serine (PS) surface antigens on exosomes [14]. Tim4 receptors on the polystyrene beads bound specifically to the PS ligands on exosomes, effectively isolating the exosomes from the rest of the solution (Figure 1-3) [14]. The beads were magnetic, which facilitated their retrieval, but their primary use was their size of 2.8 μm [14]. Due to their size, the beads were large enough to be processed in the flow cytometer. Thus, fluorescent-conjugated antibodies were added to the bead-exosome complexes, which fluoresced based on the exosomes' relative expression of the antigen of interest (Figure 1-3).

1.1.3.3 Relationship with Cancer Progression & Treatment

Previous research indicates that tumor-derived exosomes can increase chemotherapeutic resistance [15]. Other studies show that cancer-derived exosomes promote cancer growth by upregulating T-regulatory cells and activating macrophages with fibronectin to create a pro-inflammatory environment [16]. Even outside these direct immune effects, tumor exosomes can promote metastasis by activating oncogenic signaling pathways, promoting genetic exchange between cells, and enabling angiogenesis [16]. A T cell migration assay conducted by Farnaz Naeemikia showed that T cells derived from peripheral blood mononuclear cells (PBMC) migrated toward cancer-derived exosomes significantly more than non-cancer-derived exosomes (Figure 1-4). These results indicate significant interaction between healthy T cells and cancer-derived exosomes.

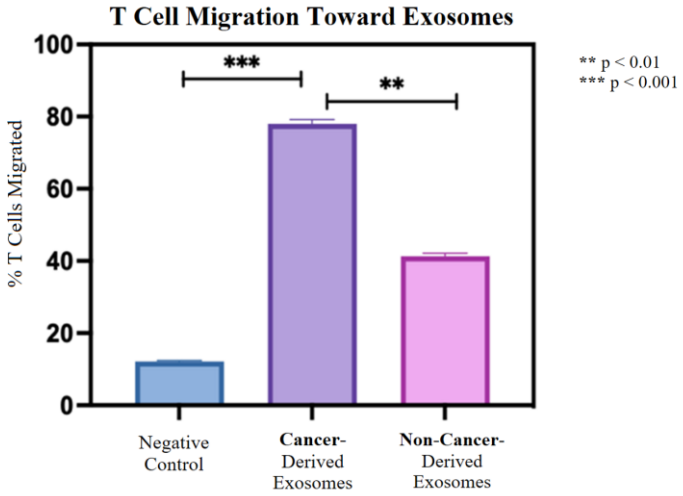

Figure **1-4**: T Cell Migration Toward Exosomes. (Credit: Farnaz Naeemikia)

Cancer-derived exosomes only represent one component of the cancer microenvironment, though. It has been determined that CAR-T cells produce their own exosomes with significant therapeutic effects, but the interaction of cancer-derived exosomes with CAR-T cells and their associated exosomes is not yet well-characterized [3]. Without the conjugated CAR molecule, CD8+ T cells are known to produce exosomes containing cytotoxic proteins like granzymes and perforin [17]. CD4+ T cell-derived exosomes produce cytokines to facilitate extracellular communication, and the number of CD4+ T cell-derived exosomes can significantly vary in correlation with different disease processes [17].

1.2 Objective

The primary objective of this thesis is to characterize exosomes in the cancer microenvironment. This thesis focuses specifically on the relative surface antigen expression between cancer-derived and non-cancer-derived exosomes. Knowledge of the biomarker differences between exosome types is directly useful in distinguishing between cancer-derived and non-cancer-derived exosomes in future *in vitro* research. The further implications of this

research stem from the hypothesis that exosome surface biomarkers could serve as additional targets for CAR-T cell therapy whose concentration gradients could lead CAR-T cells to cancerous cells. Thus, the secondary objective of this thesis is to evaluate whether any discovered biomarker differences between cancer-derived and non-cancer-derived exosomes are significant enough to warrant future *in vivo* research for CAR-T therapy development.

1.3 Thesis Organization Summary

This thesis is comprised of four chapters. The first chapter introduces background information that drives a deeper understanding of the project and its biomedical context. The second chapter details the materials and methods utilized during experimentation. The third chapter describes the experimental results, which show how biomarker expression varied on cancer-derived and non-cancer-derived cell surfaces and exosome surfaces. It also provides a discussion of the implications of these results. The fourth and final chapter summarizes conclusions and outlines future directions informed by this project.

Chapter 2

Materials & Methods

The following materials and methods yielded the results discussed in Chapter 3. Sections 2.1 and 2.2 detail cell culture and cell counting, which were useful in all experiments. The methods detailed in Section 2.3 directly yielded the results shown in Chapter 3.

2.1 Cell Culture

BV-173 and LICO D-23 cell lines were cultured. BV-173 cells are Philadelphia chromosome-positive undifferentiated blast cells derived from a patient with acute leukemia [18]. Thus, these BV-173 cells were cultured to represent acute lymphocytic leukemia cells during future experimentation. LICO D-23 cells are B cells derived from 23 different healthy human cell lines, which were combined because they each shared a clumped cell type [19]. These spontaneously immortalized cells were cultured to represent healthy B cells during future experimentation.

Each cell line was cultured in 15-centimeter tissue treated dishes. The dishes were placed in an incubator with a constant temperature of 37°C and a constant CO_2 concentration of 5%. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Exosome-depleted FBS was used before exosome isolation experiments. Before passaging or freezing, cells and media were pipetted into 15 mL centrifuge tubes and centrifuged for 5 min at 500 relative centrifugal force (RCF) before supernatants were aspirated. Cells were frozen in a -80°C freezer in the culture medium described above with an additional 10% dimethyl sulfoxide (DMSO).

2.2 Cell Counting

After cell cultures were centrifuged and the cells were resuspended in medium, 10 µL of this cell suspension would be pipetted into a microcentrifuge tube. If this suspension was highly concentrated with cells, 20-80 µL of phosphate-buffered saline (PBS) was pipetted into the microcentrifuge tube to dilute the sample. Regardless of whether the solution was diluted, 10 µL of Trypan Blue solution would be added to the cells in the microcentrifuge tube. If a dilution was performed with PBS, the dilution factor was calculated by dividing the final volume of solution in the tube by the initial volume of cell suspension (10 µL).

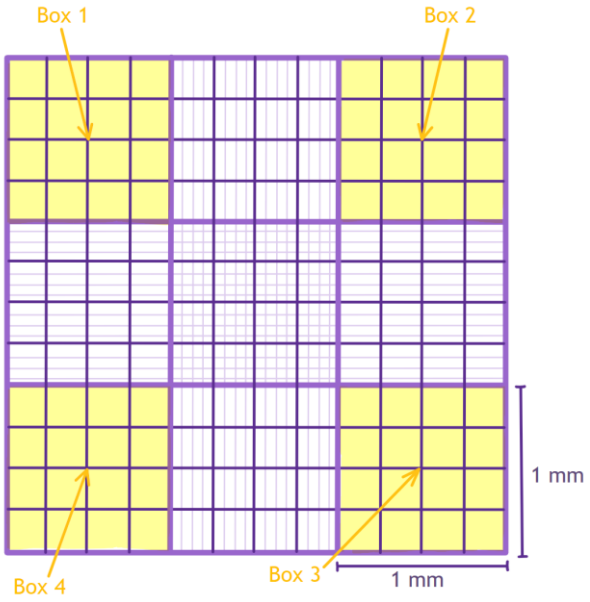

Figure **2-1**: Improved Neubauer Hemocytometer for Cell Counting.

