In Vivo Detection of CAR-T cell Immunotherapy using 3 Tesla Fluorine-19 MRI

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Abstract

Chimeric antigen receptor (CAR) T cell therapy is an emerging cancer treatment that has shown remarkable success in the treatment of B cell malignancies. However, this therapy still has the potential to cause severe toxicities or poor treatment responses in some patients. An imaging tool for tracking CAR-T cells could provide important patient-specific data on CAR-T cell fate. In chapter 2, fluorine-19 magnetic resonance imaging ($^{19}$F MRI) was evaluated as a method to track the location of $^{19}$F perfluorocarbon (PFC) labeled CAR-T cells non-invasively in a mouse model of B cell leukemia. We show for the first time that PFC labeled CAR-T cells can be detected with a 3 Tesla clinical MR scanner and that PFC labeled CAR-T cells show no significant difference in treatment response compared to unlabeled CAR-T cells as evaluated with bioluminescence imaging. Chapter 3 summarizes the study and discusses the limitations and future work.
Keywords

Chimeric Antigen Receptor T (CAR-T) Cell, Fluorine-19 Magnetic Resonance Imaging ($^{19}$F MRI), Perfluorocarbon (PFC), Bioluminescence Imaging (BLI)
Summary for Lay Audience

Chimeric antigen receptor T (CAR-T) cell therapy involves taking immune cells from a patient’s blood, reprogramming them to bind and kill cancer cells, and then injecting them back into the patient. This treatment has shown a lot of success in patients battling blood cancers such as leukemia, but current therapies still face problems such as harmful side effects and ineffective treatment of solid tumours. My project is to implement imaging techniques to track the fate of CAR-T cells after they have been injected into the body. Our technique will use fluorine-19 magnetic resonance imaging to allow us to see the location of the cells over time. Our methods will be useful for learning more about the negative treatment responses to CAR-T cell therapy and for building and assessing new CAR-T cell therapies that are safer and more effective against cancer. Importantly, this CAR-T cell tracking method should be highly translatable for use in patients receiving CAR-T cell therapies. This may eventually allow doctors to determine if the therapy will be effective at earlier time points so that they can continue or change the treatment plan, to better determine if a patient will have harmful side effects, as well as to better understand why the treatment might fail or succeed in certain patients.
Co-Authorship Statement

This thesis contains information that has been submitted for publication and that has been presented at multiple conferences.

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List of Abbreviations

19F Fluorine-19
1H Hydrogen-1
ALL Acute Lymphoblastic Leukemia
APC Antigen Presenting Cell
ATP Adenosine Triphosphate
BLI Bioluminescence imaging
bSSFP Balanced Steady State Free Precession
CAR Chimeric Antigen Receptor
CCD Charged Couple Device
cGMP current good manufacturing practice
CLL Chronic Lymphocytic Leukemia
CR Complete Remission
CRISPR/Cas9 Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR associated protein 9
CRS Cytokine Release Syndrome
CTC Circulating Tumour Cells
CTL Cytotoxic T Lymphocytes
D2O Heavy Water
DARPins Designed ankyrin repeat proteins
DLBCL Diffuse Large B-Cell Lymphoma
FA Flip Angle
FLuc Firefly Luciferase
Gd-EOB-DTPA Ethoxybenzyl Diethylenetriaminepentaacetic Acid
GE Gradient Echo
GFP Green Fluorescent Protein
HL Hodgkin's Lymphoma
hNIS Human Sodium Iodide Symporter
ION Iron Oxide Nanoparticle
IT Intratumoural
IV Intravenously
JAK-STAT Janus Kinases – Signal Transducer and Activator of Transcription Proteins
MHC Major Histocompatibility Complex
MRI Magnetic Resonance Imaging
NEX Number of Excitations
NFAT Nuclear factor of activated T cells
NHL Non-Hodgkin's Lymphoma
NK Natural Killer
NMR Nuclear Magnetic Resonance
NSG NOD-Prkdc<sup>scid</sup>-Il2rg<sup>tm1Wjl</sup>
OATP1B3 Organic Anion Transporting Polypeptide 1B3
PBMCs Peripheral Mononuclear blood cells
PBS Phosphate Buffer Saline
PCPEs Perfluoropolyethers
PCR Polymerase Chain Reaction
PET Positron Emission Tomography
PFC Perfluorocarbon
PFC+ PFC Labeled
PFOB Perfluorooctylbromide
PSMA Prostate Specific Membrane Antigen
RF Radiofrequency
ROI Region of Interest
RR Response Rate
ScFv Single Chain Variable Fragment
SCID Severe Combined Immunodeficiency
Sdev Standard Deviation
SE Spin Echo
SNR Signal-to-noise
SPIO Superparamagnetic Iron Oxide
STAT3 Signal Transducer and Activator of Transcription 3
T Tesla
TAA Tumour Associated Antigen
TAT Trans-activator of Transcription
TCR T Cell Receptor
tdT tdTomato
TE Time to Echo
TIL Tumour Infiltrating Lymphocyte
TR Repetition Time
TRUCKS T cells redirected for antigen-unrestricted cytokine-initiated killing)
Chapter 1

1 Introduction

This thesis develops and advances methods for tracking CAR-T cell therapies using fluorine-19 ($^{19}$F) magnetic resonance imaging (MRI) in a mouse model of B cell leukemia. The introductory chapter discusses B cell malignancies, T cell therapies, proton ($^{1}$H) MRI and $^{19}$F MRI, and bioluminescence imaging (BLI) techniques for cell detection. The purpose of the introductory chapter is to provide an introduction and motivation for the research project presented in this thesis.

1.1 Motivation and Overview

Cancer is a devastating disease with over 617 Canadians diagnosed every day$^{1}$. Current standard of care cancer treatments have improved patient outcomes in many cases, but there is still a large proportion of patients that do not survive for more than 5 years after their initial diagnosis. Cell-based immunotherapies are a relatively newer way to treat cancer by taking advantage of the natural functions of viable immune cells to target and kill cancer cells. These therapies often aim to boost the immune system’s response to cancer by having immune cells travel to cancer sites and mount an anti-tumor immune response. Chimeric antigen receptor (CAR) T cell therapies in particular have shown tremendous potential in patients leading to increased excitement about the potential for cell therapies to be a powerful option to treat many cancer subtypes$^{2,3}$. Unfortunately, these therapies still have a tendency to cause unwanted and sometimes serious side effects. In addition, some patients with blood cancers still show weak or no response to CAR-T cells and they are still showing disappointing results against solid tumours.
Current cell therapy monitoring in clinical trials relies heavily on blood tests and indirect measures of tumour size over time. These measures are unable to provide any information on the biodistribution of therapeutic cells or the number of cells in various important organs during treatment. Imaging methods to detect the locations and number of cells in the body would be extremely valuable for learning about therapeutic cell behaviours during negative side effects and treatment outcomes.

In this thesis, $^{19}$F MRI is used to detect CAR-T cells over time after intratumoural administration. Chapter 1 provides an overview of the background and motivation relevant to the work presented in this thesis. Chapter 2 examines the use of $^{19}$F MRI to detect perfluorocarbon (PFC) labeled CAR-T cells in a mouse model of B cell leukemia using a 3 Tesla (T) clinical scanner. It also compares the treatment response in mice administered PFC labeled (PFC+) CAR-T cells compared to mice that received unlabeled CAR-T cells using BLI. Chapter 3 summarizes the work in this thesis and its significance, expands on the limitations of this work, and discusses future work related to the results presented in this thesis.

1.2 B Cell Malignancies

Hematological (blood) cancer, including B cell malignancies, are the fourth most commonly diagnosed cancer type in Canada. Additionally, B cell malignancies are starting to affect more Canadians each year as the prevalence of these cancers increased by 25% between 2014 and 2016. These cancers are particularly devastating as they are one of the most common cancers found in children. B cell malignancies are characterized by their origin in B lymphocytes and are often referred to as “liquid cancers” due to their occurrence in the blood and lymph. B cells are an important
leukocyte population in the body that protect against pathogens by producing antibodies and releasing cytokines\textsuperscript{6}. B cell malignancies develop when B cells begin to expand rapidly and continue to grow past their normal life cycle, leading to the crowding of healthy cells. Patients often experience fever, frequent infections, and fatigue due to the accumulation and improper functioning of B cells in their body\textsuperscript{7}. B cell malignancies are subdivided into B cell leukemia and B cell lymphoma based on the characteristics of the disease.

1.2.1 B Cell Leukemia

B cell leukemia subtypes usually start in the bone marrow and are found in the blood, bone marrow, and spleen of patients\textsuperscript{8,9}. The two main types of B cell leukemias are B cell acute lymphoblastic leukemia (ALL) and B cell chronic lymphocytic leukemia (CLL). They are classified according to their rate of growth and their origin in the body. Chronic lymphocytic leukemia is characterized by abnormal mature B cell behaviour in the blood and develops more slowly\textsuperscript{10}. In comparison, acute lymphocytic leukemia develops more quickly as it is characterized by genetic mutations in immature B cells that cause them to stop maturing and continually proliferate\textsuperscript{11}.

When patients present with symptoms such as bone pain, bleeding gums or nosebleeds, fever, or frequent infections they may be tested for leukemia. Leukemia is most often diagnosed using blood tests or bone marrow biopsies. There are approximately 6 000 new diagnoses of leukemia per year in Canada alone. Staging for ALL is dependent on the maturity of the B cell and staging for CLL is based on the Rai system\textsuperscript{12}. The Rai system for CLL is dependent on the number of cancer cells in the blood, the degree to which the
spleen and lymph nodes are involved, and whether the patient has developed co-
morbidities such as anemia and thrombocytopenia. Currently, after diagnosis most
patients with leukemia undergo chemotherapy, radiation therapy, or stem cell
transplants. Leukemia tends to progress more quickly than lymphoma giving patients a
slightly worse prognosis. The success rates for leukemia treatments show that
approximately 61% of patients recover after treatment. The success rates are much less
promising if the cancer recurs though. In addition, the 5-year survival rate for patients
diagnosed with leukemia is only about 58% which is quite low compared to other cancers
such as breast cancer (80%), thyroid cancer (95%), and prostate cancer (81%).

1.2.2 B cell Lymphoma

B cell lymphomas are cancers that are found in the lymph nodes or other lymphatic
tissues when too many abnormal B cells are developed. There are two classifications of
lymphoma called Hodgkin’s lymphoma (HL) and Non-Hodgkin’s lymphoma (NHL). B
cell Hodgkin’s lymphoma is characterized by abnormal large B cells called Hodgkin and
Reed-Sternberg cells in the lymphatic system. Non-Hodgkin’s B cell lymphoma is
simply characterized by the abnormal growth of B cells that are not classified as Hodgkin
and Reed-Sternberg cells in the lymphatic system.

When patients present with symptoms such as fatigue, weight loss, fever, or many other
non-specific symptoms they may be tested for lymphoma. Lymphoma is most often
diagnosed by taking a biopsy of the affected lymph node(s). There are approximately 9
000 new diagnoses of lymphoma per year in Canada making it more common than
leukemia. Lymphoma is staged according to the number of sites that it has reached. Stage
I lymphoma is only present in one lymphatic site, stage II is found in two or more sites above the diaphragm, stage III is found in sites below the diaphragm, and stage IV is found in organs outside of the lymphatic system. After diagnosis patients with lymphoma may undergo chemotherapy, radiation therapy, stem cell transplants, surgery, or be administered drugs that prevent additional growth of abnormal cells. Patients diagnosed with B cell lymphoma have a slightly better prognosis than patients diagnosed with leukemia due to the slower progression of the disease. In general, approximately 70% of patients with lymphoma recover after treatment and the 5-year survival for HL is 85% and NHL is 66%\(^\text{15}\). Unfortunately, like leukemia, if the cancer recurs the chance of survival is greatly diminished. Treatments that could allow even larger proportions of patients diagnosed with B cell malignancies to recover and improve the outcomes of patients that experience recurrence would be extremely valuable. For this reason, it is important to continue to study therapies for B cell malignancies so that we can further improve the treatment outcomes of these patient populations.

