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Options for Low-Cost Manufacturing and Safer Cell Therapies

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OPTIONS FOR LOW-COST MANUFACTURING AND SAFER CELL THERAPIES

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ABSTRACT

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The proposed work aims to overcome the economical and feasibility-related limitations of the chimeric antigen receptor therapies by developing an artificial cell signaling pathway whose design transforms K562 cells into *in vivo* living vectors to synthesize therapeutic proteins upon engaging diseased cells in the treatment of ovarian cancer. There are various advantages of using K562 cells throughout this process. First, Food and Drug Administration approves the reinfusion of K562 cells into patients' bodies. Second, K562 cells are more affordable than T lymphocytes, and finally, these cells can be easily manipulated with any desired genetic material and can keep the expression of engineered genes stable. However, they do not express a chemokine receptor, a type of cytokine controlling the traffic of the immune cells to a desired site of the body. Therefore, these cells must be manipulated with chemokine receptors to enable them to migrate directly towards the tumor microenvironment to prevent harm to the healthy parts of the body. For the manipulation of all cells used in this study, lentiviruses were produced to transduce them. Nanoluc luciferase reporter was used as an effector protein to evaluate whether K562 cells can synthesize these enzymes *in situ* upon interacting with diseased cells. K562 cells lack necessary molecules that would drive them to form an immunological synapse to produce engineered proteins. As a result, they were not able to produce the Nanoluc enzyme. On the other hand, this study shows that the chemokine system presents an excellent potential for immunotherapies, and it may help prevent damage to healthy tissue.

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ALL - acute lymphocytic leukemia
APC - antigen presenting cell
CAR - chimeric antigen receptor
CLL - chronic lymphocytic leukemia
CRS - cytokine release syndrome
DLBCL - diffuse large B-cell lymphoma
FRα - folate receptor alpha
HLA - human leukocyte antigen
ICAM-1 - intercellular adhesion molecule-1
IL-2 - interleukin 2
IS - immunological synapse
MCL - mantle cell lymphoma
MHC - major histocompatibility complex
NHL - non-Hodgkin lymphoma
SLL - small lymphocytic lymphoma
SMAC - supramolecular activation cluster
TCR - T cell receptor
TIL - tumor-infiltrating lymphocyte
TRUCK - T cells redirected for antigen-unrestricted cytokine-initiated killing
WHO - World health organization

1 Introduction

The oldest description of cancer dates back to about 3000 BC and is found in ancient Egyptian manuscripts. Cancer is the second leading cause of death, behind heart diseases, in the United States, and the World Health Organization (WHO) attributes an estimated 9.6 million deaths to cancer worldwide (1). Research into the risk factors for developing cancer is considered a priority. Several factors have been identified: cancer can be hereditary, or it can occur because of 'lifestyle factors, including but not limited to tobacco smoke, alcohol consumption, and excessive sun or radiation exposure (2).

The treatments currently available for cancer include surgery, chemotherapy, radiation therapy, and immunotherapy. The selection of treatment is dependent on the type of cancer and its location, as well as how advanced it is. Some patients may receive only one type of therapy, while others receive a combination simultaneously or in sequence. The treatment goal is to eradicate as many tumor cells as possible while minimizing the harm to the surrounding healthy cells. However, all treatments have potential disadvantages and have the potential to cause harm. For example, chemotherapy, radiation therapy, and surgery may damage nearby tissues or may be unable to kill all the cancerous cells. Thus, it should be noted that none of these therapies may be able to cure cancer completely (3).

Immunotherapy is an emerging area of cancer research that shows tremendous advantages in relation to increasing survival rates and the maintenance of patients' life quality when compared to the other types of treatments mentioned above (4). Immunotherapy is a biological therapy that teaches the body's immune system to fight

cancer. There are two lines of defense of the body against foreign substances: innate immunity and adaptive immunity. Innate immunity is the first line of defense and is present from birth, protecting the body from infections even before they start. Most infections can be stopped by innate immunity. However, if they cannot, the second line of defense, adaptive immunity, becomes active. As such, lymphocytes, the key elements of adaptive immunity, would recognize pathogens with their cell surface antigens and proliferate to clear the body of foreign substances (5,6). Lymphocytes are mainly divided into T lymphocytes and B lymphocytes. While B lymphocytes are programmed to produce antibodies, T lymphocytes are programmed to recognize antigens and respond to them (7). T lymphocytes have T cell receptors (TCRs) on their cell surface that would allow them to engage the major histocompatibility complex (MHC) on the target diseased cells. MHC is a genetic system that binds peptide fragments of pathogens and expresses them on the cell surface to be recognized and destroyed by immune cells. However, many cancer cells do not express MHCs on their cell surface as a result of an evolutionary mechanism, avoiding immune cells and spreading to other parts of the body (6,8). To overcome this limitation, scientists manipulate patients' T cells with a protein called chimeric antigen receptor (CAR). CARs enable T cells to bind cancer cells even though they don't have MHCs on their surface (9).

In CAR T cell therapy, a patient's white blood cells are collected, and the T cells are separated. These T cells are then genetically engineered in a laboratory to express CARs on their cell surface and are then grown into the hundreds of millions to be given to the patient by infusion. The CARs enable T cells to bind to the antigens on cancer cells and

kill them (10). Since each cancer type has its specific antigen, CARs must be specific and generated for each cancer type respectively to attach to the different receptors of different cancer cells. CAR T cell therapy is a process in which each patient's immune cells are meticulously treated and engineered to express specific receptors depending on the cancer type. However, Primary T cells are highly challenging to grow in the laboratory as they need to be activated first. Given these challenges, CAR T cell therapies are costly and not affordable for many cancer patients, even though there are two approved CAR T cell therapies by Food and Drug Administration (FDA). The two treatments, Kymriah and Yescarta, cost $475,000 and $373,000, respectively (11). As a result of being very expensive, a cheaper alternative to CAR T cell therapy must be found to provide more affordable and accessible treatments for cancer patients.

Further to high treatment costs, current protein-based cancer therapies have side effects, such as systemic toxicity, and the dose-response relationship varies from patient to patient. To overcome these limitations, T cell biofactory technology represents a great potential to synthesize and deliver appropriate amounts of therapeutic proteins for cell-based diseases like cancer, viral infections, and autoimmune diseases. T cell biofactory technology refers to developing an artificial cell-signaling pathway whose design transforms a T cell into a living vector that would synthesize engineered therapeutic proteins during the engagement with the antigen-presenting target cells. Reprogramming of a biofactory to recognize different targets depending on the cancer type or disease type is also possible. A previous study performed by Bhatnagar et al. (45) showed that T cell biofactory has a great potential for targeting diseased cells with maximal specificity. This

technology was first applied to Jurkat cells (12). Jurkat cells, an immortalized line of T cells, have all the machinery that Primary T cells have, except they are cancer cells. Jurkat cells are preferred for new application evaluation because their culture and growth in the laboratory are more manageable compared to Primary T cells. Although this system has worked, and Jurkat cells have produced the engineered proteins, it is not possible to reinfuse Jurkat cells into the patient's body as they are cancer cells. Also, implementing this system in Primary T cells would cause the therapy to be costly, which is unlikely accessible for all cancer patients. Also, for the best results of this therapy, the effector cells must migrate directly to the diseased cells. To do this, T cells are engineered to express chemokine receptors that drive them to the site of infection in the body. Chemokines, being chemotactic cytokines, control the homing, retention, and migration of immune cells towards the tumor microenvironment (13). Thus, the chemokine system represents a potential target for CAR T cell therapies as it enables effector cells to go directly to target cells.

In order to find a cheaper alternative to CAR T cell therapies, one of the aims of this study was to test if K562 cells can be used as a cellular chassis for developing a biofactory technology to produce therapeutic proteins for ovarian cancer. It is important to note that it is possible to replace the antibodies that recognize antigens and the proteins that can be synthesized *in situ* upon the engagement of effector and target cells, depending on the disease type. Therefore, biofactory technology has a great potential for targeting different cell-based diseases. The rationale for choosing the K562 cell line is that it has been clinically approved to be safely administered to patients with acute

myeloid leukemia after being genetically modified, and it is cheaper and easier to expand than Primary T cells. As the price of CAR T cell therapies is an obvious barrier, this study may result in economic benefits. Another aim of this study was to test the hypothesis that Jurkat cells migrate directly to the tumor microenvironment. Neither Jurkat nor K562 express chemokine receptors. As a result, these cells may target and harm healthy parts of the body after reinfusion. Therefore, to have effector cells migrate directly to the diseased site, Jurkat cells were engineered to express appropriate chemokine receptors whose corresponding chemokines were produced in the ovarian tumor microenvironment. If Jurkat cells successfully migrate towards the tumor cells, the same system can be implemented in K562 biofactories in later studies. This implementation would provide safer therapy in which K562 biofactories directly migrate towards the tumor microenvironment without harming the healthy cells.

2 Literature Review

This literature review first discusses different types of cancer immunotherapy, building background information. Second, CAR T cell therapies and their challenges are addressed to indicate the need for this study. Third, the importance of forming an immunological synapse and chemokine system for T cells' migration, differentiation, and activation is discussed. Finally, different viral gene delivery techniques for delivering genetic material to CAR T cells are reviewed.

2.1 Cancer Immunotherapy

Immunotherapy, also known as biological therapy, is the fight of our immune cells against cancerous cells (4). Dr. William Coley, known as the father of immunotherapy, was moved to research immunotherapies for cancerous tumors after a 17-year-old patient, who did not recover and died weeks later despite the amputation of the sarcoma. Coley documented his successes through experimentation and published case series, achieving remission for sarcoma, lymphoma, and testicular cancer. However, his method was met with initial skepticism, and other oncologists were concerned about deliberately injecting patients with pathogenic bacteria to treat malignant cells (14).

Since Coley's early work, immunotherapy has progressed significantly, and there are now multiple types of immunotherapies available. One such method is adoptive T cell therapy, also known as cellular adoptive immunotherapy or T cell transfer therapy (7). There are three types of adoptive T cell therapy; tumor-infiltrating lymphocyte (TIL) therapy, engineered T cell receptor (TCR) therapy, and chimeric antigen receptor T cell

(CAR T cell) therapy. Researchers have recently begun to incorporate natural killer cells, another type of immune cell, for an alternative adoptive therapy (15).

TIL therapy was pioneered in the late 1980s to treat metastatic melanoma. In this type of therapy, T cells are isolated from a patient's tumor and expanded ex vivo with interleukin 2 (IL-2), a type of cytokine that enhances T cells' activation and contributes to their differentiation into effector T cells (15,16). The effector T cells are then infused intravenously into the patient's body. Even though studies show promising results, not every patient has T cells recognizing their tumors, meaning such patients' T cells cannot bind to antigens and destroy cancer cells. To address this challenge and support a wider range of cancer patients, TCR therapy has been developed. In TCR therapy, patients' T cells are isolated too, but instead of just activation and expansion, doctors and scientists can manipulate T cells and target specific tumor antigens. Once manipulated, T cells are grown in the laboratory and are then reinfused into the patient's bloodstream. This method provides more personalized treatments compared to TIL therapy (7,15).