Cells were counted using a hemocytometer with Improved Neubauer ruling [20]. Using 10x magnification, the number of cells in each of the four boxes highlighted in Figure 2-1 was counted and recorded. The average number of cells in each 1 mm^2 box (Box 1, Box 2, Box 3, and Box 4 in Figure 2-1) was multiplied by 10^4 to determine the number of cells in 1 mL of the cell suspension being evaluated [20]. When the initial cell suspension was diluted, the value was also multiplied by the dilution factor to determine the correct cell concentration.

2.3 Flow Cytometry

In order to determine the expression of exosome pseudo markers and three biomarkers of interest on cell surfaces and exosome surfaces, flow cytometry was utilized. Section 2.3.1 explains the cell surface staining process. However, exosomes (with a size of 30-150 nm) are significantly smaller than B cells and undifferentiated blast cells (both with a size of 8-10 μm), so they cannot be detected through traditional flow cytometry methods [1,21]. Thus, Section 2.3.2 explains how exosomes can be collected via Tim4-conjugated beads, which are large enough to be visualized in a flow cytometer with their diameter of 2.8 μm [14]. Section 2.3.3 details the post-staining flow cytometry steps that led to the results for both cell and exosome samples.

2.3.1 Cell Surface Staining

The biomarker expression on the surfaces of both LICO D-23 cells and BV-173 cells was analyzed using the same staining method. A sample of each cell line was created by counting cells and resuspending in PBS to yield a final concentration of 5 million cells/mL. Next, 200 μL of blocking buffer (5% bovine serum albumin (BSA) in PBS) were added to 100 μL of resuspended cells in a 1.5 mL microcentrifuge tube and left to incubate at room temperature for 20 minutes. The blocking buffer was subsequently removed after 5 minutes of centrifugation at 1500 RCF.

When staining with CD19 and CD44, 1 mL of blocking buffer and 2 μL of fluorescent-conjugated antibody solution were mixed in with the cells and left to incubate for 30 minutes. Then, the cells were washed 3 times with PBS and the staining was complete. For CD47, this process was completed first with the primary CD47 antibody, then repeated (including the 3 wash steps) with the secondary antibody before staining was complete.

2.3.2 Exosome Isolation & Staining via PS-Conjugated Beads

Source LICO D-23 cells and BV-173 cells were each seeded at a concentration of 1-2 million cells/mL and cultured in RPMI-1640 medium with 10% exosome-depleted FBS for 48 hours. Next, the cells and supernatants of each cell type were pipetted into separate 10 mL centrifuge tubes. Each underwent a series of centrifugation steps to remove any cells and larger debris that would interfere with the exosome-bead binding process. The initial solution was centrifuged for 5 minutes at 300 RCF, then the supernatant of that step was centrifuged for 20 minutes at 100 RCF, then the supernatant of that step was centrifuged for 30 minutes at 10000 RCF. All centrifugation steps were performed at 4°C.

Next, the exosomes were isolated from this solution using the FUJIFILM PS Capture™ Exosome Flow Cytometry Kit. The supernatant of the 10000 RCF centrifugation was combined with exosome capture beads according to the manufacturer's instructions. For 1 hour, the beads and exosomes were incubated, stirring every 20 minutes. Subsequently, the exosome-bound beads were washed 3 times with a washing buffer + exosome binding enhancer solution, as instructed by the manufacturer. These exosome-bound beads were stored overnight at 4°C.

Once the exosomes were bound to the beads, they were able to be stained in preparation for flow cytometric analysis. When staining with CD19 and CD44, 5 μL of fluorescent-conjugated antibody solution were added to the exosome-bound beads and left to incubate for 1 hour, stirring every 20 minutes. Then, the cells were washed 3 times with the washing buffer + exosome binding enhancer solution and the staining was complete. For CD47, this process was completed first with the primary CD47 antibody, then repeated (including the 3 wash steps) with the secondary antibody to complete the exosome isolation and staining process.

2.3.3 Flow Cytometric Analysis

The fluorescence of each of the stained samples was analyzed using the Attune™ NxT Flow Cytometer. Cell surface and exosome surface experiments were performed separately, so gating was only performed once per experiment using the negative control, unstained cells or exosomes. Figure 2-2A shows the first gating strategy used to define the group of interest, non-aggregated cells or beads. The side scatter vs. forward scatter graph was utilized to find this non-aggregated group of events, and the gate was created so that only the events within the gate were plotted on future graphs. Fluorescence intensity gates were then created, starting just above the highest fluorescence intensity measured in the negative control, as shown in Figure 2-2B.

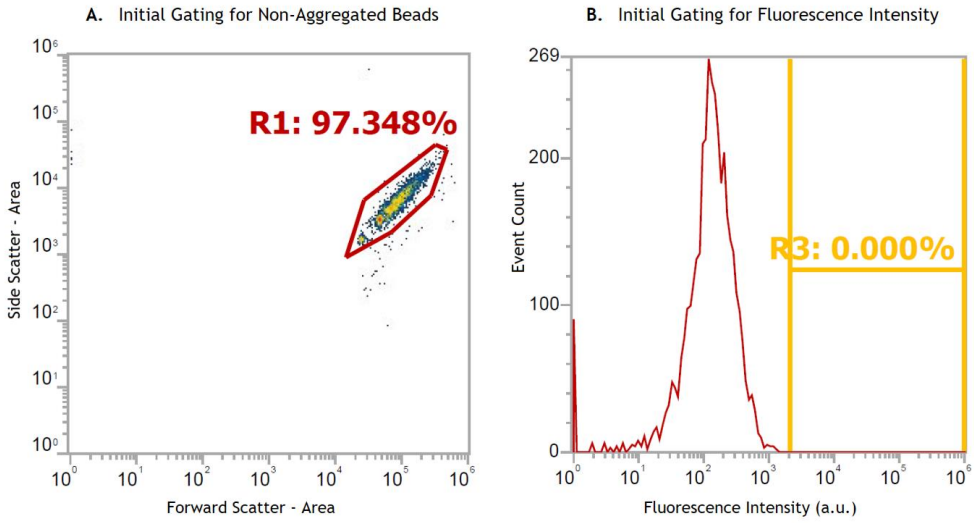

Figure **2-2**: Two Main Gating Strategies for Flow Cytometric Analysis.