1.3 T Cells

T cells are another type of leukocyte or white blood cell that acts as a specialized immune cell in the body\(^\text{19}\). The immune system provides our body with protection from bacteria, viruses, fungi, and other toxins. T cells specifically respond to foreign antigens present in the body to protect against virus infected and transformed cells. T cells are made in the bone marrow and then differentiate into cytotoxic (CD8) or helper (CD4) T cells in the thymus, hence their name. Figure 1.1 illustrates the mechanisms of action that T cells take to kill infected cells in the body. CD4 T cells protect the body from infection by
releasing cytokines such as IFN-γ, TNFα, IL-12, IL-4, IL-5, and IL-13 when they encounter antigens, which then activates other immune cells to orchestrate an immune response specific to the invader. CD8 T cells directly bind to cancer cells or infected cells to kill them through the Fas/Fas ligand pathway or, more commonly, the perforin/granzyme pathway. Briefly, the perforin/granzyme pathway is when activated cells secrete the membrane disrupting protein perforin and the serine proteases called granzymes by exocytosis causing apoptosis of the target cell. The Fas/Fas ligand pathway occurs when the Fas ligates to FasL and starts a cascade that leads to cell apoptosis. These cytotoxic functions only occur after the T cells have been activated which naturally occurs when two conditions are met. The first condition is that the T cell receptor (TCR) must bind to an antigen presented on a major histocompatibility complex (MHC) of an antigen presenting cell (APC). The second condition is that a CD28 molecule on the T cell must bind to a CD80 or CD86 molecule on the APC, or in some cases CD8 T cells can be activated by co-stimulation with CD70 or CD137 (4-1BB). In addition, co-stimulatory receptors such as 4-1BB, OX40, and ICOS can ensure survival of the T cell as long as the T cell strongly recognizes the antigen on the APC. The ability of T cells to recognize and respond to transformed cells is crucial for humans to remain cancer free. Unfortunately, cancer cells can evade T cell responses by downregulating the MHC class 1 molecules, inhibiting apoptotic pathways, and inducing anergy in T cells which allows them to tolerate cancer cell presence. Once this occurs, T cells cannot continue to recognize cancer cells and mount an effective cytotoxic response leading to uncontrolled cancer cell growth.
Figure 1.1: Effector T cell killing mechanisms. (1) Perforin (PFN)/granzyme (GzmB) pathway. (2) Fas Ligand (FasL)/Fas receptor pathway. (3) Release of cytokines such as IFNγ and TNFα.
1.3.1 T Cell Immunotherapies

T cell immunotherapies are a relatively new class of cancer treatment that takes advantage of the natural cytotoxicity of T cells. T cells are removed from a patient and modified ex vivo to give them advantages over naturally occurring T cells in the body before they are adoptively transferred back into patients\textsuperscript{26}. T cell therapies were first used to treat cancer patients in 1988 and since then many variations of T cell therapies have been developed and tested\textsuperscript{27}. Most notably there is tumour infiltrating lymphocyte (TIL) therapy, cancer antigen-induced specific T cell therapy, and engineered TCR T cell therapy. TIL therapy was the first T cell therapy developed and tested in patients\textsuperscript{28, 29}. They are produced by taking T cells from surgically resected tumours, isolating the TILs that show reactivity to cancer cells ex-vivo, and then expanding these TILs to produce a large population of tumour fighting T cells. This population of TILs is then re-infused into the patient to boost the T cell response against cancer cells. They have shown moderate responses in patients with melanoma with up to 50% response rates in patients when lymphodepleting chemotherapy was used, but only a 20% complete response rate was achieved\textsuperscript{30}. Similarly, cancer antigen-induced specific T cells can be used to fight cancer by making a cancer specific population of T cells ex vivo for adoptive transfer\textsuperscript{31, 32}. Cancer antigen-induced specific T cells are made by isolating T cells from patient peripheral mononuclear blood cells (PBMCs) or from resected tumours and then activating them with antigen presenting cells that present cancer antigens specific to the patient’s tumour type. Like TILs, this therapy has shown some success in patients with metastatic melanoma\textsuperscript{33}. The advantage of antigen-induced specific T cells over TILs is that they are easier to produce as patient T cells can be isolated from blood instead of a
tumour biopsy. Finally, engineered TCR T cell therapies are most often produced by isolating T cells from patient PBMCs, cloning an antigen specific TCR that was found in the patient’s PBMCs or TILs, engineering the T cells to express the cancer specific TCR using lentivirus or gamma retroviruses, and then expanding this population before re-infusion. The antigen specific TCR can also be produced by treating transgenic mice with cancer antigen. Clinical trials treating melanoma and colorectal cancer have shown some promise in the past with few reported toxicities. Unfortunately, the natural binding affinity of TCRs against cancer specific antigens is generally still low and there is the potential for severe side effects depending on the TCR chosen. Altogether, although there have been some successful outcomes using these T cell therapies in clinical trials, they are very time consuming to produce and tend to only elicit an effective response in a small portion of patients. CAR-T cells were recently developed to overcome some of the main limitations of previous T cell therapies by using custom designed receptors to specifically target cancer antigens.

1.4 Chimeric Antigen Receptor (CAR) T cells

CARs were first proposed by Gross et al. in 1989. The goal of the CAR is to accomplish specific targeting and killing of cancer cells by allowing for MHC independent target recognition and activation of T cells. As mentioned above, T cells are usually activated by an APC and then recognize cancer cells by binding to an antigen presented on an MHC molecule. This is often exploited by cancer cells as they can evade the immune system by downregulating their MHC-associated antigen presentation. In contrast, CAR-T cells recognize cancer cells independently of the MHC by directly
binding to a tumour associated antigen (TAA) that is specific to the CAR\textsuperscript{40}. CARs are fusion proteins that mediate T cell activation using a recognition domain and T cell signaling domains independent of the TCR (Figure 1.2). This allows for more timely activation of T cells directly in the presence of cancer cells and fewer instances of tumour escape\textsuperscript{41}. After activation, CAR-T cells can either directly kill cancer cells using the perforin and granzyme axis or the Fas and Fas ligand axis, or indirectly kill cancer cells by releasing cytokines that recruit other immune cells. Studies have shown that both CD4 and CD8 CAR-T cells most often exhibit cytotoxicity through the perforin and granzyme pathway\textsuperscript{42}. CD4 CAR-T cells express lower amounts of perforin and granzyme causing their cytotoxic response to be delayed compared to CD8 CAR-T cells. Despite their delayed target cell response, studies have shown that both subsets are important for proper target cell killing and that they kill equal amounts of tumour cells \textit{in vivo}\textsuperscript{43}. The ability of a CAR-T cell to persist in the body and continuously fight cancer is highly dependent on the components of their CAR which is further discussed below.
Figure 1.2: T cell and CAR-T cell activation and survival signaling. (a) T cells are activated by MHC interaction with the TCR (signal 1) and co-stimulation by CD80-CD28 interaction (signal 2). (b) CAR-T cells are more easily activated as they only require antigen specific interaction with a tumour associated antigen (eg. CD19).
1.4.1 CAR Design

Generally, CARs accomplish antigen specific activation using an extracellular domain, a transmembrane domain, and an intracellular signaling domain. The extracellular domain contains the antigen recognition domain that most often consists of a single chain variable fragment (scFv) derived from monoclonal antibodies to target a TAA. Recently a synthetic version of an antibody targeting domain has been developed that could be used in place of an scFv called designed ankyrin repeat proteins (DARPins)\(^4\). They may be advantageous because they are smaller, extremely stable, and can be used to make multispecific CARs which is a growing area of research. The transmembrane domain spans the cell membrane and is essential for transmission of the receptor-binding signal after the extracellular domain binds a TAA. Finally, the signaling domain is made up of a CD3-ζ subunit derived from the signaling domain in a TCR. It undergoes conformational changes when the CAR binds to a TAA allowing for downstream activation of the T cell\(^4\). The CAR signaling domain may also include the signaling endodomains of co-stimulatory molecules to mimic the co-stimulation (signal 2) that occurs during APC dependent T cell activation.

1.4.2 Generations of CAR-T cells

There are five generations of CAR-T cells to date with each generation offering additional features than the generation before it. First-generation CARs only contain an scFv and CD3-ζ signaling domain. Their lack of co-stimulatory molecules, a hallmark part of T cell activation, caused them to have limited activation potential. Without proper activation, first generation CAR-T cells had limited cytotoxicity and persistence\(^4\). This
limitation led researchers to develop the next generation CARs. Second generation CARs were designed to include a co-stimulatory molecule in the intracellular signaling domain to improve their activation potential and persistence\textsuperscript{47}. The most common co-stimulatory molecules used in CARs are 4-1BB and CD28\textsuperscript{48}. The 4-1BB co-stimulatory molecule is better at promoting long-term persistence of CAR-T cells and stimulating memory T cell generation\textsuperscript{49}. The CD28 co-stimulatory molecule is ideal for potent CAR-T cell cytotoxicity and IL-2 production. Third generation CARs were developed next in an attempt to further improve the signaling and survival of CAR-T cells. They contain two co-stimulatory domains such as OX40 and CD28 instead of just one costimulatory domain to enhance the survival of the CAR-T cells\textsuperscript{50}. Current data is showing that including two co-stimulatory domains does not appear to increase the efficacy of the therapy compared to second generation CARs\textsuperscript{51}. Fourth generation CAR-T cells, often called TRUCKS (T cells redirected for antigen-unrestricted cytokine-initiated killing), have been developed in an attempt to improve CAR-T cell efficacy against solid tumours\textsuperscript{52}. Fourth generation CARs use the framework from second-generation CARs that contain only one co-stimulatory molecule. They combine the CAR response with a nuclear factor of activated T cells (NFAT) domain that releases cytokines such as IL-12 to make CAR-T cells more resistant to the immunosuppressive tumour microenvironment\textsuperscript{53}. Recently, fifth generation CARs were developed to try to further improve the survival of CAR-T cells. They use the framework of a second-generation CAR with the addition of a fragment of IL-2 receptor β (IL-2Rβ) that can bind to STAT3\textsuperscript{54}. This domain is able to activate the JAK-STAT pathway which is important for preventing terminal differentiation of the T cells. Overall, there are still many studies
needed to fully understand the mechanisms of activation and cell death achieved by CAR-T cells, but their potential is undeniable based on clinical trials.

1.4.3 CAR-T Cell Production

CAR-T cell therapies are produced in current good manufacturing practice (cGMP) facilities that are equipped to ensure patients are receiving sterile and high-quality therapies\textsuperscript{55}. Figure 1.3 illustrates the CAR-T cell manufacturing steps. Autologous T cells are most often used for CAR-T cell production to avoid graft versus host disease\textsuperscript{56}. T cell collection begins with leukapheresis which is a process that removes leukocytes including T cells from the patient’s blood before returning the blood back to the patient\textsuperscript{57}. Once T cells have been isolated, they are expanded by activating them through their T cell receptor (signal 1) and their co-stimulatory domain (signal 2). Multiple companies sell products for T cell activation such as CD3/CD28 antibody coated magnetic beads, antibody coated nanobeads, anti-CD3 antibodies, expamer technology, and antigen presenting cells. CD3/CD28 coupled magnetic beads called Dynabeads were used in this thesis as a convenient and easily removable method for T cell activation\textsuperscript{58}. In addition, T cells are cultured in media supplemented with IL-2 to encourage proliferation. After activation, T cells are genetically modified to express a CAR specific to the patient’s cancer type. Stable CAR expression in T cells is most often accomplished using lentiviral or gamma retroviral vector transduction\textsuperscript{59}. Gamma-retroviral vectors were the first method used to generate CD19 CAR-T cells for clinical use. Gamma-retroviruses are a valuable option for clinical CAR-T production because they are easier to produce, but
they can only transduce dividing cell populations and they pose a larger risk for insertional oncogenesis\textsuperscript{60}. Currently, the most popular method for CAR transduction in clinical trials is lentiviral vectors\textsuperscript{61}. Lentiviral transduction can be performed in any phase of the cell cycle and their gene integration occurs in safer locations compared to other viral transduction methods. After transduction, the engineered CAR-T cells need to be expanded to obtain large populations for adoptive transfer. For clinical manufacturing of CAR-T cells, billions of CAR-T cells are required which has led to the use of bioreactors that mimic ideal body conditions for cell growth\textsuperscript{62}. Bioreactors are capable of consistently feeding the cells, removing waste, and rocking the cells back and forth to achieve gas transfer allowing for rapid expansion of CAR-T cells. After expansion, the CAR-T cells are used for treatment as long as they pass all of the safety, identity, and purity testing checkpoints during production\textsuperscript{63}. The production and characterization of CAR-T cells is largely the same regardless of the CAR that is being used in the study.
Figure 1.3: CAR-T cell production process. Blood is removed from a patient and then T cells are isolated from the blood. Next, T cells are engineered to express an antigen specific CAR and then expanded. After expansion, the CAR-T cells are delivered to the patient for cancer treatment.
1.4.4 CAR-T Cell Administration

Patients are often treated with chemotherapy for lymphodepletion prior to CAR-T cell adoptive transfer\textsuperscript{64}. Lymphodepletion is the destruction of lymphocytes including T cells, B cells, and NK cells in the body. It helps in adoptive cell transfer-based therapies by removing regulatory T cells, suppressing the patient’s immune system, and activating antigen presenting cells. Once lymphodepletion is performed, that patient is ready for their CAR-T cell infusion. The CAR-T cell dose that patients receive ranges from $1 \times 10^4$ to $1 \times 10^{10}$ cells/kg in clinical studies and they are most often administered intravenously (IV) in one dose using a slow infusion\textsuperscript{65,66}. Studies have also begun to test the efficacy of local or intratumoural (IT) injections of CAR-T cells or injecting the CAR-T cells in multiple slow infusions at different time points\textsuperscript{67,68}. Ideally, CAR-T cells should remain in the patient after injection until the cancer is eradicated\textsuperscript{69}. Tests have shown that they can survive anywhere from 6 weeks to 5 years in patients, although more consistent follow ups with patients after treatment will continue to improve this data\textsuperscript{70-72}.