Although these two therapies have led clinical oncologists to expect more significant tumor regression, results have been disappointing and below expectations. One explanation for the lackluster results may be the downregulation of major histocompatibility complex I (MHC-I) on the tumor surface. The downregulation of MHCs causes cancer cells not to be recognized and destroyed by T cells. Therefore, cancer cells can grow and spread to other parts of the body (8). To have promising results from TCR therapy, MHC or human leukocyte antigen complex (HLA complex), a chromosomal region with different genes, must present intracellular tumor-related

antigens on cancer cells' surface to TCRs on T cells (5). MHC-I or MHC-II should be present on the cancer cells' surface to be detected or destroyed by immune cells (8). To overcome this limitation, scientists equip patients' T cells with a protein called chimeric antigen receptors (CARs). CARs enable T cells to bind to cancer cells despite not having MHCs on their surface and enable immune cells to better identify cancer cell antigens. Since each cancer type has its specific antigen, each CAR is generated specifically for each cancer type. CAR T cells contain extracellular, intracellular, and transmembrane regions. Whereas the extracellular region has a single-chain variable fragment (scFv) domain to recognize tumor-associated antigens, the intracellular domain is composed of immunoreceptor tyrosine-based activation motif (ITAM) of the cluster of differentiation-3 zeta (CD3ζ), which activates co-stimulatory molecules (9,17).

2.2 CAR T Cell Therapies

CAR is a hybrid antigen receptor, which includes a part of an antibody and a part of a TCR. The antibody part is found in the transmembrane domain, and the T cell receptor part is found in the intracellular domain. The antibody part of CAR enables it to bind to an antigen, and the TCR part helps activate the CAR when encountering antigen-presenting cells (18). Over the past years, there have been five generations of CAR T cells, which have been generated to target a specific protein independent of MHC Class I or MHC Class II, which depends upon the type of T cell (either $CD8^+$ or $CD4^+$ T lymphocyte, respectively) on an antigen-presenting cell's surface (9,17).

In 1993, Eshhar et al. were the first to show the first-generation CARs, consisting of scFv extracellular domain as an antigen recognition motif and a cluster of differentiation-

3 zeta (CD3ζ) ITAM domain as an activation motif. They expected these CARs would promote IL-2 secretion upon encountering cancer cells independent of MHC (19). However, two years later, Brocker et al. found that resting T cells and effector T cells had different signaling capabilities. The chimeric receptor ζ, which would activate T cells, expressed by transgenic mice was not sufficient to activate resting T cells. Nevertheless, if the T cells were preactivated, the chimeric receptor ζ was able to activate them. Also, the activated T cells were able to proliferate and had cytotoxic effects upon encountering antigen-presenting cells (20).

In the late 1990s, Gong et al. conducted a study with patients at different stages of prostate cancer. They showed that if T cells were engineered to express the Pz-1 receptor, which lyses prostate cancer cells, they could release cytokines and target tumor cells (21). Upon completion of these studies (19, 20), it was concluded that the first-generation of CAR T cells had insufficient proliferation, secretion of cytokines, and cytotoxic activity *in vivo* (22).

Going forward, co-stimulatory molecules identified as vital for T cell activation, proliferation, and survival were added to second and third-generation CARs. T cell activation needs two signals, one from a TCR and the other from a co-stimulatory molecule such as CD28. This co-stimulatory molecule improves IL-2 synthesis, a cytokine necessary for the T cell stimulation, as proved by Finney et al (23). Thus, the absence of co-stimulatory molecules prevents naive T cells' functions, which would lead to T cells' anergy (24, 25). The co-stimulatory molecule used to generate the second-generation CARs is not limited to CD28. Song et al. and Homback et al. demonstrated

that CD27 or 4-1BB (CD137) and OX40 (CD134) can also promote T cell proliferation and improve CAR T cell function (26,27). The third-generation CARs are different in that they are made by combining CD3ζ-CD28-OX40 or CD3ζ-CD28-41BB to enhance the CARs' potential in terms of killing and cytokine secreting. This means that the third-generation CARs have two co-stimulatory molecules rather than just one as in second-generation CARs (24).

The fourth-generation CARs, also known as TRUCKs (T cells redirected for antigen-unrestricted cytokine-initiated killing), were generated by adding Interleukin 12 cytokine (IL-12) to the base of the second-generation CARs. IL-12 cytokine produced by different cells in response to an antigenic stimulation recruits macrophages that would destroy bacteria or other harmful microorganisms. Thus, TRUCKs can also treat innate system disorders such as viral infections. Studies exploring different cytokines such as IL-7, IL-15, IL-18, and IL-23 to generate the fourth-generation CARs are still going on. Each has different features, but all ultimately aim to enhance T cell functions (28). These successive generations of CARs bring high expectations and hope in cancer treatment.

2.3 CAR T Cell Therapy Challenges

Over the decades, CAR T cell therapies have been under investigation due to their unprecedented and unparalleled results in cancer treatments. Even though many investigations aim to use CAR T cell therapies to treat different kinds of cancer, only a few have been approved by Food and Drug Administration (FDA).

Clinical trials of CAR T cell therapies have shown remarkable results in B cell malignancies. B cell malignancies include relapsed and refractory chronic lymphocytic

leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell lymphoma (MCL), non-Hodgkin lymphoma (NHL), diffuse large B-cell lymphoma (DLBCL), and acute lymphocytic leukemia (ALL). B cell malignancy is a type of cancer formed in B cells. This disease causes the formation of many abnormal B lymphocytes that cannot fight germs as mature B cells do. B cells have a transmembrane glycoprotein, CD19, on their cell surface from early pro-B development to the end of their differentiation into mature B lymphocytes. B lymphocyte antigen CD19 is an attractive therapeutic target for CAR T cell therapies to treat B cell lymphoma because its expression is restricted to B cell lineage (29,30). Kochenderfer et al. treated a patient with advanced follicular lymphoma with a combination of chemotherapy and genetically engineered T cell therapy. They engineered T cells with retroviral vectors to express anti-CD19 CAR that was able to recognize CD19. They reinfused those T cells into the body, and 64% of those expressed the CAR as measured by flow cytometry. After nine weeks of the reinfusion, the patient's lymphoma showed partial remission, and thirty-six weeks after anti-CD19 CAR transduction, $CD19^+$ cells were completely eradicated from their blood (31). After this first effective CAR T cell therapy, Kochenderfer et al. reported on the first clinical trial with 15 patients with DLBCL by using anti-CD19 CAR T cell therapy. Eight of the 15 patients showed complete remission, and four of them achieved partial remission. These results demonstrated this treatment's broader effectiveness in treating B cell malignancies (29,32).

Based on these successful results, many more studies have been conducted over the years, and the first CAR T cell therapy Kymriah, marketed by Novartis, was approved by

the FDA in 2017 for children and young adults up to age 25 with relapsed and refractory (r/r) ALL, or for adults with r/r DLBCL (33,34). Before the approval of this therapy, Maude et al. conducted Phase II ELIANA trial, the first pediatric global CAR T cell therapy, with 63 pediatric and young adults with r/r ALL. Upon completing this study, they found the overall remission rate as 83% within three months of infusion (35). Another study conducted by Schuster et al. was the international Phase II JULIET trial, the first multi-center global registration study, with 93 adult patients. The overall response rate was 52%, including 40% of complete responses and 12% of partial responses (36). One year after the approval, Kymriah was also authorized for use in Europe (30).

Another CAR T cell therapy, Axicabtagene ciloleucel (AXI-CEL/KTE-C19), marketed by Kite as Yescarta, received FDA approval in 2019 for patients with aggressive r/r NHL including DLBCL. Locke et al. conducted phase I of the ZUMA-1 trial to support the KTE-C19 approval. Nine patients were enrolled in this study to evaluate the safety and efficacy of anti-CD19 CAR T cells (37). After getting promising results from this study, Neelapu et al. conducted phase II with 111 patients who have had histologically large B cell lymphoma. Phase II of the ZUMA-1 trial showed an objective response rate of 82% and a complete response of 54%. These results have made doctors and scientists believe that Axicabtagene ciloleucel provides clinical benefits to patients (38).

Despite the successful results for ALL patients, there are still challenges to overcome for CAR T cell therapies. Today, two main challenges remain: selecting the right antigen

to be targeted and ensuring T-cell migration to the solid tumor. An ideal antigen for solid tumors should be highly expressed on them and remain unexpressed on other vital tissues. As solid tumors' antigens may also be found on healthy tissues, it is very hard to find the specific antigen for solid tumors (29,30).

Lack of a specific target antigen for solid tumors may cause on-target off-tumor toxicity. This complication occurs when CAR T cells attack healthy cells that express the intended target antigen. Although this toxicity may be clinically manageable and may not be considered a life-threatening side effect for B cell malignancies, it is yet another challenge for solid tumors (30). For instance, Morgan et al. discuss a case that came to a catastrophic end. A 39-year-old female patient with metastatic colon cancer was infused with the CAR T cells targeting HER2 (ERBB2) antigen, which is overexpressed in the tumor microenvironment of some cancer types, including breast, colon, and ovarian cancers. After the infusion, the patient's epithelial cells of the lung were attacked by the CAR T cells, and she died five days after the infusion. Her death was attributed to the low levels of HER2 in the epithelial cells (39). Since this fatal event, scientists have been trying to find other cancer markers that are safer and more effective for solid tumors. One such marker is ICAM-1 (intercellular adhesion molecule-1), and it bears CARs with micromolar affinity rather than nanomolar affinity. Since ligands with nanomolar affinity would bind more tightly to the proteins when compared to those of micromolar affinity, ICAM-1 markers are expected not to bind to healthy cells tightly. The affinity of CARs influences cytokine release. Thus, a reduced affinity targeting strategy would allow rapid

tumor elimination while preventing the toxicity that would occur because of tight binding (40).