When each stained experimental sample was analyzed, the number of events at each fluorescence intensity was compared to its respective negative control. CD9 and CD44 antibodies were conjugated with PE, CD63 & CD19 with FITC, and CD63 with APC. The secondary antibody for CD47 was conjugated with AlexaFluor 546, which has the same properties as PE. Compensation was added to clarify the difference between FITC and PE signals. Mean fluorescence intensity (MFI) was also calculated within the Attune™ NxT software.

Chapter 3

Biomarker Expression

The expression of three selected biomarkers—CD44, CD47, and CD19—was evaluated both on cell surfaces and on exosome surfaces. The differences in biomarker expression were compared between cancerous cells, cancer-derived exosomes, non-cancerous cells, and non-cancer-derived exosomes. Section 3.1 shows the detection of exosome pseudo markers. Sections 3.2, 3.3, and 3.4 investigate the relative expressions on cell and exosome surfaces of CD44, CD47, and CD19, respectively. Section 3.5 explores further points of discussion.

3.1 Exosome Pseudo Marker Expression

Exosome pseudo markers CD9, CD81, and CD63 were utilized to ensure that exosomes were successfully collected and analyzed through flow cytometry [12]. Since each exosome sample expressed these pseudo markers, the other detected biomarkers (CD44, CD47, and CD19) were proven to be on exosome surfaces rather than other extracellular vesicle or cell surfaces (Figure 3-1).

The non-cancerous LICO D-23 cell-derived exosomes consistently yielded lower fluorescence intensities for all three pseudo markers compared to the cancerous BV-173 cell-derived exosomes (Figure 3-1). Even when the source cell concentration was doubled for both cell types, the non-cancer-derived exosomes still expressed lower levels of exosome pseudo markers (Figure 3-1). Thus, these results indicate that LICO D-23 cells produce fewer exosomes than BV-173 cells. This finding is consistent with other recent research showing that cancerous cells release significantly more exosomes than non-cancerous cells [22].

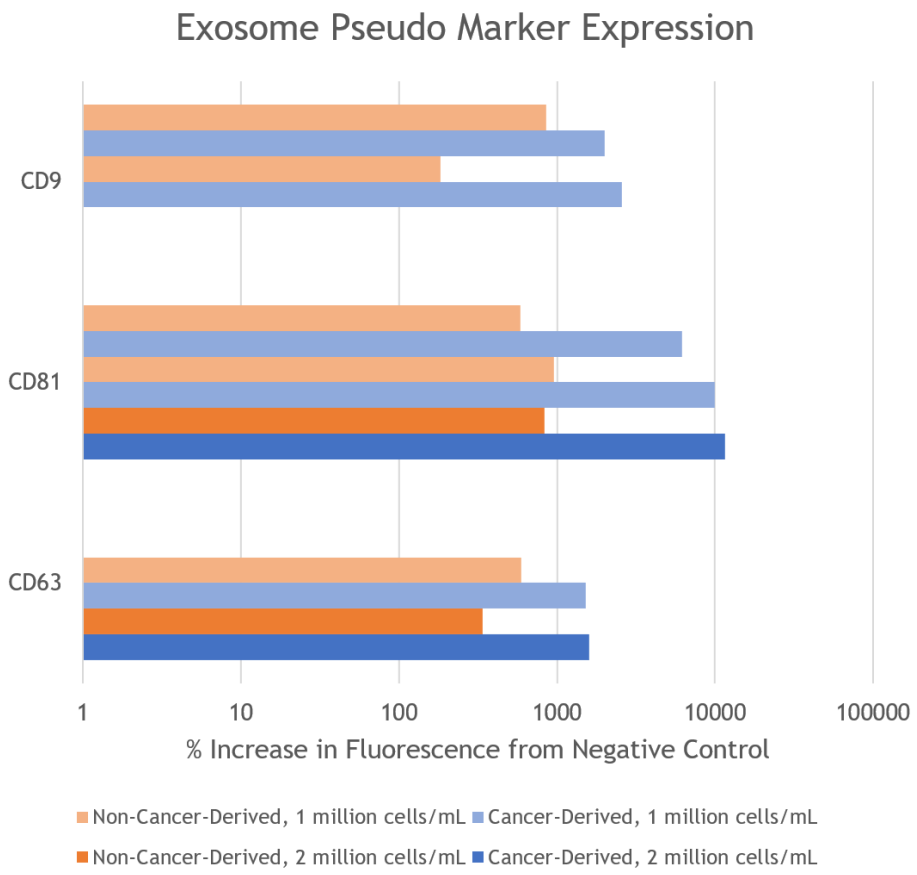

Figure **3-1**: Exosome Pseudo Marker Expression in Bead-Isolated Exosome Samples.

Exosome pseudo marker expression was verified in each exosome surface marker experiment, but the finding that the cancerous cells released more exosomes than the non-cancerous cells was a useful secondary result.

3.2 CD44

Cluster of Differentiation 44 (CD44) is a cell surface marker that is associated with many different cancer types. In ALL particularly, CD44 is associated with an increase in lymphoblast circulation, which contributes to leukemic cell spread [23]. The results of this experimentation showed CD44 as being expressed more highly in the non-cancerous cells and their associated

exosomes than the cancerous cells and their associated exosomes, which was not initially predicted. There are two possible explanations for these unexpected results: time-dependent CD44 upregulation and high CD44 concentration in the LICO D-23 cell line [19,24].

3.2.1 Cell Surface CD44

CD44 was found to be highly expressed on both cancerous and non-cancerous cell surfaces, as detected by flow cytometry. 77% of the analyzed cancerous cells and 86% of the analyzed non-cancerous cells were measured to be CD44-positive as compared to a non-stained negative control (Figure 3-2A). The mean fluorescence intensity for the cancerous cell sample was lower than the mean fluorescence intensity for the non-cancerous cell sample when compared to the same negative controls (Figure 3-2B).

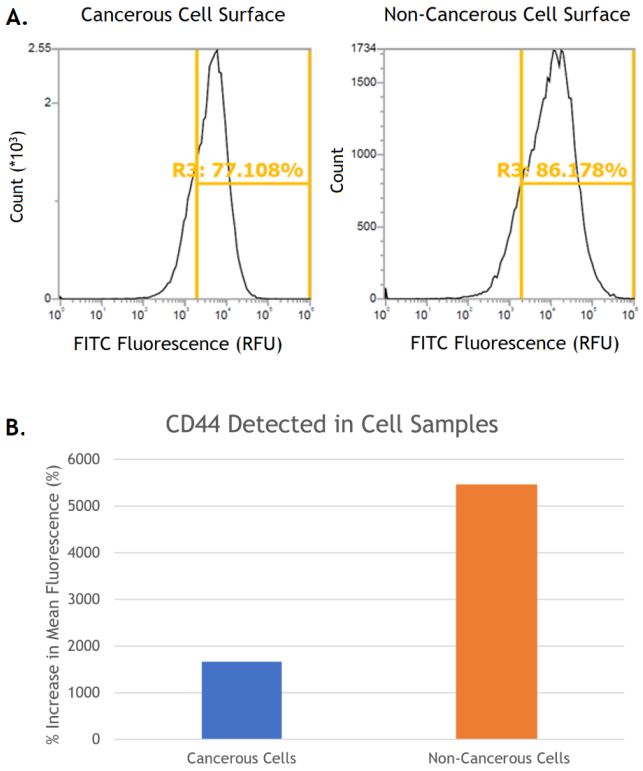

Figure **3-2**: Flow Cytometric Detection of CD44 on Cell Surfaces.