1.4.5 CAR-T Cell Therapy Outcomes

There have been many TAA specific CAR-T cells studied to target a large variety of cancer types since the first development of CAR-T cells. The most notable CAR-T cell therapies to date are second generation CD19 targeting CAR T cells as they were the first to make it to large scale clinical trials in patients. They target the CD19 antigen that is found in low levels on normal B cells but is over expressed on B cell malignancies. CD19
is an ideal target because it is only expressed by B cells which means off-target cell killing during treatment will be contained to the B cell population. Most clinical studies use different variations of CD19 CAR-T cells which has an effect on the outcome of the study. In general, CD19-CAR-T cells are showing a response rate (RR) of 72% in patients with B cell malignancies across all clinical trials which is especially impressive as these patients did not respond to or relapsed after traditional cancer treatments. In particular, patients with B cell ALL have shown amazing results with complete remission (CR) rates of up to 93% in recent clinical trials. Interestingly, comparisons between CR rates in a recent systematic review of CD19 CAR-T cell clinical trials treating ALL showed a difference between the treatment outcomes in patients treated with 4-1BB containing CARs (CR=86%) compared to CD28 containing CARs (CR=74%). In addition, diffuse large B-cell lymphoma (DLBCL), which is a form of NHL, has shown impressive CR rates of approximately 54% in patients with refractory disease using CD19 CAR-T cells. These trials resulted in Health Canada approval of two second generation CD19 CARs for the treatment of specific B cell malignancies. Kymriah (CTL019, Tisagenlecleucel) developed by Novartis was the first CAR approved by Health Canada in 2018 to treat children and young adults with B cell ALL who relapse or are not eligible for stem cell transplants. Yescarta (KTE-C19, Axicabtagen ciloleucel) developed by Kite/Gilead was approved next by Health Canada in 2019 to treat patients with relapsed or refractory DLBCL. These therapies differ slightly as Kymriah is 4-1BB driven and engineered using lentivirus and Yescarta is CD28 driven and engineered using retrovirus. Since their approval, developments have begun to create opportunities for the production and use of these therapies in Canada. In addition to CAR-T cells that
target B cell malignancies, there have also been minor successes in clinical trials using CAR-T cells that target solid tumours. For example, the use of GD2 targeting CAR-T cells to fight neuroblastoma in a recent clinical trial led to response rates of approximately 33%\textsuperscript{80}. Overall, CAR-T cell therapies are still not showing very much efficacy against solid tumours with an average response rate among clinical trials of 9%.

1.4.6 CAR-T Cell Limitations

CAR-T cell therapies tend to cause a range of side effects during treatment. Studies have shown that over 85% of patients treated with CAR-T cells experience at least one adverse event\textsuperscript{77}. One of the common side effects during treatment is cytokine release syndrome (CRS). CRS causes a range of symptoms that can be lethal in severe cases\textsuperscript{81}. The symptoms of CRS include fever, hypotension, organ dysfunction, and increasing oxygen requirements of the body. The cause of CRS has been linked to high levels of cytokines such as interleukin-6 which may be elevated directly due to the anti-tumour response of CAR-T cells. Another common side effect after CAR-T cell transfer is tumour lysis syndrome\textsuperscript{82}. Tumour lysis syndrome describes the metabolic complications that occur due to the breakdown of a large number of dying cells. Most often patients experience acute kidney failure, cardiac dysfunction, seizures, or nausea. Patients may also experience neurotoxicity during CAR-T cell treatment which also ranges in severity but tends to cause encephalopathy, seizures, delirium, agitation, and headaches\textsuperscript{83}. In addition to the side effects caused by CAR-T cells, there are also direct cytotoxicities caused by CAR-T cells acting on healthy cell populations in patients\textsuperscript{84}. On target/off tumour CAR-T
cell responses are extremely common as most TAAs are also expressed at lower levels on healthy cells in the human body. In the case of CD19 targeting CAR-T cells, CD19 is an excellent TAA because it is overexpressed on B cell malignancies, but it is also expressed on healthy B cells. Therefore, during treatment with CD19 CAR-T cells the patients will experience B cell aplasia\textsuperscript{85}.

Another limitation of CD19 CAR-T cells is that they do not mount an effective response in every patient. For example, there are still approximately 30\% of patients with B cell malignancies that do not respond to CD19 CAR-T cell therapies. Even in successful cases, relapse still occurs in up to 50\% of patients that responded to the therapy\textsuperscript{86}. These sub-optimal CAR-T cell responses may be due to a lack of CAR-T cell expansion or persistence after injection, but there is limited patient specific data to support this\textsuperscript{87}. Although these values are still much more encouraging than the response rates of CAR-T cells targeting solid tumours, further optimization of CD19 CAR-T cell therapies could continue to improve patient outcomes.

1.4.7 CAR-T Cell Monitoring

In order to effectively optimize current and emerging CAR-T cell therapies, accurate data on the fate of T cells needs to be available after injection. CAR-T cell monitoring is required to gain knowledge of CAR-T cell biodistribution and persistence in patients and learn how these characteristics correspond to treatment outcomes and side effects. Currently, blood tests are used to monitor CAR-T cell response after adoptive transfer
into patients. These blood tests employ flow cytometry, polymerase chain reaction (PCR), or a combination of both to determine the persistence and expansion of CAR-T cells in the blood after injection. Blood tests are a relatively easy and cost-effective method for determining number of circulating T cells, but they cannot provide any real time information on the location of CAR-T cells in the body. This leads to a lack of data on the number of CAR-T cells that have made it to cancer locations compared to the number of CAR-T cells in off target locations. Minn et al. showed that the number of circulating CAR-T cells detected using blood tests did not correlate to the number of CAR-T cells that were present in tumours based on positron emission tomography (PET) images. This suggests that the number of CAR-T cells detected in peripheral blood may not be a reliable method for predicting treatment response. In addition, blood tests are unable to predict neurotoxicity or CRS severity at this time due to limited access to IL-6 testing and no current available markers for neurotoxicity.

Studies have begun to look at molecular imaging techniques to complement current clinical blood tests. The rationale is that they can provide patient specific information on the location(s) and persistence of CAR-T cells during treatment. PET is a widely used clinical imaging modality because of its high sensitivity and tissue penetration in patients. Sellmyer et al. used PET to track escherichia coli dihydrofolate reductase enzyme (eDHFR) expressing CAR-T cells in a mouse model of osteosarcoma after an infusion of [18F]-trimethoprim (TMP). They successfully detected the presence of CAR-T cells in cancer locations in mice over time. They also noticed that they could detect CAR-T cells in the spleen at earlier timepoints before tumour invasion occurred. Minn et al. used PET to track prostate-specific membrane antigen (PSMA) expressing CAR-T
cells in a model of leukemia after an infusion of $^{18}$F-DCFPyL\textsuperscript{89}. Early imaging time points showed expansion of CAR-T cells in the bone marrow and later images showed accumulation of CAR-T cells in the tumour proving that this imaging technique can monitor treatment progression over time. A clinical study has also been performed by Yaghoubi et al. to detect cytotoxic T lymphocytes (CTLs) that were labeled with herpes simplex virus 1 thymidine kinase (HSVtk1) using $^{18}$F-FHBG and PET in a patient with glioblastoma\textsuperscript{93}. They were able to detect the intracranially injected CTLs 2 hours after an injection of $^{18}$F-FHBG. They found that the CTLs had localized in the location of a resected tumour as well as the new tumour that they were targeting. The images confirmed that their injection was effective and that the cells were able to migrate to tumour locations. Despite promising results with PET, there is often background uptake of the tracer in organs such as the stomach, thyroid, and kidneys which may mask the signal of CAR-T cells in these locations. In addition, longitudinal cell tracking studies are limited using PET due to radiation dose concerns for both the patients and the adoptively transferred cells\textsuperscript{94}.

MRI may be advantageous over PET for CAR-T cell tracking as scans are performed using non-ionizing radiation leading to fewer concerns when planning longitudinal cell tracking ventures. MRI also has excellent soft tissue differentiation and resolution compared to PET. Brewer et al. used MRI to track super paramagnetic iron oxide (SPIO) nanoparticle labeled CTLs in a mouse model of cervical cancer\textsuperscript{95}. They were able to detect CTLs in the tumours 24 hours after injection and distinguish differences in the location of the CTLs based on whether or not the mouse was given a cancer vaccine prior to labeled CTL treatment. In addition, Lui et al. tracked T cells using MRI by labeling
them with nano-sized iron oxide nanoparticles prior to injection. Their nanoparticle achieved an impressive labeling efficiency of over 90% in T cells and enabled the detection of the T cells in vivo\(^96\). Limitations of using iron oxides are that they cause hypointensities in images. Hypointense regions are not specific to iron labeled cells and, therefore, do not allow for quantification of adoptively transferred labeled cells. There have not been any clinical studies tracking T cells with MRI to date, but there is a lot of potential in this field as other therapeutic cell populations have been successfully tracked using cellular MRI\(^97-99\). One of the most recent promising methods for tracking CAR-T cells in clinical settings is fluorine-19 (\(^{19}\)F) MRI which combines the anatomical information of \(^{1}\)H MRI with the detailed “hotspot” imaging of \(^{19}\)F MRI.

### 1.5 Magnetic Resonance Imaging

MRI provides excellent soft tissue contrast allowing for the generation of highly detailed anatomical images. MRI was successfully used to image human anatomy for the first time in 1977 based on the principles of nuclear magnetic resonance (NMR)\(^{100}\). NMR relies on the principle that nuclei in the presence of a magnetic field can absorb energy from radiofrequency (RF) pulses that are generated at frequencies specific to the nuclei of interest called the larmor frequency\(^{101}\). The absorbed energy is then dissipated to form signal when the RF pulse is turned off. This detected signal can then be used to determine the number of nuclei present in a sample as long as the properties of the sample such as relaxation and chemical shift are known. MRI most commonly relies on the excitation of protons (\(^{1}\)H) to form signal\(^{102}\). \(^{1}\)H are the most widely used nuclei for MRI because they make up a large portion of the human body and they have a high gyromagnetic ratio of 42.58 MHz/T. The three main components of an MR scanner are the magnet, the gradient
coils, and the RF coil. Each of these parts work together to manipulate nuclei and in turn obtain signal.

Many nuclei such as $^1$H naturally possess a quantum mechanical property called “spin” (Figure 1.4A)$^{103}$. These nuclei have a net magnetization vector ($M$) which is the sum of all of the magnetic moments of each nuclei in a given region. When nuclei are not in a magnetic field, they have a net magnetization vector of zero because the individual magnetic moments are randomly oriented$^{104}$. The magnet which can range between clinical field strengths of 1.5-3 Tesla (T) or pre-clinical field strengths of 7-11.7T creates a magnetic field that causes the nuclei to obtain a small non-zero net magnetization. Once the majority of the nuclei align with the main magnetic field in the scanner, a RF pulse can be applied to excite the nuclei, changing the orientation of the net magnetization vector (Figure 1.4B)$^{105}$. This net magnetization vector then relaxes back to equilibrium via transverse relaxation and longitudinal relaxation which generates detectable signal when the RF pulse is turned off (Figure 1.4C). Transverse relaxation is described by $T_2$ which is the transverse relaxation time constant or spin-spin relaxation time constant. $T_2$ relaxation describes the dephasing of spins caused by energy transfer between neighbouring nuclei resulting in the decay of transverse magnetization. The transverse magnetization decays exponentially with time as a result of the properties of tissues. The longitudinal relaxation of the nuclei is described by $T_1$ which is the longitudinal relaxation constant or spin-lattice constant. Longitudinal magnetization recovers back to equilibrium independently of the transverse magnetization. $T_1$ relaxation describes the transition of excited spins back to a lower energy states to achieve thermal equilibrium$^{106}$. 
Figure 1.4: Nuclear precession and T1 and T2 Relaxation. (a) The behaviour of a proton placed in a magnetic field ($B_0$) where $\omega_0$ is the Larmor frequency. (b) An RF pulse causes excitation of the protons. When the RF pulse is removed, the protons relax through T1 and T2 relaxation. (c) A graphical representation of T1 (Longitudinal) and T2 (Transverse) relaxation.
1.5.1 Image Contrast

$^1$H nuclei in different tissues relax at different rates which can be taken advantage of to create contrast in MR images\textsuperscript{107}. Contrast in MR images can be weighted depending on proton density (PD), T1 relaxation time, or T2 relaxation time. PD weighted images have higher signal in areas that are proton dense. T1 weighted images have higher signal in regions with shorter T1 relaxation such as fat and lower signal in areas with longer T1 relaxation times such as water. T2 weighted images have higher signal in areas with a longer T2 relaxation time such as fluid and lower signal in areas with shorter T2 relaxation times such as fat. Many tumours for example appear darker in T1 weighted images and brighter in T2 weighted images.