The most frequently reported life-threatening toxicity associated with CAR T cell therapy is cytokine release syndrome (CRS). After the infusion of CAR T cells into the bloodstream, the number of cytokines, IFNγ, TNFα, and IL-2, in the body rises and causes CRS. The symptoms of CRS include high fever, nausea, headache, and or mild to severe tachycardia (30,41). Another CAR T cell-mediated fatal toxicity is neurotoxicity, co-occurring or after CRS. Some common manifestations of neurotoxicity are headache, confusion or delirium, and rarely acute cerebral edema (41,42). To date, both neurotoxicity and CSR occur in many patients who are treated with CD-19 CAR T cell therapy (30). Teachey et al. measured cytokine numbers in 51 patients' bloodstream after receiving CD-19 CAR T cell therapy for ALL. They realized a peak in the number of 24 cytokines including IFNγ and IL-6 (43). To identify risk factors of neurotoxicity resulting from CD-19 CAR T cell therapy, Gust et al. studied 133 patients' neurological adverse events after the infusion. Fifty-three patients experienced neurological adverse events, from mild symptoms to death. Also, the majority of the 53 patients had CRS as well. These results led researchers to that CRS causes a higher risk of neurotoxicity development (44).

2.4 Immunological Synapse

To activate the cell signaling pathway developed for this study, the formation of an immunological synapse between the effector cells and the antigen-presenting cells (APCs) is vital. An immunological synapse (IS) is a junction between APCs and

lymphocytes, such as T cells, where the interaction of T cell receptors (TCRs) and peptide major histocompatibility complexes (MHCs) occurs (46,47). IS is also known as a supramolecular activation cluster (SMAC), and it is formed of three major compartments: central SMAC (cSMAC), peripheral SMAC (pSMAC), and distal SMAC (dSMAC) (46,48). The external ring, dSMAC, is composed of large membrane glycoproteins such as CD43 and CD45 (48). It is the site where TCR microclusters (MC) first engage with peptide-MHCs. Once TCR-MCs are formed, they are translocated toward pSMAC, which is formed of integrins and the proteins involved in cell adhesion, such as lymphocyte function-associated antigen-1 (LFA-1), the cytoskeletal protein talin, and ICAM-1 (49,50). The inner circle, cSMAC, is the center area of this structure in which TCRs, CD2, CD4, CD8, CD28, protein kinase Cθ (PKCθ), and lymphocyte-specific protein tyrosine kinase (Lck) are concentrated (48,51). The proteins TCRs, CD2, CD4, CD8, and CD28 are expressed on the surface of immune cells (such as T cells), while Lck and PKCθ are expressed inside T cells. Whereas Lck associates with the cytoplasmic tails of CD4 and CD8, PKCθ plays a key role in the T cell activation. Each molecule mentioned here has an essential part in the function of IS, which integrates antigens, adhesion molecules, and co-stimulatory molecules. The co-stimulatory receptors CD4, CD8, and CD28 synergize to activate T cells, while the interaction between LFA-1 on the T cell surface and ICAM-1 on APCs allows rapid T cell migration and differentiation (46).

The formation of IS initiates the differentiation of naive T cells into effector T cells, which defend the host against various pathogens and cancer cells (46,49). Upon the

formation of IS, Lck phosphorylates and activates ZAP-70, a crucial protein kinase for the three T cell signaling pathways. The first signaling pathway is NFAT (nuclear factor of activated T cells), also known as the Calcium pathway. It can initiate gene expression by itself or with other transcription factors (52). The second pathway, NFκB (nuclear factor-κB), is essential for regulating immune responses and activates PKCθ, which is vital for T cell activation (53,54). The third pathway is Activator protein 1 (AP-1), which regulates gene expression, proliferation, differentiation, and even cell death (55). Though each pathway has its essential role, their combined effect is necessary for T cells to proliferate, differentiate, and have effector functions.

2.5 Chemokine System

Severe tissue damage occurs when immune cells migrate to the incorrect site and attack healthy parts of the body. The chemokine system represents a potential target for immunotherapy, and it is vital to prevent tissue damage. Besides their chemotactic ability, chemokines can also regulate T cell development and their effector functions. The four subfamilies of chemokines, differentiated by their initial cysteine residues position, are CXC, CC, CX3C, and XC structural motifs (56,57,58). Chemokines exert their biological effects by binding their corresponding G protein-coupled chemokine receptors. Chemokines are secreted in the tumor microenvironment, and their cognate receptors are expressed by tumor cells. There are almost 50 distinct chemokines and 20 chemokine receptors identified in humans. Some chemokine receptors are specific to one chemokine, and others may bind to many (58). Table 1 shows chemokines (ligands) and their corresponding receptors (59).

Table 1. Chemokines and Corresponding Chemokine Receptors

Chemokine Receptor	Ligand
CCR1	CCL3, CCL4, CCL5, CCL7, CCL13, CCL14, CCL15, CCL16, CCL23
CCR2	CCL2, CCL7, CCL8, CCL12, CCL13, CCL16
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL24, CCL26, CCL28
CCR4	CCL2, CCL3, CCL5, CCL7, CCL22
CCR5	CCL3, CCL4, CCL5, CCL8, CCL11, CCL14, CCL16
CCR6	CCL20
CCR7	CCL19, CCL21
CCR8	CCL1, CCL4, CCL17
CCR9	CCL25
CCR10	CCL27, CCL28
CXCR1	CXCL6, CXCL8

CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
CXCR3	CXCL9, CXCL10, CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL11, CXCL12
CX3CR1	CX3CL1
XCR1	XCL1, XCL2

In terms of the chemokines' function, there are two subfamilies, homeostatic and inflammatory chemokines. Homeostatic chemokines mainly direct the flow of leukocyte traffic throughout the body to screen for an invasion of pathogens. This family includes, but is not limited to, CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12, and CXCL13 (57). In different parts of the body, different chemokines recruit immune cells. For instance, CCL27 recruits T leukocytes in the skin, and CXCL12 mediates leukocytes homing in the brain (59). Some chemokines promote the formation of new blood vessels (angiogenesis). Although it is a part of the healing and growth of tissues, angiogenesis inhibitors can be essential for several diseases, including cancer. Since tumor cells need oxygen and nutrients to grow and spread to the other parts of the body, angiogenesis

inhibitors could block them from going to tumor cells. As a result, tumor cells starve and cannot grow or spread (60). Another chemokine family, inflammatory chemokines, is secreted to infection, tissue injury, or tumors. They control the inflammatory response of immune cells to infectious sites. CXCL8, CCL2, CCL3, CCL4, CCL5, CCL11, and CXCL10 are examples of inflammatory chemokines (61). However, these classifications are flexible, and some homeostatic chemokines may also be considered part of the inflammatory chemokines' family (62).

As one aim of this study was to have effector cells migrate to the tumor microenvironment directly, target and effector cells were engineered to express chemokines and their respective corresponding receptors. Chemokines and their corresponding receptors tend to bind each other. As a result, effector cells engineered to express chemokine receptors should be expected to go directly to the tumor microenvironment where the corresponding chemokines are secreted. Chemokines that are secreted in the tumor microenvironment depend on the type of cancer.

2.6 Gene Therapies

Genes maintain the production of proteins and enzymes within cells, providing essential body functions. Gene therapies to treat or prevent diseases have been investigated by scientists for decades. Gene therapy is the introduction of genomic material into a specific cell to give it new functions or the introduction of genomic material into a defective cell to correct abnormalities. There are many aspects of successful gene therapy, such as an effective therapeutic gene or an animal model simulating disease.

Gene therapy is divided into two categories: somatic gene therapy and germline gene therapy. Some consider germline therapy to be unethical as it replaces an offspring's defective genes with healthier ones and is used to treat genetic disorders. It is controversial because it changes someone's genetics who is not yet born, so they do not say whether they want treatment (63,64). Somatic gene therapy is considered to be ethically safer, as it affects only the patients' genes, not future generations. There have been two delivery techniques for somatic gene therapy, viral and non-viral. Both delivery techniques have one goal: delivering genetic material across cell membranes and reaching the cell's nucleus (63).

In the 1990s, the potential of gene therapy created excitement, and many studies were conducted. However, the lack of experience in the clinical applications of viral vectors and their safety issues caused two catastrophic events. In 2003, Raper et al. reported the death of an 18-year-old male with partial ornithine transcarbamylase (OTC) deficiency, which is not a life-threatening disease. Adenovirus type 5 was used to deliver the gene therapy, and he was the 18th subject who received it. Before him, no one showed the symptoms he showed. Approximately 18 hours after the delivery, he reported not feeling well and died. His death was attributed to systemic inflammatory response syndrome and multiple organ system failures. After this experience, animal studies' limitations in predicting human responses and subject-to-subject symptom variations were determined (65,66). Although the unsuccessful results led to a decrease in funding for gene delivery, there were, and still are, many scientists who believed in it. With this, gene delivery has become one of today's promising therapies.

Viral gene delivery uses viruses to deliver genetic material; the viruses include adenoviruses, gamma retroviruses, lentiviruses, and adeno-associated viruses. Lentiviruses are a subclass of retroviruses. They have been used widely in scientific research as they have unique features, including the ability to integrate with both non-dividing and dividing cells. Lentiviral vectors are derived from human immunodeficiency virus type 1 (HIV-1), becoming nonpathogenic and safe to use after some modifications (67). Naldini et al. proved that lentiviral vectors can be used in in vivo gene therapy in non-dividing cells, and they predicted that their feasibility in human gene therapy would improve (68). Lamers et al. conducted a study using gamma retroviruses instead, using γ-retroviruses to modify the genes ex vivo of patients with metastatic renal cell carcinoma. In some patients, immunity against γ-retroviral vectors occurred, concerning researchers about γ-retroviral ex vivo applications (69). Different viral vectors have their advantages and disadvantages, and the selection should be specific to the study. In this study, lentiviruses were used to transfer the intended genetic materials to cells due to the advantages mentioned above.

3 Research Hypotheses

3.1 Specific Aim 1: Developing a K562 Biofactory to Synthesize Therapeutic Proteins

In this study, to find a cheaper alternative to CAR T cell therapies, K562 cells were explored as a biological chassis to evaluate their ability to synthesize engineered therapeutic proteins when engaged with ovarian cancer cells. In this part of the study, K562 cells were engineered with an artificial cell-signaling pathway whose design transforms them into antigen-specific effector cells producing Nanoluc luciferase enzyme, a quantifiable non-human effector protein. This pathway contained three constants (receptor, actuator, secretor) and two variable (sensor, effector) domains. Whereas the constant domains gave biofactory functionality, the variable domains expanded the possibility of targeting different cell-based diseases.

The artificial cell signaling pathway developed here was a third-generation CAR. It was developed to be activated by an immunological synapse, which was to be formed between K562 cells and ovarian cancer cells when they engage with one another. When the pathway was activated, it was expected K562 cells synthesize the Nanoluc luciferase enzyme. In order to have quantifiable results and demonstrate if this system has worked, a NanoGlo luciferase assay system was used to measure the production of Nanoluc luciferase reporter. The null hypothesis was that K562 can be used as a cellular chassis for developing a biofactory technology as an alternative to T cells in the treatment of ovarian cancer, and the alternate hypothesis was the other way around.