These results were surprising because CD44 is a common cancer biomarker that was predicted to be upregulated in the cancerous cell line [23]. The first possible explanation for this result is that the cancerous cell line, BV-173, simply does not always upregulate CD44 [24]. This is possible because surface levels of CD44 change in response to intracellular and extracellular signaling cascades [24]. However, since the BV-173 cells' CD44 levels were not just equal, but less than, the LICO D-23 cells' CD44 levels, it seems that there is another explanation for this finding. LICO D-23, despite not being a cancerous cell line, could upregulate CD44 compared to BV-173. Since CD44 is known to be present on all types of leukocytes, it is likely that many different B cell lines would have significant levels of CD44 present on their surfaces [25]. One reason CD44 could be upregulated in LICO D-23 specifically, though, is because the LICO D-23 cell line was created from various lines selected specifically for their clumped cell types [19]. CD44 is known to be involved in adhesive cell-cell and cell-matrix interactions, so it could be participating in the clumping of the LICO D-23 cells [25]. This could be responsible for the higher measured levels of CD44 in the LICO D-23 cells as compared to the BV-173 cells.

3.2.2 Exosome Surface CD44

CD44 was found to be expressed more highly on the non-cancer-derived exosome surfaces than the cancer-derived exosome surfaces (Figure 3-3). This result agrees with the finding that CD44 was expressed more highly on the non-cancerous cell surfaces than the cancerous cell surfaces. The difference in fluorescence intensity between the two different types of exosomes was heightened by increasing the exosome source cell concentration from 1 million cells/mL to 2 million cells/mL, which reinforces the validity of the result (Figure 3-3).

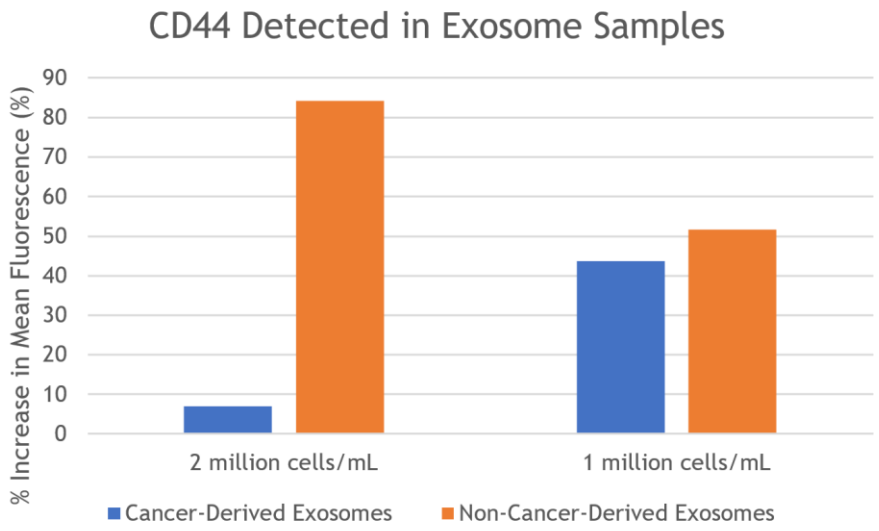

Figure **3-3**: Flow Cytometric Detection of CD44 on Exosome Surfaces.

Since exosomes are derived from endosomes, many of their surface biomarkers are found on the cell surface. However, some of the original surface biomarkers expressed on the microvesicles' membranes are enriched and others are excluded by the Endosomal Sorting Complexes Required for Transport (ESCRTs) [16,26]. Thus, the finding that CD44 is expressed more highly in the non-cancerous-derived exosomes than the cancerous-derived exosomes, just as on their associated cell surfaces, indicates that CD44 is useful for intercellular signaling especially for the LICO D-23 cells. This especially corresponds with the hypothesis that LICO D-23 cells express more CD44 because the protein is associated with the formation of cell clumps, which would be facilitated by intercellular signaling [19,25].

3.3 CD47

Exosome surface CD47 has been shown to increase exosomes' immune escape ability [27]. Additionally, anti-CD47 antibodies have been found to be therapeutic in treating ALL in humans [28]. Thus, the levels of CD47 expressed on cancer-derived and non-cancer-derived

exosomes were measured via flow cytometry. Contrary to hypothesized, CD47 was expressed more highly in non-cancer-derived exosomes than cancer-derived exosomes, even though CD47 was overexpressed on cancerous cell surfaces as expected.

3.3.1 Cell Surface CD47

CD47 was found to be highly expressed on both cancerous and non-cancerous cell surfaces. Of the analyzed cancerous cells, 91% were measured to be CD47-positive as compared to a non-stained negative control (Figure 4-2A). Only 52% of the analyzed non-cancerous cells were measured to be CD47-positive compared to an analogous negative control (Figure 4-2A). Thus, the mean fluorescence intensity for the cancerous cell sample was higher than the mean fluorescence intensity for the non-cancerous cell sample (Figure 3-4B).

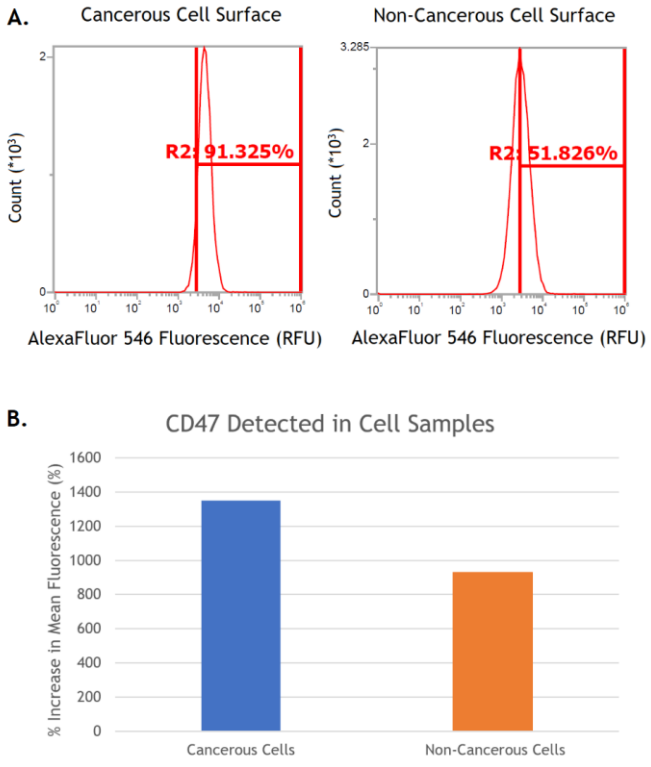

Figure **3-4**: Flow Cytometric Detection of CD47 on Cell Surfaces.