1.5.2 Magnetic Field Gradients

Magnetic field gradients are essential for MRI because they allow for spatial localization and encoding of information\textsuperscript{108}. Gradient coils are independently pulsed to create small, localized field gradients that can be used in conjunction with RF pulses to target the excitation of spins to certain locations. To form an image, an anatomical slice is excited in a plane of interest and then information encoding is applied along the other two directions using frequency and phase encoding gradients. Generally, the plane of interest is along the z direction, the phase encoding is along the y direction, and the frequency encoding is along the x direction. Frequency encoding gradients change the larmor frequency of the nuclei in a gradient along the x direction to elicit a signal that is based on the location of the nuclei along the slice. Phase encoding gradients are turned on independently of the frequency encoding gradients to change the larmor frequency in the
y direction to provide additional information on the location of the nuclei based on their phase. When RF pulses are turned off, all of this information is collected by radiofrequency coils to create MR images.

### 1.5.3 Radiofrequency Coils

RF coils are responsible for the excitation and subsequent detection of energy from the nuclei\(^{109}\). The two main RF coils are surface coils and volume coils\(^{110}\). Volume coils are more commonly used because of their ability to obtain uniform signal that does not depend on depth. In cases where deep tissues are being probed such as the head or knee it is especially important to use a volume coil. Surface coils have higher sensitivity but there is signal drop off as the distance between the coil and tissue is increased. Surface coils are most often used for imaging superficial anatomy such as the spine or temporomandibular joint. In this thesis, a surface coil was used as our imaging was focused on subcutaneous tumours so signal drop off with imaging depth was not of concern. In addition, the coil we used is dual tuned to enable \(^1\)H MRI and fluorine-19 (\(^{19}\)F) MRI so it was important to maximize signal.

### 1.5.4 Pulse Sequences

Pulse sequences are a set of instructions for the RF coil and gradients to follow to generate specific MR images. The main characteristics of a pulse sequence are time to echo (TE), repetition time (TR), and flip angle (FA). Generally, TR is the time between the start of an RF pulse and the start of the next RF pulse applied to the same slice, TE is the time between the start of an RF pulse and the peak of the echo detected, and FA is the angle of the pulse applied to flip the net magnetization vector. Spin echo (SE) and gradient echo (GE) sequences are the most common pulse sequences. SE sequences are
generally used to create T2 weighted images\textsuperscript{111}. They are insensitive to magnetic field inhomogeneity, which is useful for avoiding image artifacts, but the image acquisition takes longer. GE sequences are faster because they have shorter TR, but they are more sensitive to susceptibility artifacts. The sequence that our lab is most interested in is a GE sequence called balanced steady state free precession (bSSFP). It is also referred to as TrueFISP, FIESTA, or balanced FFE depending on the manufacturer of the MRI. The bSSFP sequence begins with a fast train of pulse sequences that do not allow time for the transverse relaxation to decay and thus, a steady state of magnetization is reached\textsuperscript{112}. This allows for high signal to noise ratio and fast image acquisition. bSSFP provides mixed T2/T1 contrast images which limits some of the diagnostic uses of the sequence but still provides useful anatomical images. Once the MRI protocol is completed, all of the information that was collected into the MR system processor is automatically processed using fourier transforms for image reconstruction. Post processing tools such as Horos or Osirix can then be used to analyze image characteristics.

\textbf{1.6 \textsuperscript{19}F Magnetic Resonance Imaging}

In addition to protons, there are other nuclei that can be used for MRI such as \textsuperscript{19}F, Carbon-13, Sodium-23, and Phosphorus-31. Of these additional nuclei, \textsuperscript{19}F is considered one of the best candidates for MRI because it has a gyromagnetic ratio of 40.08 MHz/T and a sensitivity of 83\% when compared to protons\textsuperscript{113}. In addition, there are only trace amounts of \textsuperscript{19}F in the bones and teeth of humans which is below the detection limits of MRI allowing for background free “hotspot” imaging of \textsuperscript{19}F. The “hotspot” data can then be overlayed onto \textsuperscript{1}H images to provide anatomical context\textsuperscript{114}. \textsuperscript{19}F MRI was successfully
developed by Holland et al. in 1977 and since then has become a popular imaging modality for cell tracking\textsuperscript{115}. It has been used by multiple groups to provide valuable information on adoptively transferred labeled cell populations\textsuperscript{116}. $^{19}$F cellular MRI has the advantage of specific, background free detection of $^{19}$F labeled cells. The “hot spot” characteristic of $^{19}$F MRI is highly valuable when compared to other MRI contrast agents including iron oxide nanoparticles that indirectly cause negative contrast in MR images or manganese and gadolinium that indirectly cause positive contrast in MR images. Although the sensitivity of $^{19}$F MRI is relatively low compared to other imaging methods, it can still be used to detect thousands of labeled cells\textsuperscript{117}. $^{19}$F MRI was proven to be valuable for clinical cell tracking ventures when Ahrens et al. used it to detect perfluorocarbon (PFC) labeled dendritic cells in patients\textsuperscript{118}.

$^{19}$F MRI is performed on standard MR scanners using an RF pulse specific to the resonant frequency of $^{19}$F nuclei\textsuperscript{119}. Specialized RF coils have been designed that can perform dual $^{1}$H and $^{19}$F MRI by emitting and receiving at both frequencies. Since $^{19}$F MRI directly detects the spin density of $^{19}$F nuclei, the contrast in $^{19}$F images is based on the density of $^{19}$F nuclei per voxel in the body. The linear relationship between $^{19}$F content in a voxel and $^{19}$F signal and the lack of endogenous $^{19}$F signal in the body allows $^{19}$F MRI to be quantitative\textsuperscript{120}. The number of $^{19}$F spins in a given region of interest can be calculated using the signal obtained from reference tubes containing a known amount of $^{19}$F. If cells are labeled ex vivo prior to adoptive transfer, there is also the potential to calculate cell number based on the $^{19}$F images. Calculating cell number requires three pieces of information: 1) The number of $^{19}$F spins per cell prior to injection as determined by $^{19}$F NMR, 2) the signal obtained from the region of interest containing the $^{19}$F labeled
cells in the MR image, 3) the signal obtained from the reference tubes with known \( ^{19}F \) content\(^{121} \). One major limitation to the cell number quantification is that dividing cell populations will not be accurately quantified over time.

### 1.6.1 Cell Labeling for \(^{19}F\) MRI

PFCs are tracer agents with a strong C-F bond making them stable at physiological pH and non-toxic in humans\(^{122} \). They have historically been used in the clinic as blood substitutes because of their ability to dissolve oxygen. They have also been co-opted by imaging scientists as a cell label to enable cell detection with \(^{19}F\) MRI. Perfluorooctylbromide (PFOB) was one of the first PFCs that was used extensively in patients as a blood substitute and in clinical trials for inflammation and bowel imaging\(^{123} \). Unfortunately, PFOBs showed 8 peaks in an NMR spectrum making SNR and image quality worse. This led to the development and use of Perfluoropolyethers (PCPEs). PCPEs have one main peak on a \(^{19}F\) NMR spectrum and they have high fluorine content\(^{124} \). The ideal PFC tracer for clinical cell tracking would be commercially available and extensively studied. While there are many different possible formulations for \(^{19}F\) PCPEs, this thesis focuses on the chemically modified PCPEs made by CelSense as they are commercially available and well-studied for \(in vivo\) cell tracking applications. CelSense \(^{19}F\) PFC emulsions have high fluorine content to enable \(^{19}F\) MRI and are available with fluorescence probes to enable detection with flow cytometry or histochemistry. They also have a small diameter of 165nm and a slightly negative charge to enable cell uptake by endocytosis\(^{125} \). They have been FDA approved in the United States and used in clinical trials in patients\(^{126} \). They show very low toxicity in patients
and no significant toxicities in many cell lines. Cell labeling is accomplished by simple co-incubation of the PFCs and the cell of interest. After labeling, PFCs stay in a labeled cell as long as the cell remains viable and is divided between daughter cells upon cell division. When a labeled cell dies the label is released and quickly cleared by the reticuloendothelial system (RES) to the liver where they will slowly be released into the blood and exhaled. There is also a chance that they may be taken up by phagocytic bystander cells such as macrophages. Recently, Helfer et al. showed that labeling T cells with $^{19}$F PFCs is possible at a large clinical scale without harming the final product.

### 1.6.2 CAR-T Cell Imaging with $^{19}$F MRI

$^{19}$F MRI has been used to detect the location and number of many immune cell types to date. In the past 5 years, CAR-T cells have been successfully labeled with $^{19}$F PFCs and detected using $^{19}$F MRI *in vivo*. Below is a summary of recent advances in imaging CAR-T cells using $^{19}$F MRI.

In 2017, Chapelin et al. were the first to PFC label CAR-T cells and confirm intracellular localization of the PFC. After successfully labeling the CAR-T cells without any negative effects on the CAR-T cell phenotype, they injected them into a mouse model of glioblastoma. The study showed that PFC+ CAR-T cells slow down the progression of cancer in their model compared to mice that received untransduced T cells or no treatment. They were also able to detect the presence of PFC labeled CAR-T cells in
tissue samples of the liver, lungs, tail, spleen, lymph nodes, thymus, tumours, and kidneys of treated mice up to 14 days post injection using ex vivo NMR.

Hingorani et al. used trans-activator of transcription (TAT) conjugated PFC nanoemulsions to label and track CAR-T cells in a mouse model of glioma\textsuperscript{133}. They imaged the cells 24 hours after an intratumoural injection using an 11.7T scanner and found that they could detect almost 100% of the injected cells. This study was the first to show detection of PFC+ CAR-T cells with \textsuperscript{19}F MRI.

Very recently, Chapelin et al. imaged PFC+ CAR-T cells over time in a mouse model of glioblastoma using an 11.7T MRI\textsuperscript{134}. They were able to show for the first time that intratumourally injected CAR-T cells could be detected in tumour sites using \textsuperscript{19}F MRI up to day 10 post-treatment using an 11.7T scanner. They also looked at the intracellular partial pressure dynamics of CAR-T cells using \textsuperscript{19}F MRI.

Each of these studies made important progress in CAR-T cell tracking using \textsuperscript{19}F MRI. The main limitation for clinical translation of these studies is that they used field strengths well above clinical standards to improve signal detection. The sensitivity is improved at higher field strengths allowing for shorter scan times and fewer cells to be detected. This thesis is focused on imaging PFC+ CAR-T cells using clinical 3 T MRI and a human surface coil. By using clinical field strengths, we continue to gain valuable information on this imaging technique that will improve the chances of clinical implementation of CAR-T cell tracking using \textsuperscript{19}F MRI.
1.7 Bioluminescence Imaging

Bioluminescence imaging (BLI) is a valuable pre-clinical imaging technique to track proliferating cells over time *in vivo*. BLI reporter genes are stably expressed in cell populations so that they are passed down to daughter cells during cell division which allows them to be continually detected. BLI has been adapted by many researchers due to its low cost, high sensitivity, and high specificity\textsuperscript{135}. The BLI system is composed of a black box imaging chamber with a heated stage where the subject is placed during imaging and a cooled charged couple device (CCD) camera that collects the emitted light from the subject. The system first acquires a photographic image for reference and then overlays the detected BLI signal onto the photograph\textsuperscript{136}. Imaging time can be automatically determined by the BLI software and usually ranges from seconds to minutes depending on the amount of signal coming from the subject. Tracking cells is of particular interest using BLI because the characteristics of signal generation cause only viable engineered cells to be detected. This is a characteristic of the enzymatic reaction that takes place between the engineered cells and the injected substrate. For cell tracking purposes, cells are engineered to stably express luciferase genes prior to injection into the animal model. Bioluminescence images are then acquired by first administering the substrate to the subject to produce signal from the engineered cells. Mice are then imaged for up to 30 minutes after substrate administration until the peak signal is obtained. For analysis, regions of interest (ROI) can be manually placed onto the images displayed in the LivingImage software to assess the signal in the region. Longitudinal images can then be assessed to determine the relative number of viable engineered cells over time and their location(s). This is especially valuable when studying cancer therapies in pre-
clinical models to determine if the relative number of viable cancer cells is decreasing after treatment.