3.2 Specific Aim 2: Engineering Jurkat Cells with Chemokine Receptors to Evaluate Their Migration Towards the Ovarian Cancer Microenvironment and Their Cytotoxic Functions on Tumor Cells

In the second part of the study, the aim was to prevent tissue damage on healthy areas of the body and to see the cytotoxic functions of Jurkat cells on tumor cells. In order to have Jurkat cells migrate directly towards the tumor microenvironment, they were engineered to express two chemokine receptors independently. When chemokine receptors interact with chemokines, they tend to bind to corresponding ligands. For this study, appropriate chemokine receptors were expected to be highly expressed on cytotoxic T cells ($CD8^+$ T cells) and helper T cells ($CD4^+$ T cells). Also, their low expression on T regulatory cells was another consideration. The reason was that Treg cells, also known as suppressor T cells, regulate and suppress other immune cells' functions. So, their activation could prevent the migration of cytotoxic T cells into the tumor microenvironment. The chemokine receptors selected here were CCR5 and CCR7 due to the reasons mentioned above. Whereas Jurkat cells were engineered to express CCR5 and CCR7, ovarian cancer cells were engineered to express CCL5 and CCL19, the corresponding ligands of CCR5 and CCR7. As a result, it was expected that CCR5 and CCR7 chemokine receptors of Jurkat cells would migrate directly towards the corresponding ligands and bind to them.

On the other hand, to give cytotoxic functions to Jurkat cells, they were modified to express CAR on their cell surface. Jurkat cells were also modified with anti-folate receptor alpha to recognize folate receptor alpha, a receptor highly expressed on ovarian cancer cells' surface. The last modification done was the engineering of Jurkat cells with

luciferase enzyme to give them quantifiable features. Here again, a NanoGlo luciferase assay system was used to obtain measurements.

4 Materials and Methods

In this part, the materials and methods used to test the hypotheses for the two specific aims are discussed.

4.1 Specific Aim 1

To test the hypothesis of the specific aim 1, Jurkat and K562 biofactories were developed by designing an artificial cell-signaling pathway. As mentioned earlier, this pathway included five domains: the receptor, the actuator, the secretor, the sensor, and the effector. The receptor part was generated by adding an activator motif, CD3ζ, and co-stimulatory molecules, 4-1BB and CD28. The CAR generated here was the third generation. Whereas CD3ζ led to a rise in the intracellular calcium (Ca^{2+}) concentration, the combination of 4-1BB and CD28 enhanced the therapeutic response of CAR T cells. The actuator part was based on six copies of NFAT-RE (NFAT-RE6X, nuclear factor of activated T cell response) that would respond to Ca^{2+} rise and upregulate luciferase activity. The secretor part was composed of human interferon alpha-2 (IFN-α2) that activates T cells to fight cancer cells and inhibits tumor cells' proliferation. The other two parts, the sensor, and the effector, were subject to change depending on the study. In this case, the sensor part was developed to detect folate receptor alpha (FRα), which is a receptor found on the surface of many cancer cells including ovarian cancer cells. Two different ovarian cancer cell lines (OVCAR-3 and A2780cis) were used in this study. OVCAR-3 human ovarian cancer cells were used as target cells because they express FRα and can be detected by the anti-folate receptor alpha on the biofactory cells' surface. On the other hand, A2780cis human ovarian cancer cells lacking FRα were used as non-

target negative controls. The fifth part of the pathway, an effector part, may comprise different reporter transgenes. In this study, a quantifiable non-human effector protein, Nanoluc luciferase reporter, was used for proof-of-concept. If biofactories can produce Nanoluc enzyme, they can be manipulated with different therapeutic proteins, instead of a quantifiable non-human protein, to give them therapeutic functions.

To generate Jurkat and K562 biofactories, lentiviruses, with the necessary genetic material mentioned earlier, were produced by the transfection of HEK293T/17 cells. Once lentiviruses were generated, Jurkat and K562 parental cell lines were transduced with these viruses. To check if the transduction was successful, engineered cells' live/dead status was compared to one of the positive control cells (parental cell lines). Both transduced and unmodified cells were placed under selection for two weeks with puromycin, a type of antibiotic used to select and maintain cells stably transfected with viral vectors expressing puromycin. It was expected that successfully engineered cells stay alive under the selection. Once alive engineered cells were selected and grown in the laboratory, they were co-cultured with target and non-target cancer cells. The Nanoluc activity of cell biofactories was measured with a microplate reader called NanoGlo Assay. Three 96-well plates were seeded with different co-culture combinations to measure the Nanoluc activity produced by Jurkat and K562 biofactories when they engaged the target and non-target cells in respect to 24hr, 48hr, and 72hr. Figure 1 illustrates the type of seeded cells in the plates.

Jurkat Biofactory and OVCAR-3
Jurkat Biofactory and A2780cis
K562 Biofactory and OVCAR-3
K562 Biofactory and A2780cis
Jurkat Biofactory cells
K562 Biofactory cells
OVCAR-3 cells
A2780cis cells

Figure 1. The combination of seeded cells in the 96-well plates

To understand the effect of cancer cells' mass on the Nanoluc synthesis, another NanoGlo assay was applied with the cell combinations shown in Figure 1. In this experiment, effector cell numbers remained constant, and target cell numbers were serially diluted. Nanoluc activity of biofactories was measured at 24 hours of co-culture.

4.1.1 Materials and Reagents

K562-BF, Jurkat-BF, OVCAR-3 (ATCC), and A2780cis (ATCC) cell lines were maintained in complete RPMI media [RPMI1640 with L-glutamine (Corning, CAT# 10-040CV), and 10% fetal bovine serum (Sigma-Aldrich, Cat# F2442-500ml)]. Puromycin dihydrochloride (Thermo Fisher Scientific, Cat# A1113803) was used for selecting engineered cells.

4.1.2 Reviving Cell Lines

Already engineered K562-BF and Jurkat-BF cell lines were provided by Dr. Parijat Bhatnagar's laboratory. All frozen cells were rapidly (< 2 minute) thawed in a 37°C water bath. Thawed cells were slowly diluted with 10ml of pre-warmth complete RPMI media in a Falcon 15 mL conical centrifuge tube. Then, 15 mL tube was vortexed and centrifuged at 200G for 8 minutes. After the supernatant was removed, cells were resuspended with 5ml of growth media. After 48 hours of revival, K562-BF and Jurkat-BF were placed in selection using 0.5 μg mL^{-1} and 0.2 μg mL^{-1} of puromycin dihydrochloride respectively for two weeks. After selection, all cell line were expanded for NanoGlo Assays and then were frozen using freezing media (complete culture media and 10% DMSO) for liquid nitrogen stocks.

4.1.3 Stimulation of Biofactories

2500 cells of OVCAR-3 and A2780cis were co-cultured with 12500 cells of Jurkat-BF and K562-BF in 100 μL of complete RPMI media in a single well of 96-well plate. Also, OVCAR-3, A2780cis, Jurkat-BF and K562-BF were also seeded as controls, which were to be chemically stimulated with PMA ionomycin to evaluate the function of biofactories. Three 96-well plate were seeded for three time points (24, 48, and 72 hours).

4.1.4 Stimulation of Biofactories with Variant Target Cells' Mass

12500 cells of Jurkat-BF and K562-BF were co-cultured with variant target and non-target cell numbers (from 125000 to 976) in 100 μL of complete RPMI media in a 96-well plate. Cell number variation was obtained by serially diluting downwards of target cells.

4.1.5 NanoGlo Assay to Measure Nanoluc Activity

Nanoluc substrate was diluted in the cell lysis buffer, which was provided with the NanoGlo assay. It was added 1:1 to cells. After 3 minutes of incubation, Nanoluc activity of biofactory cells was read on a microplate reader (Perkin Elmer, Envision Multilabel Plate Reader Model: 2104-0010A).

4.2 Specific Aim 2

In this part of the study, the aim was to have effector cells, Jurkat cells, migrate towards the ovarian cancer cells, OVCAR-3 cells. Jurkat parental cells were manipulated with lentiviruses to recognize FRα on OVCAR-3 cells' surface. Also, they were engineered, independently, to express CCR5 and CCR7 chemokine receptors, and not to express CCR5 and CCR7. The cells engineered with CCR5 and CCR7 expressed these chemokine receptors on their cell surfaces. On the other hand, the cells engineered with CCR5 endogenous shRNA and CCR7 endogenous shRNA did not express these receptors. shRNA molecules knocked down gene expression. To check their chemotactic ability Jurkat cells were co-cultured with OVCAR-3 cells (target cells), which were engineered to express $CCL5^{+}$, $CCL5^{-}$, $CCL19^{+}$, and $CCL19^{-}$, the corresponding ligands of $CCR5^{+}$, $CCR5^{-}$, $CCR7^{+}$, and $CCR7^{-}$. Figure 2 illustrates different combinations of chemokine receptors expressed by Jurkat cells and chemokines secreted in the ovarian tumor microenvironment.

$CCR5^-$ and $CCL5^-$
$CCR5^+$ and $CCL5^-$
$CCR5^-$ and $CCL5^+$
$CCR5^+$ and $CCL5^+$
$CCR7^-$ and $CCL19^-$
$CCR7^+$ and $CCL19^-$
$CCR7^-$ and $CCL19^+$
$CCR7^+$ and $CCL19^+$

Figure 2. Combinations of chemokines and their receptors

4.2.1 Materials and Reagents

Parental Jurkat and OVCAR-3 cells (ATCC) were maintained in complete RPMI media [RPMI1640 with L-glutamine (Corning, CAT# 10-040CV), and 10% fetal bovine serum (Sigma-Aldrich, Cat# F2442-500ml)] to be engineered. HEK293T/17 cells were maintained in complete DMEM media [DMEM with L-glutamine and 10% fetal bovine serum) to produce lentiviruses. Puromycin dihydrochloride (Thermo Fisher Scientific, Cat# A1113803) or G418 (Corning, Cat# 30 234-CR) was used for selecting engineered cells.

4.2.2 Lentivirus Production

HEK293T/17 cells were seeded and maintained in complete DMEM. In a 1.5mL microtube, 150mM NaCl, pAdv, pMD2.G, psPAX2, transporter, and the corresponding transfer plasmid [LentiStarter 2.0 kit (System Biosciences)] were mixed. This mixture was added on the plated HEK293T/17. Viral supernatant was collected 24, 48, and 72 hours after transfection. Viral supernatant was filtered and ultracentrifuged by using Polypropylene Konical ultracentrifugation tube (Beckman Coulter #C14291) in Beckman Coulter ultracentrifuge (Beckman Coulter #369650).