These results aligned with the expectation that CD47 would be upregulated in cancerous cells due to its role in the immune escape mechanism. CD47 is additionally known to inhibit other leukocytes' phagocytosis pathways, to induce apoptosis of other lymphocytes, and to block monocytes' cytokine release [27]. Notably, the fluorescence peaks had a smaller spread than the CD44 and CD19 cells' peaks. This indicates low variability in expression between cells of the same type. Overall, the clear overexpression of CD47 on cancerous cell surfaces in comparison to non-cancerous cell surfaces indicates that CD47 is a potential target for CAR-T therapy that should be explored through further research.

3.3.2 Exosome Surface CD47

CD47 was expressed more highly on non-cancer-derived exosomes than cancer-derived exosomes (Figure 3-5). This trend is the opposite of the trend in the cell surface samples. The difference in CD47 expression between exosome types was slightly more significant when the source cell concentration was increased, just as with the CD44 samples (Figure 3-5).

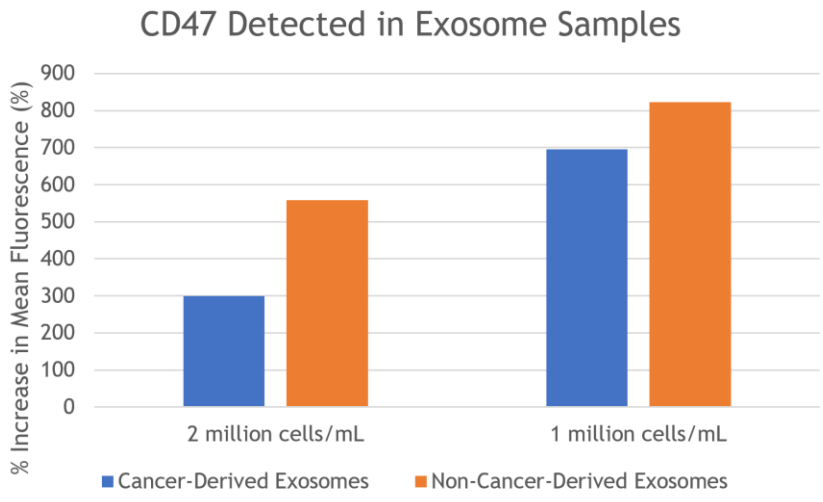

Figure **3-5**: Flow Cytometric Detection of CD47 on Exosome Surfaces.

CD47 is clearly highly expressed in both cancer-derived and non-cancer-derived exosomes. The percent increase in fluorescence due to CD47 was higher than that of CD19 and CD44, indicating that CD47 was expressed highly on exosomes of both origins. This is feasible because CD47 is known to play a role in adaptive immunity and is a common surface marker on B cells [29]. Although these results were unexpected, they could indicate that the non-cancerous cells were specifically producing exosomes with higher CD47 expression than their average cell membrane expression. However, there is also a chance of error in these results. For example, the washing of the secondary antibody could have been insufficient in these trials (because CD44 and CD19 did not require secondary antibody), causing a higher apparent concentration of CD47. Future research is needed to validate or refute these results.

3.4 CD19

Cluster of Differentiation 19 (CD19) is a surface antigen known to be highly expressed on malignant B cells due to its role in B cell development [4]. CD19 is also one of the earliest CAR targets to have been utilized in CAR-T cell therapeutics [4]. As predicted, CD19 was more highly expressed in both the cancer-derived BV-173 cells and their associated exosomes compared to the non-cancerous control cells.

3.4.1 Cell Surface CD19

Flow cytometric analysis showed that CD19 is highly expressed on both cancerous and non-cancerous cell surfaces. 94% of the analyzed cancerous cells and 85% of the analyzed non-cancerous cells were measured to be CD19-positive as compared to a non-stained negative control (Figure 3-6A). The mean fluorescence intensity for the cancerous cell sample was higher

than the mean fluorescence intensity for the non-cancerous cell sample, as expected (Figure 3-6B).

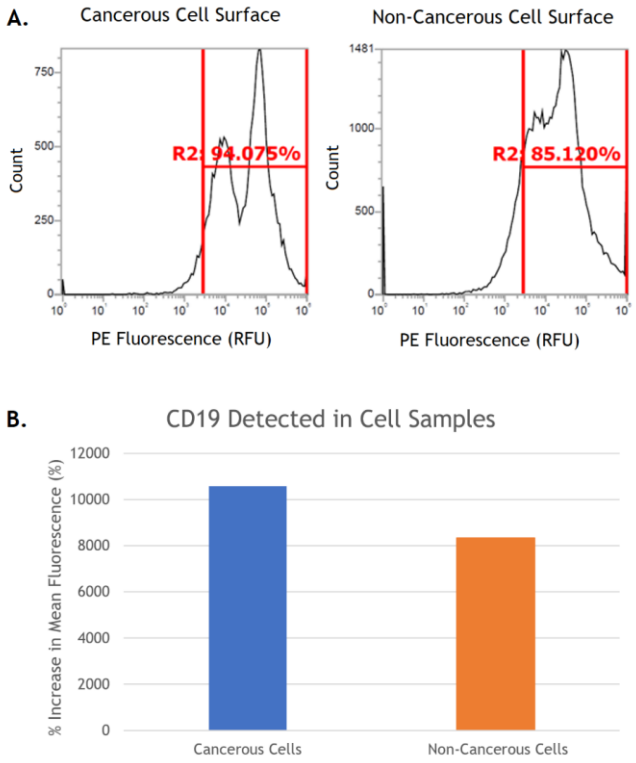

Figure **3-6**: Flow Cytometric Detection of CD19 on Cell Surfaces.

The multi-peak distribution in Figure 3-6A was unexpected. One explanation for this finding is that a specific subset of the BV-173 cells expressed CD19 more highly than another subset. CD19's active participation in a host of signaling pathways—including Src, Ras, Abl, PI3K, and MHC class II-mediated signaling—indicates that its surface expression can be modulated in response to changing intracellular states [30].

Overall, these results indicate that both cancerous and non-cancerous cells express CD19, but that cancerous cells express CD19 more highly than non-cancerous cells. The cancerous cells' upregulation of CD19 compared to other cell types is also well documented, which is why it was selected as a CAR-T therapy target [4]. CD19 is also a reliable marker for all B cells, though, since non-cancerous B cells express it in increasing concentrations across their stages of

development [30]. Since BV-173 cells maintain the phenotype of undifferentiated blast cells, the upregulated CD19 is more significant because if the cells were not cancerous, they would be predicted to have lower CD19 expression compared to the more developed LICO D-23 cells [18,30].