1.7.1 Genome engineering

For cell tracking studies, cells of interest are cultured ex vivo and engineered to stably express a luciferase gene. Many luciferase enzymes have been isolated from different species and made available for imaging purposes\(^{137}\). Renilla luciferase and firefly luciferase (FLuc) are the most common transgenes for BLI. This thesis focuses on FLuc as it has a more favourable injection route and has better light penetration through tissues because its emission spectrum is more red shifted. When expressed, FLuc produces an enzyme that reacts with the substrate D-luciferin in the presence of oxygen and adenosine triphosphate (ATP) to produce light\(^{138}\) (Figure 1.5). To accomplish stable gene expression, cell populations need to be engineered prior to their injection. In the past, my colleagues and I successfully used clustered regularly interspaced short palindromic repeats and crispr associated protein 9 (CRISPR/Cas9) to integrate FLuc into a safe harbour site in cells\(^{139}\). The greatest advantage of CRISPR/cas9 genome editing is that gene integration is site specific which means safe sites can be targeted to avoid behavioural modifications caused by insertional mutagenesis. Unfortunately, this process is still fairly inefficient compared to largely used methods such as lentivirus and gammaretrovirus transduction. Our lab and others have used lentivirus extensively to make stable cell lines because it is cost-effective, efficient, and relatively safe. In this
thesis we used lentivirus to produce FLuc expressing leukemia cells to enable the tracking of their relative viability with longitudinal BLI.
Figure 1.5: Bioluminescence Imaging (BLI) diagram depicting the reaction between viable firefly luciferase expressing cancer cells and the substrate D-luciferin in the presence of oxygen and ATP to produce light, AMP, carbon dioxide, and oxyluciferin. The light is detected by the CCD camera to create an image.
1.7.2 Cancer Cell Imaging with BLI

Cancer researchers rely heavily on methods that measure tumour burden when studying new therapies. Caliper measurement is a commonly used metric for measuring tumour volumes and is often used to determine the response of tumours to a new therapy\textsuperscript{140, 141}. Although this method is easy to implement and very cost effective, tumour necrosis can occur much before a tumour changes in size. Therefore, if researchers are relying solely on changes in size to evaluate a response to treatment it might prolong the study and provide inaccurate estimates of how long it takes for the treatment to begin working. In contrast, BLI is an ideal method for cancer detection in pre-clinical models because it is cost-effective, specific to engineered cell populations, and only detects viable cells. BLI is an extremely valuable imaging method for studying cancer cell growth and subsequent treatment with therapies as signal is only produced by viable luciferase expressing cells\textsuperscript{142}. Cancer cell growth can be monitored prior to treatment to determine a baseline for cancer signal. Downstream BLI post-treatment can then be used to monitor for cancer cell death as there will be less signal if cancer cells die. Therefore, as necrosis occurs tumour signal will decrease even if the volume does not change. Below are examples of studies that have successfully used BLI to detect cancer cell viability and growth over time in the presence of therapeutic cells.

Rehemtulla et al. used BLI as a method to assess the cancer treatment response to chemotherapy\textsuperscript{142}. They successfully imaged FLuc expressing glioma growth over time in rats using BLI to detect differences in signal before and after treatment. Their data
suggests that BLI may be a better measure of tumour burden over time compared to MRI tumour volume measurements because BLI only measures metabolically active cells.

Parkins et al. used dual BLI to track RLuc expressing circulating tumour cells (CTC) that were engineered to express a suicide gene and then adoptively transferred into mice bearing FLuc expressing breast cancer. We were able to compare the locations of the therapeutic cells and the cancer cells over time. In addition, we could determine the CTC treatment effect by comparing the BLI signal measurements over time.

Finally, two of the previously discussed studies that imaged PFC+ CAR-T cells used BLI to evaluate the tumour response after treatment in combination with F MRI to evaluate the CAR-T cell location. They found that PFC+ CAR-T cells cause significant cancer cell death in mouse models of glioblastoma. Currently, no groups have looked at the difference in treatment response between PFC+ CAR-T cells and unlabeled CAR-T cells in vivo using BLI. This thesis focuses on determining if PFC+ CAR-T cells show the same treatment effect in vivo as unlabeled CAR-T cells using BLI.

1.8 Acute Lymphoblastic leukemia Mouse Model

There are many models developed to study human leukemia treatments in mice. In this thesis, we used NOD- Prkdc<sup>scid</sup>-II2rg<sup>tm1Wjl</sup> (NSG) mice because of their ability tolerate injections of human cells. NSG mice are genetically modified to be immunodeficient in mature T cells, B cells, and nature killer cells. In addition, they lack other signaling pathways that are important for proper immune system functioning. Specifically, the NOD mutation causes abnormal dendritic cell and macrophage function and removes the
complement system that is important for innate immunity. Generally, innate immunity is the first line of defense against pathogens that enter your body. The Prkdc<sup>scid</sup> severe combined immunodeficiency (SCID) mutation disrupts the development of mature T and B cells which comprise the adaptive immune response. The adaptive immune response is responsible for fighting viral, bacterial, and fungal infections in the body and is required for memory of infections which helps improve future immune function in the body. The Il2rg<sup>tm1Wjl</sup> mutation removes functioning interleukin 2 receptor gamma which causes natural killer (NK) cells to be unable to differentiate and mount a proper immune response. NK cells recognize infected cells in the body by their lack of MHC I molecule and kill them to ensure that virally infected cells and early cancer cells do not persist. They have been shown to assist in the rejection of transplanted tissues, so it is very important that their function is blocked to allow for human cells to be injected into mice while avoiding severe side effects such as graft versus host disease and rejection<sup>146</sup>.

Altogether, these mutations allowed us to implant the NALM6 cell line into mice to form acute lymphoblastic leukemia bearing mice. NALM6 cells are a human precursor B Cell leukemia cell line that originated from a 19-year-old man with ALL. NALM6 cells have been used by many researchers to study leukemia both in vitro and in mouse models<sup>147-149</sup>. By implanting the NALM6 cells with Matrigel we were able to form solid subcutaneous tumours in the left hind flank of mice. This technique is ideal as it allows researchers to study human cancer in a model that can be easily injected with therapeutic cells and imaged<sup>89</sup>. Flank tumours were also ideal to minimize the effects of breathing artifacts in our MR images.
1.9 Purpose of thesis

This thesis uses $^{19}$F-based cellular MRI techniques to detect CAR-T cells in a murine model of B cell leukemia. The objectives of this work were to determine if PFC+ CAR-T cells could be detected using a 3 T clinical MRI scanner and to evaluate the performance of PFC+ CAR-T cells compared to unlabeled CAR-T cells using BLI. Our hypotheses are: 1) $^{19}$F based MRI performed on a 3T clinical system will be able to detect PFC labeled CAR-T cells in a mouse model of leukemia, and 2) PFC+ CAR-T cell cytotoxicity will not be significantly different from unlabeled CAR-T cell cytotoxicity in vivo. Chapter 2 demonstrates the application of $^{19}$F MRI to detect PFC labeled CAR-T cells in a mouse model of leukemia.

1.10 References


126. Celsense, Inc Files FDA & EMEA Regulatory Submissions: FDA accepts Drug Master File and EMEA receives Active Substance Master File for Cell Sense in vivo imaging agent


2 Visualizing CAR-T Cell Immunotherapy Using 3 Tesla Fluorine-19 MRI

**Purpose:** Chimeric antigen receptor (CAR) T cell cancer immunotherapies have shown remarkable results in patients with hematological malignancies and represent the first approved genetically modified cellular therapies. However, not all blood cancer patients respond favourably, serious side effects have been reported, and the treatment of solid tumours have been a challenge. An imaging tool for visualizing the variety of CAR-T cell products in use and being explored could provide important patient-specific data on CAR-T cell location to inform on potential success or failure of treatment as well as off-target toxicities. Fluorine-19 ($^{19}$F) magnetic resonance imaging (MRI) allows for the non-invasive detection of $^{19}$F perfluorocarbon (PFC) labeled cells. Our objective was to visualize PFC labeled (PFC+) CAR-T cells in a mouse model of leukemia using clinical field-strength (3 Tesla) $^{19}$F MRI and compare the cytotoxicity of PFC+ versus unlabeled CAR-T cells.

**Procedures:** NSG mice (n=17) received subcutaneous injections of CD19+ human B cell leukemia cells (NLM6) expressing firefly luciferase in their left hind flank ($1\times10^6$). Twenty-one days later, each mouse received an intratumoural injection of $10\times10^6$ PFC+ CD19-targeted CAR-T cells (n=6), unlabeled CD19-targeted CAR-T cells (n=3), PFC+ untransduced T cells (n=5), or an equivalent volume of saline (n=3). $^{19}$F MRI was performed on mice treated with PFC+ CAR-T cells days 1, 3, and 7 post-treatment. Bioluminescence imaging (BLI) was performed on all mice days -1, 5, 10, and 14 post treatment to monitor tumour response.
Results: PFC+ CAR-T cells were successfully detected in tumours using $^{19}$F MRI on days 1, 3, and 7 post-injection. *In vivo* BLI data revealed that mice treated with PFC+ or PFC- CAR-T cells had significantly lower tumour burden by day 14 compared to control cohorts (p<0.05). Importantly, mice treated with PFC+ CAR-T cells showed equivalent cytotoxicity compared to mice receiving PFC- CAR-T cells.

Conclusions: Our studies demonstrate that clinical field-strength $^{19}$F MRI can be used to visualize PFC+ CAR-T cells for up to 7 days post intratumoral injection. Importantly, PFC labeling did not significantly affect *in vivo* CAR-T cell cytotoxicity. These imaging tools may have broad applications for tracking emerging CAR-T cell therapies in preclinical models and may eventually be useful for the detection of CAR-T cells in patients where localized injection of CAR-T cells is being pursued.

2.1 Introduction

Cancer is a devastating disease with over 220 000 Canadians receiving a cancer diagnosis each year [1]. Despite enormous effort, cancer continues to be one of the leading causes of death in the world [2]. There continues to be an urgent need to develop new cancer therapies that allow for a greater number of cancer patients to survive for significantly greater lengths of time after their diagnosis. Chimeric antigen receptor (CAR) T cell therapy was first proposed in 1989 and is now the first genetically modified cellular therapies to be approved for the treatment of B cell leukemia and lymphoma [3]. CAR-T cells are produced using a patient’s own T cells that have been isolated and engineered to express a cancer antigen-specific CAR [4]. The CAR redirects the T cells to bind and kill
the patient's cancer cells after injection. Multiple CD19-targeted CAR-T cell therapies have been approved in Canada after showing remarkable results in patients with B cell malignancies, providing a transformative, potentially curative therapeutic option [5].

Despite the success that CAR-T cells show against B cell malignancies, major challenges remain. Up to 30% of blood cancer patients do not respond to these therapies, many patients can relapse, and patients can also suffer from life-threatening side effects such as cytokine release syndrome or neurotoxicity [6]. In addition, CAR-T cells continue to show disappointing results against solid tumours [7]. Many of the disparate outcomes among patients receiving this therapy are thought to be due to CAR-T cells not proliferating and persisting in the body, proliferating and activating excessively, or homing to normal organs such as the brain [8]. However, due to the inadequate information provided by serial blood tests currently used by clinicians, we have limited evidence about the behaviour of CAR-T cells over time in individual patients. Therefore, methods to track the fate of adoptively transferred T cells would be extremely valuable for both pre-clinical and clinical studies to learn about the behaviour of CAR-T cells after injection.

Cellular imaging is a potential complementary technology to blood tests involving non-invasive imaging of cells labeled with imaging technologies to achieve information on cell fate after adoptive transfer. Ex-vivo labeling is a particularly invaluable approach for CAR-T cell therapies as they require ex-vivo processing for production regardless of whether or not imaging is implemented. The breadth of cellular imaging technologies available spans from preclinical imaging modalities such as fluorescence and bioluminescence imaging (BLI) to clinical modalities such as magnetic resonance
imaging (MRI), photoacoustic tomography, and positron emission tomography (PET) [9]. Importantly, successful PET imaging of intracranially infused cytotoxic T cells co-expressing a PET reporter gene was demonstrated in glioma patients [10], [11].

MRI is also being explored extensively as a clinical cell tracking tool. MRI provides images with fine spatial resolution and high soft tissue differentiation, uses non-ionizing radiation that can be beneficial for longitudinal studies, numerous MRI probes and reporter genes have been developed for ex vivo cell labeling, and MRI is broadly available within the healthcare system in most developed countries. Currently, most immune cell tracking studies utilizing MRI have been accomplished by labeling cells with iron oxide nanoparticles (IONS) [12]. Clinical imaging of ION labeled dendritic cells in melanoma patients was achieved by De Vries et al in 2005 [13]. IONs allow labeled cells to be detected with high sensitivity, even single cells in preclinical models, but detection of the cells can be difficult in locations such as the lungs as IONs cause hypointensities in images [14]. In contrast, fluorine-19 perfluorocarbons (PFC) are a tracer agent that is easily taken up by cells and can be detected directly by fluorine-19 (19F) MRI [15]. 19F MRI cell tracking provides positive contrast which enables improved quantitation in comparison to ION imaging and has high specificity because there is no detectable endogenous 19F in the body. This technique is clinically relevant as PFCs were used off label in patients for many years as a blood substitute [16]. In addition, 19F MRI has been successfully used in the clinic to detect PFC labeled (PFC+) dendritic cells in patients [17]. Previous preclinical studies have shown the feasibility of labeling CAR-T cells with PFC and detecting them with high field-strength 19F MRI [18]-[20]. However, data supporting the ability to image PFC+ CAR-T cells using a clinical field-strength
scanner is lacking, which is important to demonstrate when assessing if translation of this technology into patients is feasible. Moreover, *in vivo* data comparing the cytotoxicity against tumors of CAR-T cells versus PFC+ CAR-T cells is lacking.

In this study we focused on using $^{19}$F PFC based imaging to monitor CAR-T cells over time using clinical field-strength 3 Tesla MRI. In addition, we used bioluminescence imaging (BLI) to evaluate whether labeling CAR-T cells with PFC affects their *in vivo* cytotoxicity towards cancer cells. Our results indicate that this technique can reliably detect PFC+ CAR-T cells post-intratumoural injection using clinical field strengths. We also show for the first time that PFC labeling does not significantly affect *in vivo* CAR-T cell cytotoxicity in a mouse model of leukemia, which is important for potential future use of this imaging technique in patients.