4.2.3 Transduction of Parental Cell Lines

Corresponding lentivirus particles were added to parental cell lines (Jurkat and OVCAR-3). After five hours of incubation, polybrene was added to flasks.

4.2.4 Boyden Chamber Assay

Respective OVCAR-3 cells engineered with ligands were seeded at 10000 per well in a 24-well plate. After 24 hours of incubation, respective Jurkat cells engineered with chemokine receptors were seeded at $1x10^6$ per well in Boyden chambers. Boyden chamber assemblies were incubated for 3 and 6 hours, and Boyden chambers were discarded. Migrated Jurkat cells were allowed to be co-cultured with OVCAR-3 cells for 48 hours.

4.2.5 NanoGlo Assay to Measure Luciferase Activity

Luciferase reagent was added to each well of 24-well plate and plates were incubated for 5 minutes. Cells in 24-well plate were transferred to 96-well plate to be read

luminescence on a microplate reader (Perkin Elmer, Envision Multilabel Plate Reader Model: 2104-0010A).

The most important safety consideration for Specific Aim 1 and 2 was to work with lentiviral vectors. While working with viruses, double gloves, double lab coats, and lab goggles must be worn. Also, ten percent of bleach must be always found in the hood to remove used pipettes.

5 Results

In this thesis, two hypotheses were evaluated to be true or false through the data obtained by various experiments explained above. The first hypothesis was that K562 cells could be used as a cellular chassis for developing a biofactory technology to produce therapeutic proteins for targeting diseased cells. In the first part (Figure 3, Figure 4, Figure 5), the hypothesis that K562 cells synthesize engineered proteins *in situ* upon the interaction with ovarian cancer cells was evaluated. In the second part of the study (Figure 6, Figure 7), the hypothesis that manipulated Jurkat cells migrate towards ovarian cancer cells and have a cytotoxic effect on them was evaluated. For all comparisons of statistical significance, two-sample t-tests with common variance were used. Values of $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered as being significant, very significant, and extremely significant respectively. Values of $P > 0.05$ were considered as being not significant. The results are expressed as mean (M) ± standard deviation (SD).

5.1 Specific Aim 1. K562 Biofactory Function

5.1.1 NanoLuc Luciferase Expression Measurement of Chemically Stimulated Biofactories

First, the function of K562 and Jurkat cell biofactories that had been chemically stimulated with Phorbol 12-Myristate 13-Acetate (PMA) ionomycin formulation were tested to see whether the cells were engineered successfully, and they were able to synthesize the NanoLuc enzyme. The comparison between chemically stimulated and non-stimulated biofactories is shown below (Figure 3).

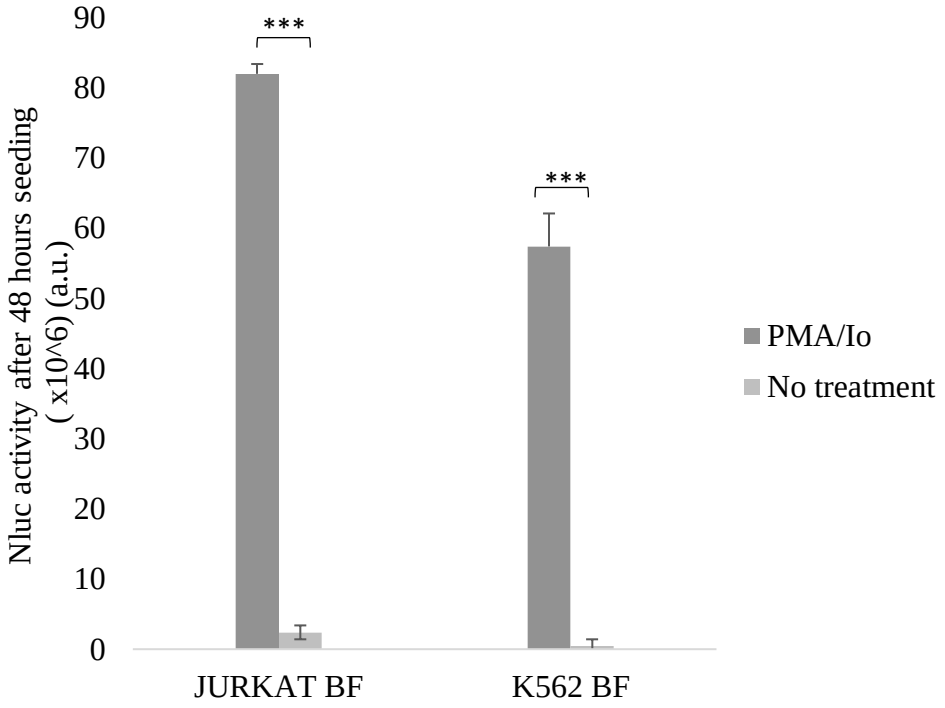

Figure 3. Demonstration of chemically stimulated K562 and Jurkat cells biofactories' function. Chemically stimulated and non-stimulated effector cells from both biofactories were compared to each other. 50,000 cells were seeded for each condition. Statistics between K562 and Jurkat biofactories were calculated by a two-sample t-test assuming equal variances. Values are expressed as mean ± SD. P values: *P < 0.05, **P < 0.01, ***P < 0.001. a.u., arbitrary unit.

Nanoluc activity of chemically stimulated and non-stimulated Jurkat biofactories (Jurkat-BF) was measured as 82×10^6 a.u. ± 1.4×10^6 and 2.4×10^6 a.u. ± 0.11×10^6; Nanoluc activity of chemically stimulated and non-stimulated K562 biofactories (K562-BF) was measured as 57.4×10^6 a.u. ± 4.7×10^6 and 0.421×10^6 a.u. ± 0.064×10^6. Statistical analysis supported that the difference between the amount of Nanoluc synthesized by chemically stimulated and non-stimulated biofactories was extremely significant ($p = 7.17 \times 10^{-18}$ for Jurkat-BF and $p = 4.32 \times 10^{-11}$ for K562-BF), meaning

chemically stimulated biofactories were able to produce a significant amount of Nanoluc compared to a non-stimulated biofactory.

5.1.2 NanoLuc Luciferase Expression Measurement of Biofactories that Interact with Cancer Cells

Second, to evaluate the K562 biofactory function *in situ* upon interaction with ovarian cancer cells, Nanoluc expression of effector cells (K562-BF and Jurkat-BF) and target cells (OVCAR-3 and A2780cis cells) co-cultures were measured and compared to each other. Co-culture of effector cells with OVCAR-3 cells were used as positive controls, and co-culture of effector cells with A2780cis cells were used as negative controls.

It was expected that both biofactories would produce the Nanoluc enzyme when engaged with OVCAR-3 cells. The Nanoluc amount expressed by K562-BF was compared to the one expressed by Jurkat-BF to evaluate whether K562 cells could be substituted for T cells as a cellular chassis for developing a biofactory technology.

The chemically stimulated biofactories were able to synthesize the Nanoluc enzyme whereas chemically stimulated target cells were not (Figure 3). When the Nanoluc expression of the positive control and negative control of Jurkat-BF (13.43×10^6 a.u. ± 1.3×10^6 and 0.95×10^6 a.u. ± 0.13×10^6) were compared to each other, it was seen that Jurkat cells synthesize Nanoluc *in situ* upon interaction with OVCAR-3 cells. This finding was also supported by a two-tail t-test ($p = 0.008$). It was concluded that the difference between co-cultures of Jurkat-BF with positive and negative controls was very significant. However, the co-cultures of K562-BF with OVCAR-3 and A2780cis (0.21×10^6 a.u. ± 0.014×10^6 and 0.17×10^6 a.u. ± 0.035×10^6) expressed almost the same amount of Nanoluc enzyme ($p = 0.46$), meaning K562-BF did not produce Nanoluc when

engaged with antigen-presenting target cells. To confirm this finding, a two-tailed t-test between co-cultures of biofactories with OVCAR-3 was applied and again K562-BF did not produce Nanoluc enzyme as Jurkat-BF did (p = 0.007, a very significant difference). All t-tests were done according to the data at 48 hours (Figure 4).

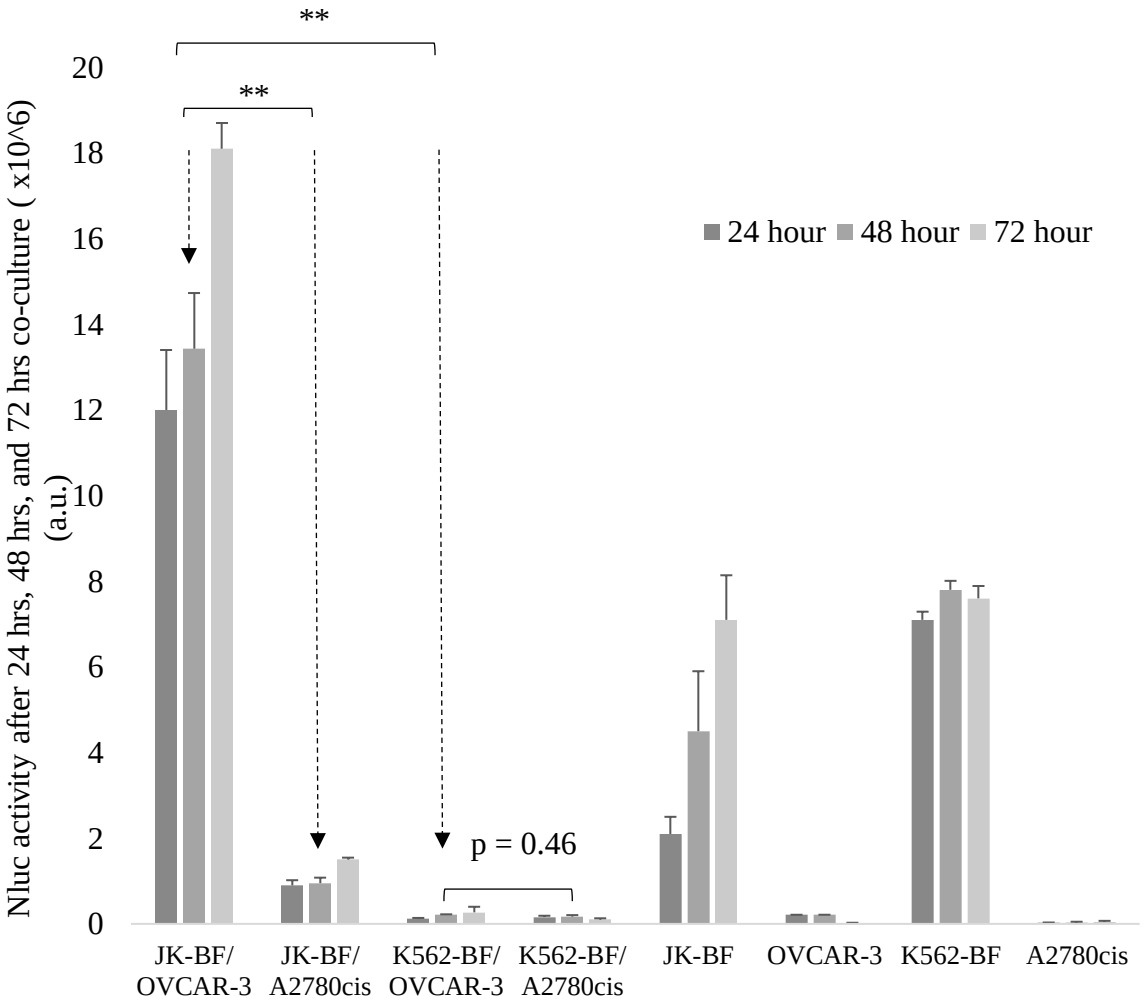

Figure 4. Nanoluc expression measurement of effector cells, JURKAT-BF and K562-BF, after co-culture with target cells, OVCAR-3 and A2780cis, and of chemically stimulated (PMA ionomycin) K562-BF, JURKAT-BF, OVCAR-3, and A2780cis cells at 24, 48, and 72 hours. 12,500 effector and 2,500 target cells were seeded for each condition. Statistics were calculated by a two-sample t-test assuming equal variances. Values are expressed as mean ± SD. P values: *P < 0.05, **P < 0.01, ***P < 0.001. a.u., arbitrary unit.