3.4.2 Exosome Surface CD19

In agreement with the cell surface findings, CD19 was expressed more highly on the cancer-derived exosome surfaces than the non-cancer-derived exosome surfaces (Figure 3-7). As observed strongly with CD44 and weakly with CD47, the difference in antigen expression between the two exosome samples increased with an increase in source cell concentration from 1 million cells/mL to 2 million cells/mL (Figure 3-3). The agreement of the results yielded from two different experimental conditions reinforces the overarching concept of CD19 upregulation in cancerous exosomes.

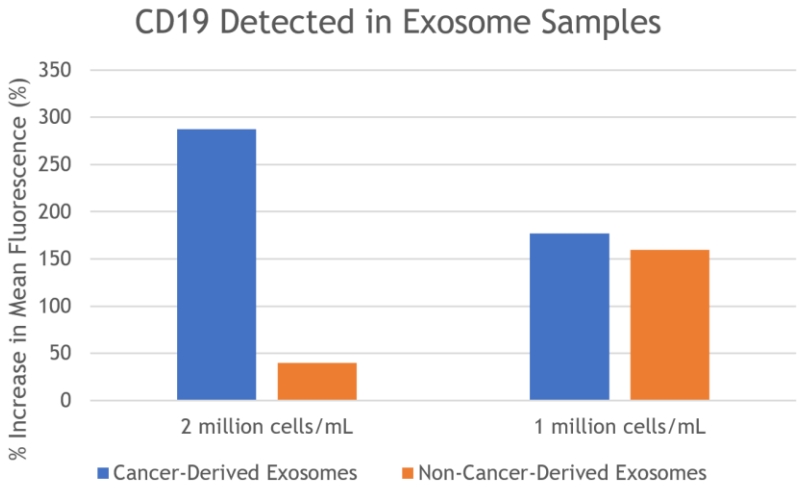

Figure **3-7**: Flow Cytometric Detection of CD19 on Exosome Surfaces.

While elevated cell surface expression of CD19 is a well-known biomarker for ALL, much less is known about the relative expression of exosomes on exosome surfaces. The higher

expression of CD19 on cancer-derived exosomes in comparison to non-cancer-derived exosomes could be due to random chance in the formation of exosomes from endosomes, but it is possible that ESCRT-mediated exosome generation also played a role in this CD19 upregulation [16,26]. Since CD19 facilitates B cell expansion in a complex of other tetraspanins, its mobilization on exosomes could lead to a wider radius of cancerous cell proliferation [30]. Additionally, these results indicate that exosome-based detection mechanisms could enable more accurate comparisons between cancerous and non-cancerous microenvironments.

3.5 Discussion

The exosome pseudo marker results indicate that the cancerous cells produced more exosomes than the non-cancerous cells. This effect could be favorable for CAR-T therapy if the CAR target is highly expressed on exosomes. In this case, the T cells could theoretically follow the exosome concentration gradient to the source cell [31]. Current research has shown that exosomes themselves are useful vehicles of targeted drug delivery because of their high binding specificities [32]. Thus, increased concentration of cancer-derived exosomes compared to non-cancer-derived exosomes could be useful in assisting CAR-T cells in locating the cancer-derived exosomes' cancerous cells of origin. Exosome secretion would not need to be artificially enhanced because the known methods of enhancing exosome secretion *in vivo* include hypoxia, low pH, and other conditions already associated with the tumor microenvironment [33]. Applying these methods *in vivo* would increase treatment toxicity more than increasing the CAR-T cells' ability to bind to tumor-specific antigens in the cancer microenvironment [33].

However, a higher concentration of cancer-derived exosomes also impairs all cancer therapeutics because cancer-derived exosomes are known to promote cancer growth and inhibit immune function [15,16,22]. Thus, an alternative cancer therapy technique could focus on

inhibiting exosome expression from cancerous cells. There are many currently available compounds that inhibit the release of extracellular vesicles, including calpeptin [34]. Treatments utilizing this exosome release-inhibiting mechanism would likely inhibit cancer cell communication more significantly than healthy cell communication because cancerous cells release more exosomes [22,34].

It is crucial to contextualize the cancer-derived exosomes, though. These exosomes are just one element of the cancer microenvironment, and they are constantly interacting with other exosomes [35]. The interactions between T cell-derived exosomes and cancer-derived exosomes are not well characterized yet, but they must be studied before broad exosome-affecting methods like exosome release inhibition are employed. Coculture of T cells and cancerous cells is critical, as it can reveal the extent of the synergistic effects between these two different exosome types. Current research indicates that exosomes interact with each other physically and through chemical signaling to relay messages [35]. For this reason, more must be known about the interaction between exosomes of cancerous and healthy immune origin and whether they are agonistic or antagonistic—which likely varies with different intracellular and extracellular conditions—with respect to each other.

Chapter 4

Conclusions & Future Directions

4.1 Conclusions

Exosomes were isolated and detected via flow cytometry, as evidenced by the detection of exosome pseudo markers in each exosome sample. Additionally, these exosome pseudo markers, which are highly expressed on exosomes of all origins, were detected at higher levels in the cancer-derived exosome samples compared to the non-cancer-derived exosome samples [12]. This indicates that the cancerous cells released more exosomes than the non-cancerous cells, an observation that agrees with recent research and has significant clinical implications, especially in cancer metastasis [22]. Additionally, a higher source cell concentration yielded more significant differences in exosome biomarker expression (for CD44, CD47, and CD19) between the two exosome types. This informs future protocols by demonstrating the concentration dependence of these flow cytometric assays.

CD44 was found to be more highly expressed in both non-cancerous cells and non-cancer-derived exosomes than cancerous cells and cancer-derived exosomes. One explanation for these results is that CD44 could be upregulated in LICO D-23 because the cell adhesion-promoting properties of CD44 could be participating in the characteristic clumping of the LICO D-23 cells [25]. These results show agreement between the cell surface and the exosome surface, which implies increased validity and implies that CD44 is useful in extracellular communication, at least between LICO D-23 cells. Future research should be conducted with other non-cancerous cell types to elucidate the cause of these results.

CD47 was upregulated on cancerous cell surfaces compared to non-cancerous cell surfaces, as expected. This finding is reinforced by the existing knowledge of CD47's role in immune escape from existing cancer therapies [27]. However, an unexpected result was also encountered: CD47 was downregulated on cancer-derived exosome surfaces compared to non-cancer-derived exosome surfaces. While there are many explanations for this result, the leading explanations are either a secondary antibody washing error or a selection of CD47-upregulated regions during the formation of LICO D-23 exosomes. Due to its clear upregulation on cancer cell surfaces, CD47 is still recommended for future research as a CAR-T therapy target.

CD19 was found to be more highly expressed in both cancerous cells and cancer-derived exosomes. These results were in agreement with the current research indicating that CD19 was upregulated in cancerous cells, and they also validate the current CAR-T therapies that target CD19 antigens [4,18,30]. With the higher source cell concentration, the difference in CD19 expression between cancer-derived and non-cancer-derived exosomes was more significant than that between the cancerous and non-cancerous cells. This indicates that exosome-based CD19 detection could be more sensitive when comparing cancerous vs. non-cancerous B cells than a cell-based biomarker detection test.