### 2.2 Materials and Methods

#### 2.2.1 Cancer Cells and Engineering

A CD19 positive B cell acute lymphoblastic leukemia cell line (NALM6 cells; Cedarlane) was utilized for this study. NALM6 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (Wisent) and 1% antibiotic-antimycotic (100X; Thermofisher). NALM6 cells were engineered to stably co-express the fluorescence reporter tdTomato (tdT) and a codon-optimized bioluminescence firefly luciferase reporter (Luc2) using a lentiviral vector previously constructed in our lab [21]. Cells were transduced with lentiviral vector using polybrene (1.6ug/ml, Sigma Aldrich). Transduced cells were analyzed and sorted using fluorescence activated cell sorting
2.2.2 Human T Cells and Engineering

Frozen PBMCs from various donors were purchased from StemCell. PBMCs cells were cultured in ImmunoCult-XF T cell expansion medium (StemCell) supplemented with 100U/mL interleukin-2 (Chiron) and 2µL (55µM) 2-mercaptoethanol (Sigma). T cell populations were obtained by thawing human PBMCs (StemCell) and activating 1x10⁵ cells per well with 2µL of 4x10⁷ beads/mL Human T-activator CD3/CD28 Dynabeads (Thermofisher) as outlined in the protocol from Hammill et al. [22]. Twenty-four hours later, T cells were engineered to co-express a CD19 targeted CAR and green fluorescent protein (GFP) using a CD19 CAR-GFP lentiviral transfer plasmid expressing a second generation CD19 targeting CAR containing the 4-1BB co-stimulatory molecule generously gifted by Drs. Robert Holt and Brad Nelson (University of British Columbia) using an MOI of 5. Transduced and untransduced T cell populations were then expanded and evaluated with flow cytometry to evaluate CAR/GFP, CD3, CD4, and CD8 expression. To produce PFC labeled (PFC+) CAR-T cells or PFC+ untransduced T cells for ¹⁹F MRI, T cell populations were labeled overnight with 5mg/ml Texas Red fluorescent dye conjugated PFC (CS-ATM DM Red, CelSense) and washed three times with phosphate buffer saline (PBS) prior to downstream applications.
2.2.3 In Vitro Imaging

To evaluate the minimum number of PFC+ CAR-T cells that could be detected at 3T, triplicates of CAR-T cell pellets containing decreasing numbers of labeled cells (2, 1, 0.5, 0.25, 0.1 (x 10^6) cells) were imaged using 19F MRI. Samples were made by mixing labeled and unlabeled CAR-T cells to obtain a total of 2x10^6 cells per Eppendorf tube, the Eppendorf tube was then spun down to form pellets, and then topped with 1% agarose prior to MRI. The resulting samples were imaged at 3T using a clinical GE 3T MR750 system following the same imaging protocols used for in vivo imaging (see below). Analysis of all 19F MRI images is described further below.

To compare the cytotoxicity of PFC+ and unlabeled CAR-T cells, 5x10^4 NALM6-tdT-FLuc cells were seeded with PFC+ or unlabeled CAR-T cells at increasing effector to target ratios (1:4, 1:2, 1:1). Twenty-four hours later, 1μL of D-luciferin was added to each well (30 mg/mL, Syd Labs) and BLI was performed immediately on an IVIS Lumina XRMS scanner (IVIS Lumina XRMS In Vivo Imaging System, PerkinElmer). BLI signal was evaluated with region-of-interest (ROI) analysis using LivingImage Software (Perkin Elmer). Quantification was performed by drawing ROIs over each well to obtain the average radiance per well (photons/second/mm^2/steradian).

2.2.4 Animal models

Animals were cared for in accordance with the standards of the Canadian Council on Animal Care, and under an approved protocol of the University of Western Ontario’s Council on Animal Care (2018-150). NOD.Cg-PrkdcscidI2rgtm1Wjl/SzJ (NSG) mice (n=17) received subcutaneous injections of 1x10^6 NALM6-tdT-FLuc cells mixed with 50 ul of Matrigel in their left hind flank. Twenty-one days later, each mouse received an
intratumoural injection of 10x10^6 PFC+ CAR-T cells (n=6), unlabeled CAR-T cells (n=3), PFC+ untransduced T cells (n=5), or an equivalent volume of saline (n=3).

2.2.5 Nuclear magnetic resonance (NMR)

To evaluate the number of average 19F spins in cells, NMR was performed on samples containing 1x10^6 PFC+ CAR-T cells or PFC+ untransduced T cells. To prepare the samples for NMR, the cells were lysed by adding 10 µl Radioimmunoprecipitation assay (RIPA) buffer (VWR, Mississauga, CAN), sonicated 3 times, and then underwent 3 freeze-thaw cycles. The lysate was then placed in an NMR tube with 0.1% trifluoroacetic (TFA) acid and heavy water (D2O). 19F NMR measurements were performed using a Varian Inova 400 spectrometer (Varian Inc, Palo Alto, USA) as described by Makela et al. [23].

2.2.6 In Vivo BLI

BLI was performed on days -1, 5, 10, and 14 post-treatment in all mice. Mice were anesthetized with 2% isoflurane in oxygen during imaging sessions. Anesthetized mice received an intraperitoneal injection of 100µL of D-luciferin (30mg/mL) and images were collected using an IVIS Lumina XRMS scanner for up to 30 minutes. Day -1 images were used as a baseline for tumour burden to determine treatment response after CAR-T cell, T cell, or saline injections. BLI signal was evaluated with ROI analysis using LivingImage Software (Perkin Elmer). An ROI was drawn around the whole mouse and the total flux (photons/sec) was measured to determine the peak signal in the 30-minute imaging session. The peak signal for each mouse at each time point was recorded and used for statistical analysis.
2.2.7 In Vivo $^{19}$F MRI

Mice bearing leukemia tumours that received PFC+ CAR-T cells or PFC+ untransduced T cells were imaged with $^{19}$F MRI on days 1, 3, and 7 post-treatment. Mice were anesthetized with 2% isoflurane in oxygen during imaging sessions. $^1$H and $^{19}$F images were acquired on a clinical 3T MRI (GE 3T MR750 system, General Electric, ON, CAN) using a custom built 4.3 x 4.3 cm$^2$ dual tuned $^1$H/$^{19}$F surface coil. In vivo $^1$H and $^{19}$F images were both acquired with a 3D balanced steady state free precession (bSSFP) pulse sequence. Two reference tubes of known $^{19}$F concentration (3.33 x 10$^{16}$ $^{19}$F/$\mu$L) were imaged alongside the mice for quantification purposes. $^1$H imaging parameters were: field of view (FOV) = 60 x 30 mm, matrix size = 150 x 75, slice thickness = 0.4 mm (0.4 x 0.4 x 0.4 mm$^3$ resolution), flip angle (FA) = 20°, bandwidth (BW) = $\pm$31.25 kHz, repetition time (TR)/echo time (TE) = 12.8/6.4 ms, phase cycles (PC) = 12, total scan time = 9 minutes. $^{19}$F imaging parameters were: FOV = 60 x 30 mm, matrix = 60 x 30, slice thickness = 1 mm (1 x 1 x 1 mm$^3$ resolution), FA = 72°, BW = $\pm$10 kHz, TR/TE = 5.6/2.8 ms and 150 NEX, scan time = 27 mins.

$^{19}$F images were analyzed using Horos software. The standard deviation (Sdev) of background signal for each $^{19}$F image was measured by drawing a region of interest (ROI) in an area of background noise. A minimum threshold of 5 times the Sdev was used to mask lower amplitude signal and yield a reliable measurement of $^{19}$F signal in cell pellets, tumors, and reference tubes. This imaging criteria is based on an $^{19}$F signal with signal-to-noise (SNR) ratio > 5. Total $^{19}$F signal in cell pellets, tumors, and reference
tubes was calculated as mean $^{19}$F signal * volume of ROI. $^{19}$F content in cell pellets and tumors was determined by comparing the $^{19}$F signal measured from these ROIs to the signal measured from the reference tubes (3.33 x $10^{16}$ $^{19}$F/µL).

2.2.8 Histology
Two mice from the PFC+ CAR-T cell treatment group and the PFC+ untransduced T cell treatment group were euthanized via overdose of isoflurane on day 10 post-treatment. Their primary tumours were excised, fixed in 4% paraformaldehyde, and cryoprotected by passaging through a sucrose gradient of 10%, 20%, and 30%. Samples were then frozen in optimal cutting temperature compound using dry ice and 10 µm sections were collected using a cryostat (Leica CM350 Cryostat). Tumour sections was stained with DAPI and imaged using fluorescence microscopy (EVOS FL Auto 2) to detect GFP expressing CAR-T cells.

2.2.9 Statistics
Statistics were performed using the GraphPad Prism 8 Software. Unpaired t-tests were performed on the in vitro BLI cytotoxicity assay data to assess the difference between the cytotoxicity of PFC+ and PFC- CAR-T cells. A simple linear regression was performed on the in vitro $^{19}$F MRI data to assess the correlation between $^{19}$F signal and cell number. A two-way ANOVA with multiple comparisons was performed on the in vivo $^{19}$F signal data to compare between the labeled treatment groups at each time point. A two-way ANOVA with Tukey’s multiple comparisons was performed on the in vivo BLI data to assess any differences in the treatment responses observed between treatment groups at each imaging time point. A nominal p-value less than 0.05 was considered significant.
2.3 Results

2.3.1 Production and characterization of treatment and target cells

Figure 2.1a shows a representation of the CD19-CAR-GFP plasmid used to make CD19 targeting CAR-T cells and the tdT-FLuc plasmid used to make firefly luciferase expressing NALM6 cells. Flow cytometry revealed that CAR-T cell populations were approximately 68.6% CAR/GFP positive after transduction. Further, the CAR-T cell populations were approximately 97% CD3 positive, 67.5% CD4 positive, and 26.1% CD8 positive prior to injection. Untransduced T cell populations showed similar characteristics with approximately 97% CD3 positive cells, 47.6% CD4 positive cells, and 45.5% CD8 positive cells (Fig 2.1c). After labeling the T cell populations with 5 mg/ml texas red fluorophore-conjugated PFCs overnight, 88.8% of the cells were positive for uptake of PFC (Fig 2.1d). On average, the labeled T cell populations contained 5.116865×10^{11} 19F/Cell. After transduction, 98.7% of NALM6 cells expressed tdT/Fluc (Fig 2.1b).
Figure 2.1: Production and characterization of CAR-T cells and their target cells. (a) Diagrams depicting a lentiviral (LV) tdT-FLuc transfer plasmid co-expressing the tdTomato fluorescent gene and firefly luciferase (FLuc2) bioluminescence reporter gene for the production of NALM6-tdT-FLuc cells and the LV-C19-CAR-GFP plasmid co-expressing the CD19 targeted second generation CAR and GFP for the production of CD19 CAR-T cells. (b) Merged histograms showing the NALM6 cell population before and after transduction with the tdT-FLuc lentivirus. (c) Bar graph showing the percent expression of CAR/GFP, CD3, CD4, and CD8 markers in CD19-CAR-GFP transduced T cell populations and untransduced T cell populations (n=3). (d) Merged histograms showing a representative CAR-T cell population before and after labeling with red fluorescent perfluorocarbons (CelSense).
2.3.2  *In vitro* assessment of PFC labeled CAR-T cells and their target cells

Figure 2.2 shows the *in vitro* characterization data for the imaging reporters and CAR-T cell cytotoxicity. BLI revealed that the NALM6-tdT-Fluc cell line had functional Fluc2 activity (Fig 2.2a). Cytotoxicity assays showed that co-culture with unlabeled CAR-T cells caused an average of 63.6%, 80.5%, and 94.5% Nalm6-tdT-Fluc cell lysis at effector to target ratios of 1:4, 1:2, and 1:1, respectively (Fig 2.2a). In comparison, PFC+ CAR-T cells caused an average of 43.3%, 73.7%, and 90.0% Nalm6-tdT-Fluc cell lysis at effector to target ratios of 1:4, 1:2, and 1:1, respectively. There was no significant difference in cytotoxicity between unlabeled and PFC+ CAR-T cells at any effector to target ratio (Fig 2.2b). *In vitro* $^{19}$F MRI of PFC+ CAR-T cell pellets showed that pellets containing only 12.5% labeled CAR-T cells ($2.5 \times 10^5$ PFC+ CAR-T cells) could be reliably detected at 3T. Quantification of the $^{19}$F spins revealed that there was a strong positive correlation between $^{19}$F signal and labeled cell number ($R^2=0.8568; p<0.05$).
Figure 2.2: In vitro characterization of the PFC labeled CAR-T cells. (a) BLI cytotoxicity assay showing the viability of NALM6-tdT-Fluc cells in the presence of saline (control), unlabeled CAR-T cells (PFC-), or PFC labeled CAR-T cells (PFC+) at increasing effector to target ratios (n=3). (b) Bar graph showing percent lysis of the NALM6-tdT-Fluc cells 24 hours after treatment with PFC labeled or unlabeled CAR-T cells. (c) 19F MRI of PFC labeled CAR-T cell pellets (2x10^6 total cells) containing decreasing numbers of labeled CAR-T cells (2, 1, 0.5, 0.25, 0.1 x10^6 cells). (d) Quantification of 19F signal compared to cell number shows a strong positive correlation (R^2=0.8568; p<0.05).
2.3.3 \(^{19}\text{F Cellular MRI Detection of PFC-Labeled T cells in Tumor-Bearing NSG Mice\)  