5.1.3 Effect of Target Cells' Mass on Nanoluc Enzyme Expression

Last, we tested the effect of target cells' mass (OVCAR-3 and A2780cis) on Nanoluc enzyme synthesis by the biofactories. Eight different amount of target cells (from 125,000 to 976 cells) were co-cultured with same amount of effector cells. For Jurkat-BF, effector protein activity was proportional to the mass of target cells. However, for K562-BF, the mass of target cells did not have any effect on the Nanoluc expression as K562-BF cells were almost unable to produce Nanoluc enzyme when encountered by the antigen presenting target cells (Figure 5). This finding was supported by statistical analysis done between the co-cultures of biofactories with OVCAR-3 cells, where the number of seeded cells of effector and target cells were 12,500 and 125,000 respectively. It was concluded that K562-BF did not produce Nanoluc as Jurkat-BF did, and there was an extremely significant difference between their Nanoluc expression ($p = 2.9 \times 10^{-6}$).

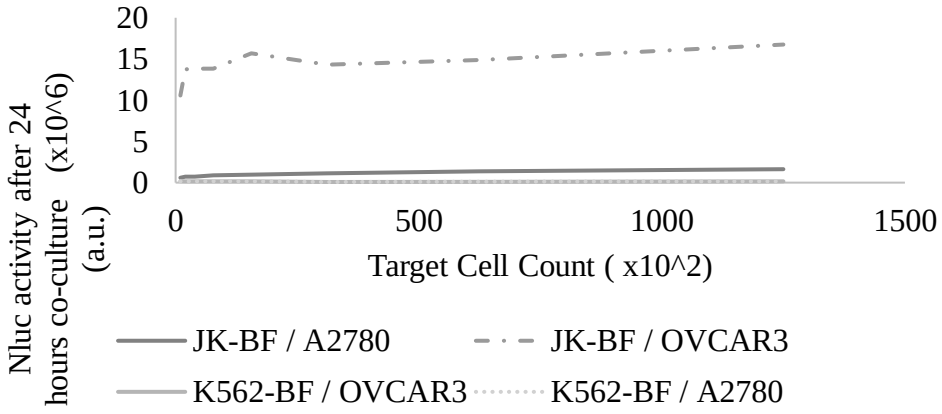

Figure 5. Evaluation of the effect of target cells' mass on the effector cells' Nanoluc expression. Target cell numbers were serially diluted from 125,000 to 976 cells whereas effector cell numbers remained constant at 12,500 cells. Statistics were calculated by a two-sample t-test assuming equal variances. Values are expressed as mean ± SD. P values: *P < 0.05, **P < 0.01, ***P < 0.001. a.u., arbitrary unit.

5.2 Specific Aim 2. Function of CAR Jurkat Cells Engineered with Chemokine Receptors

In this part of the study, the migration and cytotoxic ability of Jurkat cells on OVCAR-3 cells were tested. Results were found by measuring luciferase (Luc2) expression of cancer cells.

5.2.1 Function of CCR5 Jurkat Cells

First, we tested the killing ability of Frα-CAR Jurkat cells expressing CCR5 on OVCAR-3 cells. Various statistical analyses were applied with different co-cultures combinations illustrated in Figure 2. Frα-CAR Jurkat cells expressing CCR5 chemokine receptor (Jurkat $CCR5^{+}$) were co-cultured with OVCAR-3 expressing CCL5 (OVCAR-3 $CCL5^{+}$) and OVCAR-3 non-expressing CCL5 (OVCAR-3 $CCL5^{-}$). Frα-CAR Jurkat non-expressing CCR5 (Jurkat $CCR5^{-}$) was also co-cultured with OVCAR-3 $CCL5^{+}$. Then, the migration and cytotoxic ability of Frα-CAR Jurkat cells on OVCAR-3 cells were measured at 3 and 6 hours. Luc2 expression of OVCAR-3 cells are illustrated below (Figure 6).

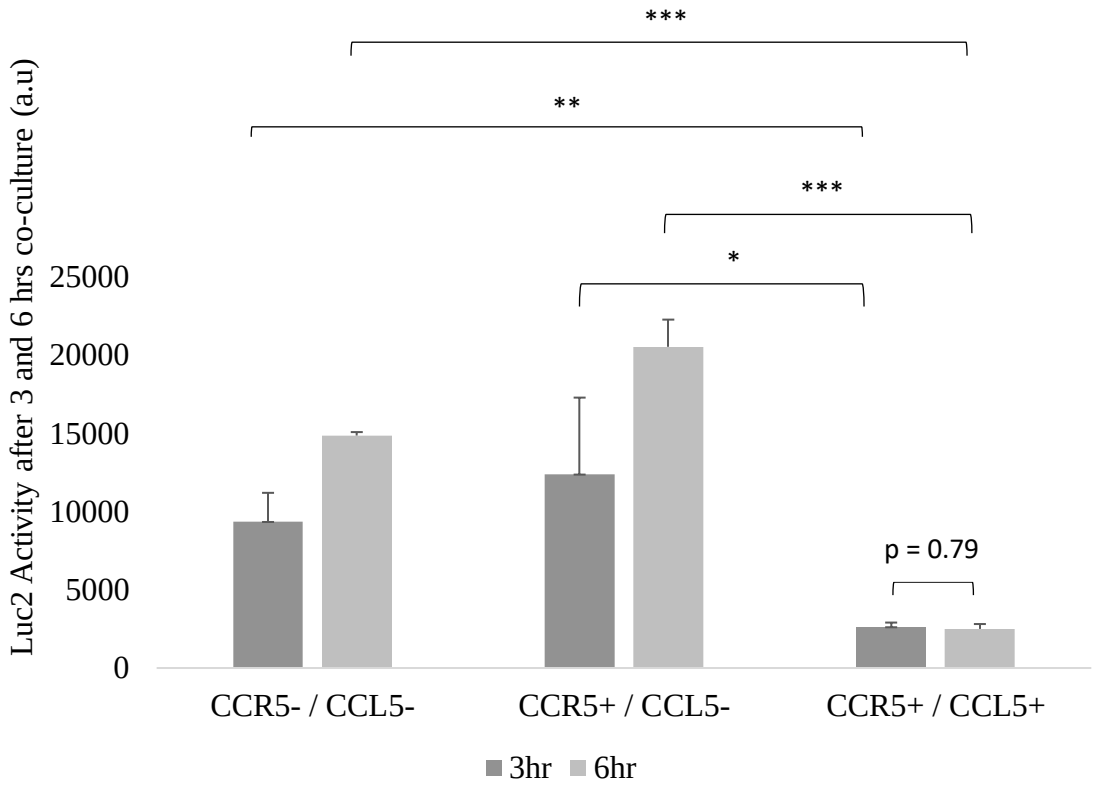

Figure 6. The migration ability and cytotoxic effect of CAR Jurkat CCR5+ and CAR Jurkat CCR5$^-$ on OVCAR-3 CCL5$^+$ and OVCAR-3 CCL5$^-$ are shown by the measurement of Luc2 expression of target cells. Luc2 expression of OVCAR-3 CCL5$^-$ cells co-cultured with CAR Jurkat CCR5$^+$ and CAR Jurkat CCR5$^-$ were measured as 12356 a.u. and 9329 a.u. at 3 hours, and 20516 a.u. and 14849 a.u at 6 hours respectively. Luc2 expression of OVCAR-3 CCL5$^+$ cells co-cultured with CAR Jurkat CCR5$^+$ was measured as 2604 a.u. at 3 hours and 2498 a.u. at 6 hours. OVCAR-3 cells and CAR Jurkat cells were seeded at 10,000 and $1x10^6$ cells for each condition. Statistics were calculated by a two-sample t-test assuming equal variances. Values are expressed as mean ± SD. P values: *P < 0.05, **P < 0.01, ***P < 0.001. a.u., arbitrary unit.

It was hypothesized that Jurkat CCR5$^+$ cells would migrate more towards OVCAR-3 CCL5$^+$ cells and have a higher cytotoxic effect on them compared to their co-culture with OVCAR-3 CCL5$^-$ cells after both 3 and 6 hours. This hypothesis was proven by statistical analyses done between Jurkat CCR5$^+$ / OVCAR-3 CCL5$^-$ and Jurkat CCR5$^+$ /

OVCAR-3 $CCL5^+$ at 3 and 6 hours ($p = 0.027$ at 3 hours and $p = 6x10^{-5}$ at 6 hours). Another analysis was done between 3 and 6 hours to understand whether time has an effect on the migration and killing ability of Jurkat cells. It was found that the cytolytic ability of Jurkat $CCR5^+$ cells on OVCAR-3 $CCL5^+$ cells remained constant after 3 and 6 hours co-culture ($p = 0.79$, no significant difference).

The second hypothesis was that Frα-CAR Jurkat $CCR5^+$ cells would migrate more towards OVCAR-3 $CCL5^+$ cells and have more killing effect on them than the migration and killing ability of Jurkat $CCR5^-$ cells on OVCAR-3 $CCL5^-$ cells. This hypothesis was supported by the statistical analysis done between Jurkat $CCR5^+$ / OVCAR-3 $CCL5^+$ and Jurkat $CCR5^-$ / OVCAR-3 $CCL5^-$ at both 3 and 6 hours. At 3 hours the difference was very significant ($p = 0.0034$) and at 6 hours the difference was extremely significant ($p = 6x10^{-7}$).