4.2 Future Directions

The knowledge of the antigen differences between cell types developed in this thesis will be useful in distinguishing between cancer-derived and non-cancer-derived exosomes in future research. For future *in vitro* research, more cancerous exosome biomarkers should be identified to create a distinct profile of cancer-derived exosomes that contrasts with non-cancer-derived exosomes. Once this profile is completed, it can be used to identify exosomes' cells of origin when they are isolated from coculture experiments. For example, if cancerous and non-cancerous

B cells are cocultured, exosomes could be isolated and identified based on their cells of origin. Studying these exosomes and their contents will lead to significant advancements in the understanding of the cancer microenvironment and the role exosomes play in cancer development and progression.

Additionally, biomarkers should be identified to distinguish T cell-derived exosomes from B cell-derived exosomes so that T cells and cancerous cells can be cocultured. T cell-derived exosomes are known to have therapeutic efficacy, but there is little knowledge of how cancer-derived exosomes interact with T cell-derived exosomes [17]. Exosomes are known to create chemotherapeutic resistance, so it is hypothesized that they could play an active role in resistance to immunotherapy, whether through immune escape or another mechanism [15,27]. Since Farnaz Naeemikia found that T cells migrated significantly more towards cancerous exosomes than non-cancerous exosomes, one future plan is to extend this work by co-culturing T cells with cancer cells in the presence of additional exosomes. If the addition of cancer-derived exosomes significantly impacts the killing capability of the T cells, the hypothesis of an active exosome response would be supported. In this case, future CAR-T therapies could focus on limiting cancerous T cell interactions with exosomes. Analysis of this killing assay would be combined with that of the biomarkers to guide future CAR-T research and development.

References

[1] Hu, C., Jiang, W., Lv, M., Fan, S., Lu, Y., Wu, Q., & Pi, J. (2022). Potentiality of Exosomal Proteins as Novel Cancer Biomarkers for Liquid Biopsy. *Frontiers in Immunology*, *13*, 2356. https://doi.org/10.3389/FIMMU.2022.792046/BIBTEX

[2] Ruggiero, C., Metter, E. J., Cherubini, A., Maggio, M., Sen, R., Najjar, S. S., Windham, G. B., Ble, A., Senin, U., & Ferrucci, L. (2007). White Blood Cell Count and Mortality in the Baltimore Longitudinal Study of Aging. *Journal of the American College of Cardiology*, *49*(18), 1841–1850. https://doi.org/10.1016/J.JACC.2007.01.076

[3] Wang, C., Fu, W., Lei, C., & Hu, S. (2022). Generation and functional characterization of CAR exosomes. *Methods in Cell Biology*, *167*, 13–21. https://doi.org/10.1016/BS.MCB.2021.06.01

[4] Sadelain, M. (2015). CAR therapy: the CD19 paradigm. *The Journal of Clinical Investigation*, *15*(9), 3392–3400. https://doi.org/10.72/JCI80010

[5] Bradshaw, A., Wickremsekera, A., Tan, S. T., Peng, L., Davis, P. F., & Itinteang, T. (2016). Cancer Stem Cell Hierarchy in Glioblastoma Multiforme. *Frontiers in Surgery*, 3, 21. https://doi.org/10.3389/FSURG.2016.00021/BIBTEX

[6] Lightfoot, T., Smith, A., & Roman, E. (2022). Leukemia. *International Encyclopedia of Public Health*, 410–418. https://doi.org/10.1016/B978-0-12-803678-5.00253-8

[7] Sheykhhasan, M., Manoochehri, H., & Dama, P. (2022). Use of CAR T-cell for acute lymphoblastic leukemia (ALL) treatment: a review study. *Cancer Gene Therapy 2021*, *29*(8), 1080–1096. https://doi.org/10.1038/s4317-021-00418-1

[8] Liu-Dumlao, T., Kantarjian, H., Thomas, D. A., O'Brien, S., & Ravandi, F. (2012). Philadelphia-Positive Acute Lymphoblastic Leukemia: Current Treatment Options. *Current Oncology Reports*, *14*(5), 387. https://doi.org/10.1007/S912-012-0247-7

[9] Luginbuehl, V., Abraham, E., Kovar, K., Flaaten, R., & Müller, A. M. S. (2022). Better by design: What to expect from novel CAR-engineered cell therapies? *Biotechnology Advances*, *58*, 107917. https://doi.org/10.1016/J.BIOTECHADV.2022.107917

[10] Nie, Y., Lu, W., Chen, D., Tu, H., Guo, Z., Zhou, X., Li, M., Tu, S., & Li, Y. (2020). Mechanisms underlying CD19-positive ALL relapse after anti-CD19 CAR T cell therapy and associated strategies. *Biomarker Research*, *8*(1), 1–17. https://doi.org/10.86/S40364-020-00197-1

[11] Gurung, S., Perocheau, D., Touramanidou, L., & Baruteau, J. (2021). The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Communication and Signaling 2021*, *19*(1), 1–19. https://doi.org/10.86/S1964-021-00730-1

[12] Khushman, M., Bhardwaj, A., Patel, G. K., Laurini, J. A., Roveda, K., Tan, M. C., Patton, M. C., Singh, S., Taylor, W., & Singh, A. P. (2017). Exosomal Markers (CD63 and CD9) Expression Pattern Using Immunohistochemistry in Resected Malignant and Non-malignant Pancreatic Specimens. *Pancreas, 46*(6), 782. https://doi.org/10.1097/MPA.0000000000000847

[13] Shirejini, S. Z., & Inci, F. (2022). The Yin and Yang of exosome isolation methods: conventional practice, microfluidics, and commercial kits. *Biotechnology Advances, 54*, 107814. https://doi.org/10.1016/J.BIOTECHADV.2021.107814

[14] Nakai, W., Yoshida, T., Diez, D., Miyatake, Y., Nishibu, T., Imawaka, N., Naruse, K., Sadamura, Y., & Hanayama, R. (2016). A novel affinity-based method for the isolation of highly purified extracellular vesicles. *Scientific Reports 2016*, *6*(1), 1–11. https://doi.org/10.1038/srep33935

[15] Zhong, Y., Li, H., Li, P., Chen, Y., Zhang, M., Yuan, Z., Zhang, Y., Xu, Z., Luo, G., Fang, Y., & Li, X. (2021). Exosomes: A New Pathway for Cancer Drug Resistance. *Frontiers in Oncology*, *11*, 3846. https://doi.org/10.3389/FONC.2021.743556/BIBTEX

[16] Taylor, D. D., & Gercel-Taylor, C. (2011). Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. *Seminars in Immunopathology*, *33*(5), 441–454. https://doi.org/10.1007/S00281-010-0234-8

[17] Shao, Y., Pan, X., & Fu, R. (2021). Role and Function of T Cell-Derived Exosomes and Their Therapeutic Value. *Mediators of Inflammation, 2021*. https://doi.org/10.55/2021/8481013