In vivo detection of PFC+ CAR-T cells using 3T clinical MRI was assessed after intratumoural injections of \(10 \times 10^6\) cells into mice bearing subcutaneous Nalm6-tdT-Fluc tumours. All mice injected with NALM6-tdT-Fluc cells developed tumours in their left hind flanks by day 21 post-injection. Figure 2.3 shows representative \(^{19}\text{F images of tumour bearing mice after intratumoural injections of either PFC+ CAR-T cells or PFC+ T cells. \(^{19}\text{F images are overlaid onto } ^{1}\text{H images for anatomical reference. In all mice, \(^{19}\text{F signal was present in the tumour on days 1, 3, and 7 post PFC+ cell injection (Fig 2.3a and 2.3b). The \(^{19}\text{F MRI data shows that the PFC+ T cells and CAR-T cells were accurately injected intratumourally in all of the treated mice and the persistent \(^{19}\text{F signal suggests that the PFC+ cells remain in the tumour over time. The total number of \(^{19}\text{F spins for each tumor on days 1, 3, and 7 were quantified and are shown in Figure 2.3c. The mean number of \(^{19}\text{F spins was not significantly different between PFC+ CAR-T cell treated tumors and PFC+ T cell treated tumours at any time point.\)\)\)\)\)\)\)\)
Figure 2.3: In vivo 19F MRI of leukemia tumour bearing mice treated with 10x10^6 PFC labeled CAR-T cells (n=6) or 10x10^6 PFC labeled untransduced T cells (n=5). 19F images are overlaid onto 1H images for anatomical reference. 19F signal is detected in the tumours over time. Scale bars represent the range of 19F signals. (a) Representative images from two PFC labeled CAR-T cell treated mice on days 1, 3 and 7 post-treatment. (b) Representative images of a PFC labeled untransduced T cell treated mouse on days 1, 3, and 7 post-treatment. (c) Quantification shows no significant differences of 19F signal over time between PFC+ CAR-T cell and PFC+ T cell groups.
2.3.4 *In vivo* BLI of leukemia bearing mice treated with CAR-T cells

To assess whether PFC labeling effected CAR-T cell therapy outcome, BLI images of NALM6-tdT-Fluc tumour bearing mice were obtained up to 14 days after intratumoural injections of $10 \times 10^6$ PFC+ CAR-T cells (Fig 2.4a), unlabeled CAR-T cells (Fig 2.4b), PFC+ T cells (Fig 2.4c), or an equivalent volume of saline (Fig 2.4d). PFC+ and unlabeled CAR-T cell treated mice showed decreased BLI signal after treatment. PFC+ T cell and saline treated mice showed continuous increases in BLI signal after treatment. The total flux from each mouse at each imaging timepoint was quantified and are shown in Figure 2.4e. Mice treated with PFC+ CAR-T cells had significantly lower BLI signal by day 14 compared to mice treated with PFC+ T cells or saline ($p<0.0001$, $p<0.0001$). There were no significant differences between the BLI signal in mice treated with saline or PFC+ T cells at any time point. Importantly, there were no significant differences in BLI signal between mice treated with unlabeled CAR-T cells compared to mice treated with PFC+ CAR-T cells at any time point.
Figure 2.4: In vivo BLI of firefly luciferase expressing NALM6 tumour bearing mice days -1, 5, 10, and 14 post-treatment with intratumoural injections of 10x10^6 PFC labeled CAR-T cells (n=6), unlabeled CAR-T cells (n=3), PFC labeled T cells (n=5), or and equivalent volume of saline (n=3). (a) Representative images of a PFC labeled CAR-T cell treated mouse showing a decrease in tumour burden over time. (b) Representative images of an unlabeled CAR-T treated mouse showing a decrease in tumour burden over time. (c) Representative images of a PFC labeled T cell treated mouse showing increases in tumour burden over time. (d) Representative images of a saline treated mouse showing increases in tumour burden over time. (e) Quantitation of BLI signal over time showing significant differences between PFC labeled CAR-T cell treated mice compared to PFC labeled T cell and saline treated mice on day 14 (P<0.0001 and p<0.0001). There is no significant difference between labeled and unlabeled CAR-T cell signal at any timepoint post-treatment.
2.3.5 Histology

Tumours from mice that received intratumoural injections of PFC+ CAR-T cells or PFC+ T cells were excised on day 10 post-treatment and analyzed to detect the presence or absence of CAR-T cells. Histological analysis confirmed that GFP positive CAR-T cells were still present in PFC+ CAR-T cell treated tumours on day 10 post injection (Fig 2.5a). No GFP positive cells were detected in tumours treated with PFC+ T cells (Fig 2.5b).
Figure 2.5: Histological analysis of GFP expression in a murine tumour treated with PFC+ GFP expressing CAR-T cells or a murine tumour treated with PFC+ untransduced T cells. (a) Images showing GFP positive CAR-T cells in the tumour treated with PFC+ CAR-T cells 10 days post intratumoural injection. (b) Images showing no GFP positive cells in tumours treated with PFC+ untransduced T cells 10 days post intratumoural injection. Images were taken at 20X magnification.
2.4 Discussion

CAR-T cell therapies have shown tremendous promise in clinical trials against B cell malignancies. Despite these successes, there are still many limitations to overcome including overcoming their potential to cause serious side effects and increasing their efficacy in some patients with hematological malignancies and patients with solid tumours. Studies have shown that intratumoural injections of CAR-T cells may improve the treatment outcome in models of solid tumours [24], [25]. One of the main barriers in intratumoural treatments is ensuring that the injection is administered accurately to tumours that may be different sizes and in different locations across patients. Acquiring adequate information on cell location and persistence after injection may improve intratumoural treatments by ensuring that each patient receives the therapy in the correct location. In this study, we demonstrate that $^{19}$F MRI at 3T provides information on PFC labeled CAR-T cell location and persistence after injection into mice bearing NALM6-tdT-FLuc tumours. Importantly, we saw no significant effect on CAR-T cell treatment due to PFC labeling.

Clinical translation of cell tracking requires a safe cell label that can be detected with imaging devices that are commonly found in hospitals. PET has been used in combination with clinically relevant radiotracers in many studies to track immune cells with high sensitivity [10],[26],[27]. Unfortunately, concerns about cost, half-life, and radioactive dose may limit longitudinal cell tracking studies using PET [28]. In contrast, MRI uses non-ionizing radiation which is ideal for longitudinal cell tracking studies as radiation dose is not of concern. For this reason, we chose to use $^{19}$F MRI as our imaging
modality and $^{19}$F PFCs as our cell label because these are both clinically applicable and, in combination, allow for direct cell detection and quantification. CAR-T cells had been successfully labeled with PFCs for detection with $^{19}$F MRI in the past, but these studies used field strengths well above clinical field strengths to enhance $^{19}$F signal [18], [19]. In addition, these studies used CAR-T cells labeled with PFC nanoemulsions that are not, at this time, commercially available nor manufactured in a manner acceptable for human use. We demonstrated that functioning CAR-T cells could be detected using 3T clinical MRI using a surface coil and SNR optimized bSSFP sequence after labeling with commercially available PFC, which is also available in GMP form for clinical translation. Additionally, we were able to perform the imaging using a clinically feasible scan time of approximately 9 minutes for the $^1$H scan and 27 minutes for the $^{19}$F scan.

Our phenotyping results for PFC+ CAR-T cells shown in Figure 1, agree with previous studies showing that CD8 expressing cells make up approximately 1/3 of the population and CD4 expressing cells make up approximately 2/3 of the population [19], [20]. Our cell labeling allowed us to image down to 250 000 cells in vitro which agrees with previously published results suggesting that thousands of PFC+ cells are needed per voxel to achieve detection [23]. Our in vivo imaging of mice treated with PFC+ T or CAR-T cells showed that $^{19}$F signal could be detected in every tumour on days 1, 3 and 7 post treatment. Our findings on PFC+ CAR-T cell detection after intratumoural injection are similar to recent results published by Chapelin et al. which looked at PFC+ CAR-T cells up to day 10 post intratumoural injection in a mouse model of glioma using an 11.7T MRI scanner [18]. The $^{19}$F signal was consistent over time and suggested that the CAR-T cells were viable and persisting in the tumour site. We also did not see significant
differences between the $^{19}$F signal detected in mice receiving PFC+ CAR-T cells compared to PFC+ T cells. This is consistent with their work and may be because T cells are surviving and remaining in the tumour site in both treatment groups.

We chose to complement our $^{19}$F MRI CAR-T cell detection with BLI to assess treatment response in our mice and determine if PFC labeling influenced \textit{in vivo} CAR-T cell cytotoxicity. Our \textit{in vitro} results showed no significant differences in cytotoxicity between labeled and unlabeled CAR-T cells, similar to previous work [18]. In addition, previous PFC+ CAR-T cell tracking studies have shown that labeled CAR-T cells cause cytotoxicity against glioma \textit{in vivo}. However, to our knowledge no studies have evaluated PFC+ CAR-T cell \textit{in vivo} cytotoxicity compared to unlabeled CAR-T cell cytotoxicity. BLI of luciferase-expressing tumors over time in mice treated with both PFC+ CAR-T cells and unlabeled CAR-T cells demonstrated that PFC labeling does not significantly affect CAR-T cell \textit{in vivo} cytotoxicity in this model.

There are still limitations to our cell detection method including $^{19}$F MRI being less sensitive compared to clinical imaging modalities such as PET and cell division preventing accurate measures of cell number over time [29]. These limitations are especially important when working with T cells because they are small and non-phagocytic which makes them more difficult to label. Nevertheless, in current clinical studies testing intratumourally injected CAR-T cells, patients receive up to $1\times10^{10}$ CAR-T cells, which is well above the detection limit of $^{19}$F MRI [30]. One advantage of $^{19}$F MRI is that it is quantitative and the number of cells in a given region can be estimated using \textit{in vitro} NMR data to determine the amount of $^{19}$F per cell. This method can be used to quantify cell numbers early after injection. However, it is important to point out that
this method is not as reliable for quantifying CAR-T cell numbers over time, as CAR-T cells have been shown to proliferate significantly after CAR interaction with their respective antigen [31]. During cell division, the PFC label should be divided between daughter cells. If these cells do not remain in the same voxels, this may decrease the $^{19}$F signal in an individual voxel below the detection limit, which would result in an underestimate of the number of CAR-T cells. Moreover, if the cells remain in the same voxel this would still underestimate the number of CAR-T cells based on $^{19}$F spins. There is also the potential for background signal caused by macrophages taking up PFCs that are lost when labeled cells die after injection. However, studies indicate that when labeled cells die the PFC is most likely broken down and released through the liver and then exhaled using the reticuloendothelial system [32]. Considering these limitations, it is therefore important to not overinterpret the $^{19}$F signal as the number of viable cells at extended periods after adoptive transfer, particularly in highly dividing cell populations.

A complementary imaging tool such as reporter genes, which are passed to daughter cells, would allow for both highly sensitive short-term imaging with $^{19}$F PFCs and long-term cell viability imaging with a reporter gene [33], [34]. We are currently exploring the usefulness of this combination of cellular imaging technologies for tracking CAR-T cells in preclinical cancer models.

Currently, our system would be useful for detecting CAR-T cells after intratumoural injections into easily accessible tumours such as glioblastoma, metastatic colorectal cancer, and metastatic breast cancer$^{24,25,31}$. It would be interesting to try imaging intravenously injected CAR-T cells in an animal model at clinical MRI field strengths in the future to determine if clinical-field strength imaging of intravenously administered
CAR-T cells would be feasible. Future work focusing on the development of larger radiofrequency coils for dual $^1$H and $^{19}$F MRI would also help advance this field. However, even if this is not feasible due to lack of sensitivity, it will still be valuable to continue to explore and develop $^{19}$F MRI of PFC-labeled CAR-T cells after intratumoral injections. In this case, localized coils with high sensitivity like the one used in our study would be valuable.

**Conclusions:** We report that PFC+ CAR-T cells can be detected over time with $^{19}$F MRI using a 3T clinical field strength scanner. In addition, we show that PFC labeling does significantly impact the *in vivo* treatment response of CAR-T cells in this model, as shown by longitudinal BLI. $^{19}$F MRI is a useful tool for determining the location and persistence of CAR-T cells in tumours after localized injection and may have utility for tracking systemically administered cells in particular tumor types. This imaging tool may be useful for optimizing current CAR-T cell therapies and may have broad applications for evaluating emerging CAR-T cell formulations *in vivo*.

### 2.5 References


Chapter 3

3 Summary and Future Work

This chapter summarizes the experimental findings, limitations, and future work associated with this thesis.