5.2.2 Function of Jurkat Cells Expressing $CCR7^+$ and $CCR7^-$

Second, we tested the migration and killing ability of Frα-CAR Jurkat cells expressing CCR7 on OVCAR-3 cells. The hypothesis for this stage of the study remained the same as the previous stage, which Frα-CAR Jurkat $CCR7^+$ cells would migrate more towards OVCAR-3 $CCL19^+$ cells and would have a higher cytotoxic effect on them compared to their co-culture with OVCAR-3 $CCL19^-$ cells. Luc2 expression of OVCAR-3 cells are demonstrated below (Figure 7).

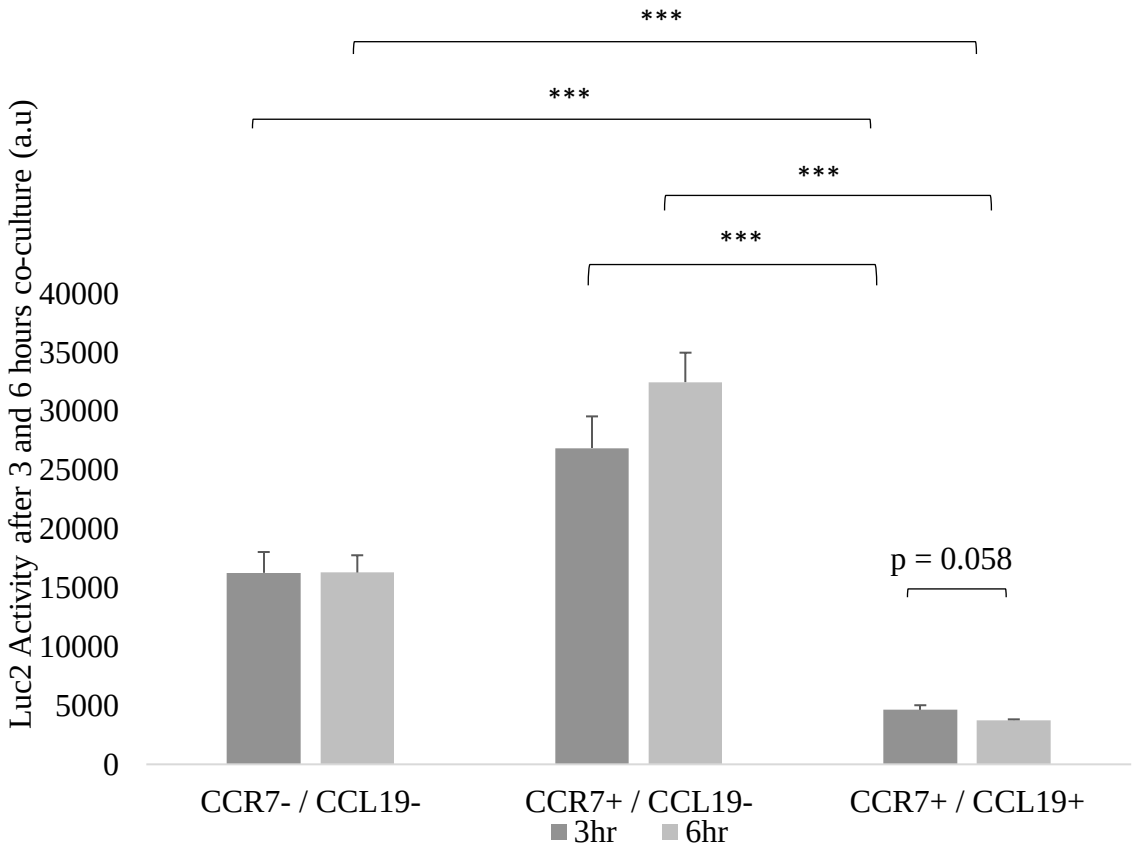

Figure 7. The migration ability and cytotoxic effect of CAR Jurkat CCR7+ and CAR Jurkat CCR7$^-$ on OVCAR-3 CCL19$^+$ and OVCAR-3 CCL19$^-$ are shown by the measurement of Luc2 expression of target cells. Luc2 expression of OVCAR-3 CCL19$^-$ cells co-cultured with CAR Jurkat CCR7$^+$ and CAR Jurkat CCR7$^-$ were measured as 26871 a.u. and 16267 a.u. at 3 hours, and 32467 a.u. and 16293 a.u. at 6 hours respectively. Luc2 expression of OVCAR-3 CCL19$^+$ cells co-cultured with CAR Jurkat CCR7$^+$ was measured as 4627 a.u. at 3 hours and 3760 a.u. at 6 hours. OVCAR-3 cells and CAR Jurkat cells were seeded at 10,000 and $1x10^6$ cells for each condition. Statistics were calculated by two-sample t-test assuming equal variances. Values are expressed as mean ± SD. P values: *P < 0.05, **P < 0.01, ***P < 0.001. a.u., arbitrary unit.

The Frα-CAR Jurkat CCR7$^+$ cells migrated more to OVCAR-3 CCL19$^+$ than OVCAR-3 CCL19$^-$ at both 3 and 6 hours. The difference at both 3 and 6 hours was extremely significant ($p = 0.00014$ and $p = 3.8x10^{-5}$). The cells' killing ability at 3 and 6 hours was same ($p = 0.058$, no significant difference). It was also found that Jurkat

$CCR7^+$ cells' killing ability on OVCAR-3 $CCL19^+$ was much higher than Jurkat $CCR7^-$ cells on OVCAR-3 $CCL19^-$. The difference between their cytolytic effect at 3 and 6 hours was extremely significant ($p = 0.00037$ at 3 hours and $p = 0.00012$ at 6 hours).

6 Discussion

Despite having unprecedented and unparalleled results in cancer treatments, CAR T cell therapies are not affordable for many patients and may have fatal side effects. Thus, Bhatnagar et al. studied T cell biofactory technology (45) and transformed T cells into living vectors to synthesize therapeutic proteins in situ when engaging with diseased cells in the body. Yet, this technology is very expensive and may have side effects as it uses Jurkat cells, an immortalized line of human T lymphocytes cells. This thesis aimed to create a cheaper and safer alternative to CAR T cell therapy by testing the hypothesis that K562 cells can be used as a cellular chassis to synthesize therapeutic proteins when encountering diseased cells. K562 cells used in this research were derived from a 53-year-old woman with chronic myelogenous leukemia in blastic crisis, and they can be easily manipulated and keep the expression of manipulated genes stable (70).

To evaluate the hypothesis, this study was divided into two parts. The first part investigated whether K562 cells would synthesize the engineered protein, Nanoluc luciferase, when interacting with antigen-presenting cells. The second part evaluated whether engineered Jurkat cells migrate to target cells with the help of chemokine receptors and if they have a cytolytic effect on them. If promising results from the second part were found, the same system could be implemented in K562 biofactories in later studies.

6.1 K562 Biofactory Function Evaluation

A brief ex-vivo stimulation of Jurkat and K562 biofactories was conducted using PMA ionomycin. PMA ionomycin formulation activates multiple intracellular signaling

pathways, resulting in the biofactories' activation. While there are other methods to stimulate cytokine production, the optimal method is to use this formulation, as found by Ai et al. in 2013 (71).

PMA activates protein kinase C (PKC), which has an essential role in mature T cell activation. When immune cells are engaged with antigens of cancer cells in normal conditions, CD3ζ, a part of CAR signaling, causes Ca^{2+} to rise within the cells. PKC enzymes are in turn activated by these signals. Consequently, T cells are activated. Alternately, ionomycin is a calcium ionophore that facilitates the calcium ion transfer into and out of cells. When ionomycin is combined with PMA, they activate NFκB and NFAT transcription factors, leading to cytokine production (72).

As previously mentioned in Materials and Methods for Specific Aim 1, the artificial cell-signaling pathway of both biofactories was composed of three constant domains (receptor, actuator, secretor) and two variable domains (sensor, effector). The receptor domain consisted of the CD3ζ signaling domain (causing Ca^{2+} to increase upon interaction with antigen-presenting cells), the CD28 that connects the intracellular and extracellular domains of CAR, and 4-1BB that enhances the therapeutic response of CAR. The actuator domain contained six copies of NFAT-RE (NFAT-RE6X), and IFN alpha-2 was selected as a secretor due to its high Nanoluc activity. Bhatnagar et al. evaluated three versions of the actuator domain (three, six, and nine copies of NFAT-RE), and five different signal peptides for the secretor domain (HSA, IL-6, IL-2, Gluc, and IFN alpha-2) (45). Specific to this study, the sensor part was derived from an anti-

Frα antibody to detect Frα receptor on target cells. The Nanoluc reporter enzyme was used as an effector.

As both biofactories were engineered with identical constant and variable domains, their structure was expected to be the same. Thus, it was anticipated that PMA ionomycin would activate them both. From the comparison of the Nanoluc expression by chemically stimulated and non-stimulated biofactories, it was concluded that biofactories activated by PMA ionomycin were able to synthesize Nanoluc whereas non-stimulated ones were not (Figure 3). This meant the engineering of K562 and Jurkat cells was successful, and the cells were ready to be co-cultured with antigen-presenting target cells and non-target cells to evaluate their function of Nanoluc synthesis *in situ* upon engaging with cancer cells.

The feasibility of the K562 cell biofactory's engineered protein synthesis for targeting diseased cells was compared to the Jurkat biofactory, the feasibility of which was already proven *in situ* upon engaging with ovarian cancer cells (45). Both biofactories were engineered to recognize a specific receptor on the cancer cells' surface, facilitating their protein expression after stimulation.

Two different ovarian cancer cell lines were used to prove the function of biofactories. The rationale behind the selection of two distinct ovarian cancer cell lines was the expression of a specific receptor, Frα. Frα is overexpressed on OVCAR-3 cells, and its expression on normal cells is limited (45, 73). Thus, OVCAR-3 cells were used as target cells (positive controls) as Frα would enable ligand-receptor recognition between

cancer cells and biofactories. A2780cis cells lacking Frα were used as non-target negative controls.

Upon engaging target cells and biofactories, it was expected that Frα on Jurkat-BF and K562-BF would engage the antigens on target diseased cells, which would, in turn, result in biofactory activation. Along with this activation, Nanoluc and IFN-α2 syntheses were upregulated. IFN-α2 (secretor peptide) was cleaved off, and Nanoluc (effector peptide) was transported in the extracellular space (45).