[18] L. Pegoraro, L. Matera, J. Ritz, A. Levis, A. Palumbo, & G. Biagini. (1983). Establishment of a Ph1-positive human cell line (BV173). *Journal of the National Cancer Institute*, *70*(3), 447–453. https://europepmc.org/article/med/6572735

[19] Hanna, M., Haspel, M. v., & Hoover, H. C. (1987). *Tumor associated monoclonal antibodies derived from human B-cell line* (Patent No. US4997762A). https://patents.google.com/patent/US4997762

[20] Thermo Fisher Scientific, *Counting Cells in a Hemocytometer*. (2006). Retrieved March 2023, from https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/counting-cells-in-a-hemacytometer.html

[21] Cano, R. L. E., & Lopera, H. D. E. (2013). *Introduction to T and B lymphocytes*. https://www.ncbi.nlm.nih.gov/books/NBK459471/

[22] Liu, S. L., Sun, P., Li, Y., Liu, S. S., & Lu, Y. (2019). Exosomes as critical mediators of cell-to-cell communication in cancer pathogenesis and their potential clinical application. *Translational Cancer Research, 8*(1), 298–311. https://doi.org/10.21037/TCR.2019.01.03

[23] Cavalcanti Júnior, G. B., Savino, W., & Pombo-de-Oliveira, M. S. (1994). CD44 expression in T-cell lymphoblastic leukemia. *Revista Brasileira de Pesquisas Medicas e Biologicas*, *27*(9), 2259–2266. https://europepmc.org/article/med/7540475

[24] Xu, H., Niu, M., Yuan, X., Wu, K., & Liu, A. (2020). CD44 as a tumor biomarker and therapeutic target. *Experimental Hematology & Oncology 2020 9:1*, *9*(1), 1–14. https://doi.org/10.86/S40164-020-00192-0

[25] Naor, D. (1998). CD44. *Encyclopedia of Immunology*, 488–491. https://doi.org/10.1006/RWEI.1999.018

[26] Larios, J., Mercier, V., Roux, A., & Gruenberg, J. (2020). ALIX- And ESCRT-III-dependent sorting of tetraspanins to exosomes. *Journal of Cell Biology*, *219*(3). https://doi.org/10.1083/JCB.2019043/23723/

[27] Ben, X. Y., Wang, Y. R., Zheng, H. H., Li, D. X., Ren, R., Ni, P. L., Zhang, H. Y., Feng, R. J., Li, Y. Q., Li, Q. F., & Yi, X. N. (2022). Construction of Exosomes that Overexpress CD47 and Evaluation of Their Immune Escape. *Frontiers in Bioengineering and Biotechnology*, *10*, 67. https://doi.org/10.3389/FBIOE.2022.936951

[28] Chao, M. P., Alizadeh, A. A., Tang, C., Jan, M., Weissman-Tsukamoto, R., Zhao, F., Park, C. Y., Weissman, I. L., & Majeti, R. (2011). Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia. *Cancer Research*, *71*(4), 274–284. https://doi.org/10.58/0008-5472.CAN-10-2238

[29] Cham, L. B., Adomati, T., Li, F., Ali, M., & Lang, K. S. (2020). CD47 as a Potential Target to Therapy for Infectious Diseases. *Antibodies*, *9*(3), 44. https://doi.org/10.3390/ANTIB9030044

[30] Wang, K., Wei, G., & Liu, D. (2012). CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. *Experimental Hematology & Oncology 2012, 1*(1), 1–7. https://doi.org/10.86/2162-3619-1-36

[31] Lee, H., Park, H., Noh, G. J., & Lee, E. S. (2018). pH-responsive hyaluronate-anchored extracellular vesicles to promote tumor-targeted drug delivery. *Carbohydrate Polymers, 202*, 323–333. https://doi.org/10.1016/J.CARBPOL.2018.08.31

[32] Liang, Y., Duan, L., Lu, J., & Xia, J. (2021). Engineering exosomes for targeted drug delivery. *Theranostics, 11*(7), 3183. https://doi.org/10.7150/THNO.52570

[33] Debbi, L., Guo, S., Safina, D., & Levenberg, S. (2022). Boosting extracellular vesicle secretion. *Biotechnology Advances, 59*, 107983. https://doi.org/10.1016/J.BIOTECHADV.2022.107983

[34] Catalano, M., & O'Driscoll, L. (2020). Inhibiting extracellular vesicles formation and release: a review of EV inhibitors. *Journal of Extracellular Vesicles, 9*(1). https://doi.org/10.1080/2002078.2019.1703244

[35] Beit-Yannai, E., Tabak, S., & Stamer, W. D. (2018). Physical exosome:exosome interactions. *Journal of Cellular and Molecular Medicine, 22*(3), 2001–2006. https://doi.org/10.11/JCMM.2479

Academic Vitae
Lauren Spadt

Education

The Pennsylvania State University, University Park, PA
Schreyer Honors College

Bachelor of Science in Biomedical Engineering
May 2023

Research & Teaching Experience

Undergraduate Research Assistant, Penn State University, November 2019 – May 2023

- Investigated exosomes in the cancer microenvironment through cell migration essays, exosome isolation via ultracentrifugation and phosphatidyl serine (PS) beads, and flow cytometry.

Women in Engineering Program Academic Facilitator, Penn State University, January 2021 – May 2023

- Organized and planned weekly review sessions for a group of undergraduates. Led reviews based on students' unique learning styles. Facilitated communication and answered questions between sessions.

Honors Chemical Principles I Grader, Penn State University, August 2020 – December 2020

- Evaluated a class of 40 students' weekly homework assignments and weekly quizzes for accuracy. Presented detailed, personalized feedback on each assignment that enabled students to learn from their mistakes.

Clinical Experience

Emergency Medical Technician, Keystone Quality Transport, May 2022 – August 2022

- Providing basic life support care and safely transporting patients with mobility-limiting medical conditions.

Medical Assistant, Pennsylvania Pain and Spine Institute, May 2021 – August 2021

- Connected patients with personalized resources and communicated with external organizations to improve care.

College Student Summer Volunteer, Doylestown Hospital, May 2021 – August 2021

- Shadowed and assisted medical professionals caring for patients in Doylestown Hospital's medical-surgical unit.

Leadership & Service

- President, Access Club at Penn State 2019-2023
- Vice President, Alpha Epsilon Delta Health Preprofessional Honor Society 2019-2023
- President, Presbyterian Student Fellowship 2019-2023
- Big Brothers Big Sisters Mentor, Centre County Youth Services Bureau 2020-2023
- Volunteer, YMCA of Centre County's Anti-Hunger Program 2022-2023

Honors & Awards

- Biomedical Engineering Student Marshal, Penn State University May 2023
- Evan Pugh Scholar Senior Award, Penn State University May 2022
- Remote Innovation Grant, Penn State Student Engagement Network May 2021
- President Sparks Award, Penn State University May 2021
- President's Freshman Award, Penn State University May 2020