3.1 Discussions and Conclusions

Despite strong evidence supporting CD19 targeting CAR-T cell therapies as an ideal cancer treatment for patients with B cell malignancies, there is still the potential for severe side effects and poor treatment responses in some patients\(^1\). Optimizing CAR-T cell therapies and mitigating their side effects could aid in the widespread clinical adaptation of these therapies. Knowledge of CAR-T cell biodistribution and persistence after injection is crucial to learn more about their behaviour during side effects and poor treatment outcomes. Molecular imaging techniques could complement current clinical blood tests to provide real time information on the locations and numbers of CAR-T cells in important sites in the body such as tumours or off target organs. In this thesis, \(^{19}\)F MRI was used to detect PFC+ CAR-T cells in a mouse model of leukemia. Below is a summary of the results found in this study.

In Chapter 2, mice bearing luciferase expressing human B cell leukemia tumours were intratumorally injected with PFC+ CD19 targeting CAR-T cells and imaged over time with BLI and \(^{19}\)F MRI. The leukemia cells express CD19 which allows them to be targeted by the CD19 targeting CAR-T cells. \(^{19}\)F MRI data was quantified to determine the number of \(^{19}\)F spins in each tumour over time. BLI data was analyzed to assess the
cancer treatment response in mice administered PFC+ CAR-T cells compared to mice that received unlabeled CAR-T cells. The main findings were:

1. PFC+ CAR-T cells could be detected in tumours up to 7 days after injection using a 3 T clinical MR scanner and custom dual tuned surface coil designed for use in humans.

2. Quantification of the BLI data showed that PFC+ CAR-T cells show no significant difference in cancer treatment response in vivo compared to unlabeled CAR-T cells.

These findings present important evidence that $^{19}$F 3T MRI could be a useful tool to detect PFC+ CAR-T cells during clinical trials exploring intratumoural injections of CAR-T cells. This method may provide information on the location of adoptively transferred cells to ensure that the injection was successfully administered into a tumour and that CAR-T cells persist in the tumour during the first week of treatment. In many cases, a limitation of $^{19}$F MRI is the low sensitivity. In our study, we detected strong signal in the tumours even at the latest timepoint of day 7 using a clinically relevant scan time highlighting the potential for this method in the clinic.

There are currently three other papers that have PFC labeled CAR-T cells and detected them with $^{19}$F NMR or MRI. In 2017, Chapelin et al. established PFC labeling techniques for CAR-T cells and ensured that the label did not affect T cell phenotype$^2$. In 2019, Hingorani et al. successfully imaged PFC+ CAR-T cells for the first time using $^{19}$F MRI two hours after an intratumoral injection into mice bearing glioma tumours$^3$. Finally, in 2021 Chapelin et al. imaged intratumourally administered PFC+ CAR-T cells up to 10 days post-injection for the first time in a mouse model of glioblastoma$^4$. These studies all
contributed important milestones to the implementation of $^{19}$F MRI for studying CAR-T cell therapies. One limitation that is common to all of these studies is that they relied on high field strength MR scanners (e.g., 11.7T) that are currently well above clinical standards. The work in this thesis successfully implemented PFC+ CAR-T cell detection using a 3T clinical MR scanner and RF surface coil designed for human use for the first time. Further, this is the first comparison of PFC labeled CAR-T cell and unlabeled CAR-T cell cytotoxicity in vivo using BLI, which is important to show before PFC labeling and $^{19}$F MRI could be translated to the clinic. It is our hope that these findings support the use of $^{19}$F MRI for clinical CAR-T cell tracking to achieve patient specific information on CAR-T cell location after adoptive transfer.

3.2 Challenges and Limitations

3.2.1 COVID-19 Pandemic

The COVID-19 pandemic was a unique challenge for graduate students over the past year. I recognize that the pandemic has had more extreme impacts on other populations and that overall, I was lucky to have this stable position with the University during this time. It was challenging to complete research activities during the lockdowns. I did not have access to the laboratory from Mid-March 2020 until June 2020 which meant that I could not make any progress on my project. In addition, access to the laboratory and research staff has continued to be reduced since we returned to campus in June-August 2020 due to strict safety measures put in place to stop the spread of COVID-19. This has caused significant delays in the completion of my project, but I was still able to complete most of the experiments that we had planned and my thesis during this time.
3.2.2 T cell detection challenges with $^{19}$F MRI

Currently, $^{19}$F MRI is less sensitive than many other forms of molecular imaging such as iron oxide nanoparticle (ION) imaging with MRI or PET$^5,6$. The sensitivity of $^{19}$F MRI is reliant on how well the cells are labeled, the imaging sequence chosen, the MR field strength, and the RF coil used$^7$. The uptake of PFC by T cells is lower when compared to larger, more endocytic/phagocytic cell populations such as dendritic cells or monocytes$^8$. This means that the signal obtained from labeled T cell populations will be lower per cell compared to other therapeutic cell populations. In addition, when CAR-T cells are activated by cancer cells they proliferate extensively to mount a proper response$^9$. As cell division occurs, the PFCs are divided between the daughter cells, diluting the amount of label in each cell. This causes the amount of $^{19}$F per voxel to decrease over time resulting in a decrease in signal over time. Eventually, with cell division, the labeled cells will fall below the detection limit of $^{19}$F MRI. Regardless, using the commercially available PFC CelSense, BSSFP pulse sequence, 3T MRI, and a human dual tuned surface coil we were able to clearly detect 10 million PFC+ CAR-T cells over time after an intratumoural injection into mice bearing leukemia tumours. Clinical trials using intratumoral or local delivery methods for CAR-T cells should expect that they would be able to detect the injected cells at clinical doses as well.
### 3.2.3 Requirements for translating $^{19}$F MRI to the clinic

Implementing $^{19}$F MRI cell tracking in a clinical setting would require specialized equipment to perform dual $^1$H and $^{19}$F imaging. Many hospitals already have an MR scanner which can be used for these imaging applications, but they would need to obtain a dual tuned RF coil for $^{19}$F MRI and have multi-nuclear imaging capabilities. In addition, the RF coil that was used in this study could easily cover the flank tumours on mice without any depth concerns. Unfortunately, as $^{19}$F MRI is moved towards patient imaging the 4.3cm diameter and depth limitations of the surface coil will reduce the number of studies that could be performed to track cell populations in patients. During the only clinical trial that used $^{19}$F MRI to image labeled cells in patients, they used a 7 cm diameter surface coil\textsuperscript{10}. They successfully imaged and quantified the number of intradermally injected PFC$^+$ dendritic cells in two patients enrolled in a dendritic cell vaccine study. This data suggests that clinical imaging of $^{19}$F PFCs in subcutaneous tumours such as breast cancer or prostate cancer should be possible.

In addition, there has still not been a study to determine whether intravenously (IV) injected PFC labeled CAR-T cells can be detected at clinical MR field strengths. As most clinical trials administer CAR-T cells IV, it is important to determine if it is possible to detect CAR-T cells as they traffic to different locations in the body following this injection route. Imaging at 3 T would limit the sensitivity of $^{19}$F MRI compared to higher field strength imaging, but the study in this thesis showed that as few as $2.5 \times 10^5$ cells could be detected \textit{in vitro}. In most studies, between $1 \times 10^6$-$1 \times 10^8$ CAR-T cells are
injected per kg of body weight into patients which should improve the chances of detecting cells that traffic to certain locations in the body.

Finally, the lower sensitivity of $^{19}$F MRI may require a higher number of excitations (NEX) to be used during an imaging session leading to longer scan times. Most current MRI scan times for patients are 30 to 40 minutes to avoid patient discomfort$^{11}$. In our study, the pre-clinical scan times were 9 minutes for the $^1$H scan followed by 27 minutes for the $^{19}$F scan. This would be feasible in the clinic but as $^{19}$F MRI is moved towards patients they may need to increase these scan times to accommodate for the depth of the PFCs in the body and the RF coil that is being used.

3.2.4 Quantification Inaccuracies

As mentioned in chapter 1 of this thesis, PFC labels are taken up by cells through endocytosis and held in the cytoplasm as long as the cell remains viable$^{12}$. Unfortunately, when cell death occurs the labeled cell can be taken up by macrophages and persist in tissues leading to background signal. This phenomenon is known as “bystander cell uptake” and can cause false positives in $^{19}$F images. Although this cannot be avoided, Fink et al. have previously confirmed that the $^{19}$F signal in their model was being produced by the PFC+ PBMCs that were injected instead of macrophages$^{13}$. 
In addition to signal accuracy concerns, there are also limitations associated with quantifying the number of cells that are detected in an $^{19}$F image. As described in chapter 1, an advantage of $^{19}$F MRI is that it is quantitative and can be used to determine the number of labeled cells in a subject. Most studies that have used $^{19}$F MRI to track ex vivo PFC+ cells have used information from $^{19}$F NMR and the signal obtained from $^{19}$F images to determine the number of cells in a given region. This data may be accurate at early imaging timepoints, especially if the cells do not divide or divide very slowly. Unfortunately, with cell populations like CAR-T cells, activation causes extensive proliferation and dilution of the PFC label. This leads to a decrease in detected $^{19}$F signal over time which would indicate that there were fewer cells in a given location. In reality, there would in fact be many more cells in the body over time due to CAR-T cell expansion. For this reason, $^{19}$F MRI cannot accurately be used to quantify the number of CAR-T cells in a tumour over time. Other groups have still used $^{19}$F MRI cell quantification to their advantage by determining the accuracy of their intratumoural injections immediately following adoptive transfer. Hingorani et al. for example determined that they could detect approximately 100% of the cells that they injected 2 hours after injection into a mouse model of glioma using $^{19}$F MRI cell quantification.$^{14}$

### 3.3 Future work

Future work will use $^{19}$F MRI cell detection to study CAR-T cell location and persistence in animal solid tumour models such as breast cancer. Additionally, future work will look
into combined $^{19}$F MRI and reporter gene-based CAR-T cell tracking to gain more information on CAR-T cell behaviour in treatment models and determine the best method for clinical cell detection.

3.3.1 Explore $^{19}$F MRI for intratumourally injected cancers such as breast or prostate cancer

Intratumoural or local delivery of CAR-T cells has been proposed as a method to improve the efficacy of CAR-T cell therapies against solid tumours. Multiple pre-clinical studies have showed that local delivery of CAR-T cells is an effective method for treating tumours and may even improve the therapeutic outcome in animal models compared to other injection methods$^{15-17}$. In addition, there are clinical trials underway to test intratumorally injected CAR-T cells in patients with breast cancer and head and neck cancer and intracranially injected CAR-T cells in patients with brain cancer$^{18,19}$. Unfortunately, studies have noted that local delivery is more difficult than IV delivery as tumours may be different sizes and in different locations making it harder to accurately inject the therapy$^{20}$. An inaccurate injection may have large implications on the efficacy of the treatment and the validity of efficacy studies in both pre-clinical and clinical trials.

In the work presented in this thesis, we were able to demonstrate that CAR-T cells can be efficiently labeled with PFC and detected in leukemia tumours over time after intratumoural injections. Future work will look at using $^{19}$F MRI to detect CAR-T cells in relevant solid tumour models such as breast cancer or breast cancer brain metastases. The use of $^{19}$F MRI in studies testing the efficacy of intratumourally injected CAR-T cells would provide important information on the location and persistence of CAR-T cells in
the tumours. It could also be used as a metric to ensure that the intratumoural injections are accurate and successfully reach the targeted location in the body. This information may be useful for determining why some subjects do not respond to CAR-T cells. Additionally, it may help in determining which injection route is best for each cancer type that is being targeted by the therapy.

3.3.2 Dual $^{19}$F and reporter gene-based cell tracking of CAR-T cells using MRI

Reporter gene-based imaging is valuable for studying highly proliferative cell populations as it relies on stable expression of a reporter gene. Stable reporter gene expression allows for longitudinal cell tracking because the reporter gene is passed onto daughter cells during cell division, leading to increased signal as cells proliferate. In addition, by co-expressing CARs and reporter genes in the same construct, reporter genes can be introduced to T cells using the same protocols that are already in place to generate current CAR-T cell therapies in the clinic. Many groups have started to employ reporter gene-based imaging methods to try to circumvent the signal loss that occurs with labels during cell division$^{21-23}$. Wu et al. developed a human derived MRI reporter gene called organic anion transporting polypeptide 1B3 (OATP1B3) that can be used in combination with the clinically approved contrast agent gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid (Gd-EOB-DTPA)$^{24}$. This method of imaging may be able to track dividing cells for longer periods of time due to stable gene expression. Using this cell detection technique in CAR-T cells in combination with $^{19}$F MRI will provide both short term and long-term information on cell location, viability, and proliferation.
using non-invasive imaging. We posit that this will also be a valuable technique to compare $^{19}$F MRI of PFC labeled cells against OATP1B3 reporter gene imaging of engineered cells. All together, these imaging techniques will provide more information on CAR-T cell behaviour after injection that may provide insight into making safer and more effective CAR-T cell therapies. Additionally, these techniques will get us closer to clinical cell tracking ventures.

### 3.4 References


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