The results obtained from the co-culture of Jurkat-BF, with positive and negative controls, demonstrated that Frα found on the surface of OVCAR-3 cells enabled Jurkat-BF's stimulation and thus synthesized the Nanoluc reporter enzyme. Nanoluc enzyme, which produces bright and sustained luminescence (74), represented a quantifiable non-human effector protein. The Nanoluc expressed by Jurkat-BF co-cultured with OVCAR-3 cells was much greater than the Jurkat-BF and A2780cis co-culture at 24, 48, and 72 hours (Figure 4). The findings of Bhatnagar et al. (45), a similar study, support these results.

Even though both Jurkat and K562 cells were engineered with the same artificial cell-signaling pathway, co-cultured K562-BF and OVCAR-3 did not elicit similar results to the co-cultured Jurkat-BF and OVCAR-3. The results of the chemically stimulated K562 cells' expression of the Nanoluc enzyme showed that they were successfully stimulated by PMA ionomycin and synthesized the Nanoluc enzyme. However, when they were engaged with antigen-presenting target cells, the pathway was not activated, and K562 cells were not able to produce the Nanoluc enzyme. In other words, when PMA, a PKC

activator, was not added to the culture media, the engagement of K562 and target antigens was not sufficient to activate the cell signaling pathway. As such, the PKC enzymes were not activated by the insufficient initial Ca^{2+}rise, which would have driven the NFAT signaling pathway activity, in turn upregulating Nanoluc activity (45, 75).

These results should be taken into account when considering how other cells line can be substituted with T cells for immunotherapies. Since the constant domains of the signaling pathway of this study give the biofactory functionality, it is highly possible that at least one of the constant domains did not perform its function. As a result, the immunological synapse was not formed between K562 cells and target cells. An immunological synapse is an interface between antigen-presenting cells and lymphocytes such as T cells. For the integration of an immunological synapse, there are three broad categories of receptors: antigens, adhesion molecules, and costimulatory molecules, which are involved in T cell activation (46). Since Jurkat cells have all the machinery that Primary T cells have, the immunological synapse between Jurkat-BF and OVCAR-3 cells successfully formed. However, K562 cells do not express all the receptors that Jurkat cells do. As a result, it was concluded that K562-BF was not a good choice to be substituted with Primary T cells to recreate biofactory technology.

The ICAM-1 and LFA-3 adhesion molecules that are required to form an effective immunological synapse are expressed by K562 cells. However, K562 cells do lack many molecules that are necessary for the formation of the immunological synapse and the activation of biofactories. The co-stimulatory signal, the second signal to activate an immune response when engaged with antigen-presenting cells, depends on two co-

stimulatory molecules expressed by T cells. One of them is CD28, which was expressed by K562-BF as it was used to manipulate both biofactories. However, ICOS (Inducible co-stimulator or CD278), the second co-stimulatory molecule for T cell proliferation and cytokine secretion, was not expressed by K562 cells (46, 70).

Furthermore, K562 cells do not express the T cell receptors that enable T cells to form the complex with CD3, the interaction of which would trigger an antigen-specific immune response. Upon the formation of an immunological synapse, Lck (lymphocyte-specific protein tyrosine kinase) phosphorylates and activates ZAP-70, a protein whose activation is required for the induction of NFAT transcriptional activity (76). All these molecules have a specific function in the formation of an immunological synapse and, in consequence, the synthesis of therapeutic proteins. It is highly possible that the lack of these molecules is the reason that K562-BF did not synthesize the Nanoluc enzyme when engaging with target cells.

6.2 Function of Jurkat Cells that are Engineered to Express Chemokine Receptors

Chemokines are vital for many biological activities. The chemokine system represents a potential target for immunotherapy, and it is vital in preventing healthy tissue damage in the body. Chemokines, a large family of cytokines, control the migration and positioning of immune cells within the body. In this part of the study, we generated a second-generation Frα specific chimeric antigen receptor. The antigen recognition motif was composed of an anti-folate receptor alpha antibody. The signaling domains consisted of CD28 and CD3ζ.

Jurkat cells were engineered to express this CAR receptor along with the CCR5 and CCR7 chemokine receptors, in order to evaluate their migration and cytolytic ability on cancer cells. Whereas the CAR receptor gave Jurkat cells the killing ability, the anti-Frα antibody enabled them to recognize human ovarian cancer antigen, folate receptor alpha, on OVCAR-3 cells. The rationale behind the selection of CCR5 and CCR7 chemokine receptors was that CCL5 and CCL19, corresponding ligands of CCR5 and CCR7, are highly produced in the ovarian tumor environment (77, 78, 79). As a proof of concept, OVCAR-3 cells were also engineered to express CCL5 and CCL19 as their receptors enable Jurkat cells to migrate and bind to their corresponding ligands. Target cells were also engineered to express luciferase (luc2), which was measured to see whether OVCAR-3 cells were alive. Since luciferase activity is ATP-dependent, dead cells cannot produce bioluminescence. If the amount of luc2 were too high, it means OVCAR-3 cells are alive and able to produce luc2 enzyme.

Figure 6 and Figure 7 show the luc2 expression of OVCAR-3 cells that were co-cultured with Jurkat cells, expressing CCR5 and CCR7. The results and analysis supported the theory that Jurkat $CCR5^+$ and Frα-CAR Jurkat $CCR7^+$ cells migrated successfully towards OVCAR-3 $CCL5^+$ and OVCAR-3 $CCL19^+$ cells at 3 and 6 hours. Both cells were found to be responsive to their corresponding ligands. Aldinucci et al. (2020) and Cheng et al. (2020) supported the idea that CCR5/CCL5 and CCR7/CCL19 could be targeted in ovarian cancer (80,81). Both Jurkat cells, which express CCR5 and CCR7, had the same cytolytic ability on target cells at different hours (3 and 6 hours). It is therefore concluded that the cytolytic ability of Jurkat cells is not time dependent.

7 Conclusion

Options for low-cost manufacturing and safer cell therapies were evaluated throughout this research. To find a safer and cheaper alternative to CAR T cell therapies, K562 cells were explored whether to be used instead of T cells. The rationale behind using this cell line is that it has been approved as safe to be reinfused into the human body by the FDA. Also, it is cheaper compared to T cells. Therefore, therapies generated by K562 cells may be more affordable and safer for cancer patients.

Even though K562 cells were engineered with the same biofactory as Jurkat cells, they were not able to synthesize Nanoluc protein as Jurkat cells did. Jurkat cells, an immortalized line of T cells, have all the machinery that T lymphocytes have. As a result, when they encounter antigen-presenting cells, an immunological synapse forms and activates the biofactory. Upon activation of the biofactory, Jurkat cells synthesize engineered proteins. K562 cells lack some of the molecules and receptors vital for the formation of an immunological synapse. Thus, *in situ* upon interacting with antigen-presenting cells, intracellular signaling pathways were not activated. Therefore, the signaling pathways whose activation is necessary to produce therapeutic proteins were not activated either.

The study's second aim was to have effector cells migrated directly towards target cells without harming the healthy sides of the body by using the chemokine system. Effector cells and target cells were engineered to express chemokines and chemokine receptors respectively to bind one another. This part of the study showed that the

chemokine system is crucial to create safer therapies as they may prevent healthy tissue damage.

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Appendices

Appendix A Specific Aim 1. K562 Biofactory Function

Table 2 shows NanoLuc expression amounts of chemically stimulated biofactories.

Table 2. Means and Standard Deviation of Nanoluc Expression Measurements of Jurkat and K562 Biofactories

Effector Cells	Mean ($x10^6$ a.u.)		Standard Deviation ($x10^6$ a.u.)	
	PMA/Io	No treatment	PMA/Io	No treatment
Jurkat BF	82	2.4	1.4	0.11
K562 BF	57.4	0.421	4.7	0.064

Table 3 shows NanoLuc expression amounts of biofactories that interact with cancer cells.

Table 3. Means and Standard Deviation of Nanoluc Expression Measurements of Effector and Target Cells, and Their Co-culture

Co-culture Combinations	Mean ($x10^6$ a.u.)			Standard Deviation ($x10^6$ a.u.)		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
JK-BF / OVCAR-3	12	13.43	18.1	1.4	1.3	0.6
JK-BF / A2780cis	0.9	0.95	1.5	0.12	0.13	0.047
K562-BF / OVCAR-3	0.13	0.21	0.27	0.0092	0.014	0.13
K562-BF / A2780cis	0.16	0.17	0.1	0.03	0.035	0.031
JK-BF	2.1	4.5	7.1	0.4	1.4	1.04
OVCAR-3	0.21	0.21	0.021	0.003	0.004	0.006
K562-BF	7.1	7.8	7.6	0.19	0.21	0.29
A2780cis	0.025	0.035	0.049	0.0075	0.015	0.022

Table 4 introduces the Nanoluc enzyme expressions of the biofactories that were co-cultured with positive and negative controls.

Table 4. Nanoluc Expression Amounts After 24 hours of Co-culture of Target and Effector Cells (a.u.)

Effecter Cell Number	Target Cell Number	JK-BF / A2780cis	JK-BF / OVCAR-3	K562-BF / OVCAR-3	K562-BF / A2780cis
12500	976	0.59	10.57	0.18	0.164
12500	1953	0.74	13.71	0.14	0.135
12500	3906	0.75	13.84	0.13	0.152
12500	7812	0.92	13.8	0.14	0.173
12500	15625	0.95	15.69	0.12	0.144
12500	31250	1.12	14.31	0.107	0.113
12500	62500	1.34	14.88	0.094	0.111
12500	125000	1.63	16.751	0.125	0.152

Appendix B Specific Aim 2. Function of CAR Jurkat Cells Engineered with Chemokine Receptors

Table 5 and Table 6 introduce the Luc2 expression of the co-cultures of OVCAR-3 and Jurkat cells at 3 and 6 hours.

Table 5. Luc2 Expression by Target Cells' Co-cultured with Effector Cells Expressing $CCR5^+$ and $CCR5^-$ at 3 and 6 hours

Receptor and Ligand Combinations	3 hours		6 hours	
	Mean (a.u.)	Standard Deviation (a.u.)	Mean (a.u.)	Standard Deviation (a.u.)
$CCR5^-$ / $CCL5^-$	9329	1860	14849	220
$CCR5^+$ / $CCL5^-$	12356	4915	20516	1738
$CCR5^+$ / $CCL5^+$	2604	297	2498	311

Table 6. Luc2 Expression by Target Cells' Co-cultured with Effector Cells Expressing $CCR7^+$ and $CCR7^-$ at 3 and 6 hours

Receptor and Ligand Combinations	3 hours		6 hours	
	Mean (a.u.)	Standard Deviation (a.u.)	Mean (a.u.)	Standard Deviation (a.u.)
$CCR7^-$/$CCL19^-$	16267	1770	1629	1467
$CCR7^+$/$CCL19^-$	26871	2687	32467	2502
$CCR7^+$/$CCL19^+$	4627	394	3760	61